Symposia Biologica Hungarica 13

NUCLEIC ACIDS AND PROTEINS IN HIGHER PLANTS

Akadémiai Kiadó, Budapest

NUCLEIC ACIDS AND PROTEINS IN HIGHER PLANTS

13

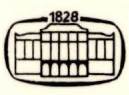
NUCLEIC ACIDS AND PROTEINS IN HIGHER PLANTS

Proceedings of the Symposium held at the Biological Research Institute in Tihany, 2 to 4 September, 1971

Edited by

G. L. FARKAS

The Symposium, the first international meeting of this kind, covered analytical, structural and metabolic aspects of nucleic acids and proteins in higher plants. The present volume contains contributions of 37 authors from 16 countries on many aspects of the synthesis and hormonal control of proteins and nucleic acids. Special attention has been devoted to the problem of nucleic acid and protein synthesis in cell particles and to the role of nucleic acids and proteins in plant development.



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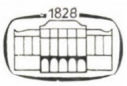

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SECTION I

PLANT NUCLEIC ACIDS

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STUDIES ON SHORT-TIME LABELLED RNAs OF SOYBEAN AND CARROT[1]

by

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In some early studies on RNA metabolism in plants (Ingle et al., 1965), we described a RNA fraction of soybean which was labelled with ^{32}-P-orthophosphate at a rate at least twice that of rRNA, which was polydisperse sedimenting in the range of 10S to greater than 50S on sucrose gradient analysis, which had a short half-life relative to rRNA and which had a base composition giving a GMP/AMP ratio of about 0·6 similar to the dGMP/dAMP ratio of soybean DNA. This fraction of RNA was termed DNA-like or D-RNA, based on the similarity to soybean DNA in base composition and because similar rapidly-labelled RNAs in animal cells were referred to as D-RNA (Soeiro et al., 1966; Darnell, 1968). A relatively small fraction of the D-RNA was isolated in association with polyribosomes (Lin et al., 1966). Since soybean D-RNA was described (Ingle et al., 1965), a large number of laboratories have described similar types of rapidly labelled, AMP-rich RNAs in plants (Chroboczek and Cherry, 1966; Ewing and Cherry, 1967; Key and Ingle, 1968; Johri and Varner, 1970; Leaver and Key, 1967; Loening, 1965; Tester and Dure, 1967). In addition, Ellem and co-workers (1964, 1966) showed that a significant amount of rapidly labelled RNA of animal cells was not eluted from MAK columns by the salt gradient. This type of RNA which remains tightly bound to MAK columns is present in a number of plant systems (Ewing and Cherry, 1967; Johri and Varner, 1970; Key and Ingle, 1968; Tester and Dure, 1967).

In this paper we report on the further characterization of AMP-rich RNAs of soybean, and to a lesser extent carrot, and show that there are at least two distinct classes of AMP-rich RNAs present in short-time labelled ^{32}P-RNA. These can be distinguished from each other and from precursors to rRNA based on fractionation on the MAK column, base composition analysis, acrylamide gel fractionation and sedimentation on sucrose gradients. Preliminary evidence indicates that these two types of AMP-rich RNA have very different half-lives and are differentially associated with polyribosomes.

[1] "D-RNA" and "TB-RNA" are used only to identify the fractions of MAK columns which elute after rRNA in the salt gradient and in SLS, respectively. D-RNA and TB-RNA (without " ") are used to identify two classes of purified AMP-rich RNA as exemplified by D-RNA No. 3 and TB-RNA No. 3 of Tables 2 and 3 and Figs 5 and 6; MAK, methylated albumin-kieselguhr; FU, 5-fluorouracil; G/A, GMP/AMP ratio.

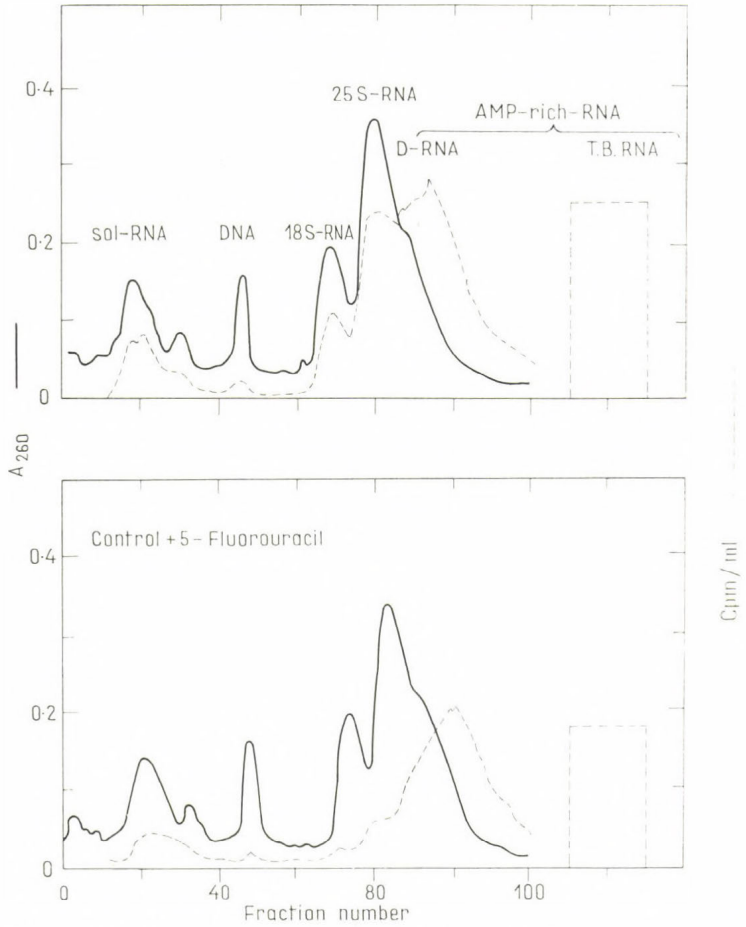

Fig. 1. MAK column fractionation of soybean nucleic acids. Total phenol detergent extracted nucleic acids (Ingle et al., 1965; Leaver and Key, 1970) from control and 5-fluorouracil-treated tissue incubated for 2 h in ^{32}P-orthophosphate were fractionated on MAK columns (Mandel and Hershey, 1960) using a 2-stage linear gradient (150 ml each of 0·4 to 0·8 M NaCl followed by 250 ml each of 0·8 to 1·2 M NaCl). After the salt gradient was completed, the RNA remaining bound to the MAK column ("TB-RNA") was eluted by "stripping" the column with 0·5% SLS. The bulk of the RNA was eluted in a 15 ml volume after a bed-volume of SLS was passed through the column. About 2 mg of carrier rRNA were added to the 15 ml eluate, and NaOAc was added to a final concentration of 0·15 M. An equal volume of phenol containing hydroxyquinoline and m-cresol was added (Leaver and Key, 1970). After vigorous agitation, the sample was centrifuged at 15,000 rpm for 15 min in a Servall type 34 rotor. The RNA was precipitated from the aqueous layer by addition of 2·4 volumes of ethanol or pelleted overnight in a Spinco type 40 rotor.

The RNA eluting in the "D-RNA" region of MAK columns was collected by sedimentation in a Spinco type 40 rotor overnight together with carrier rRNA. The pelleted RNA was dissolved in 0·5% SLS containing 0·15 M NaOAc and precipitated by addition of 2·4 volumes of ethanol.

The "D-RNA" or "TB-RNA" samples were then subjected to rechromatography on MAK columns for two additional cycles. After 3 cycles through the MAK column, the D-RNA and TB-RNA samples were fractionated by acrylamide get electrophoresis and sucrose gradient centrifugation and base compositions were determined for each fraction

Auxin was shown to enhance incorporation of ^{32}P-orthophosphate into rRNA precursors as well as into both types of AMP-rich RNAs. Additionally, auxin increases the proportion of D-RNA relative to TB-RNA in the total population of polydisperse AMP-rich RNAs.

RESULTS

A typical MAK column fractionation of total and ^{32}P-labelled soybean RNA is shown in Fig. 1. In tissue labelled for 2 h a large proportion of the ^{32}-P-RNA elutes in the "D- and TB-RNA" regions, although there is considerable labelled 4, 5, 18 and 25S RNAs by 2 h. Fluorouracil (FU) markedly depresses the accumulation of ^{32}-P-labelled 4, 5, 18 and 25S RNAs without significantly affecting the level of ^{32}-P-AMP-rich RNAs. Figure 2 presents acrylamide gel profiles of control (i.e. auxin-treated) samples of RNA labelled for 15 min (A), 30 min (B) and 60 min (C) and FU-treated samples (D, E, F) labelled for the same times, respectively. Again it is clear that FU blocks the accumulation of 18 and 25S (0·7 and 1·3 M molecular weight, respectively) rRNA while also depressing the

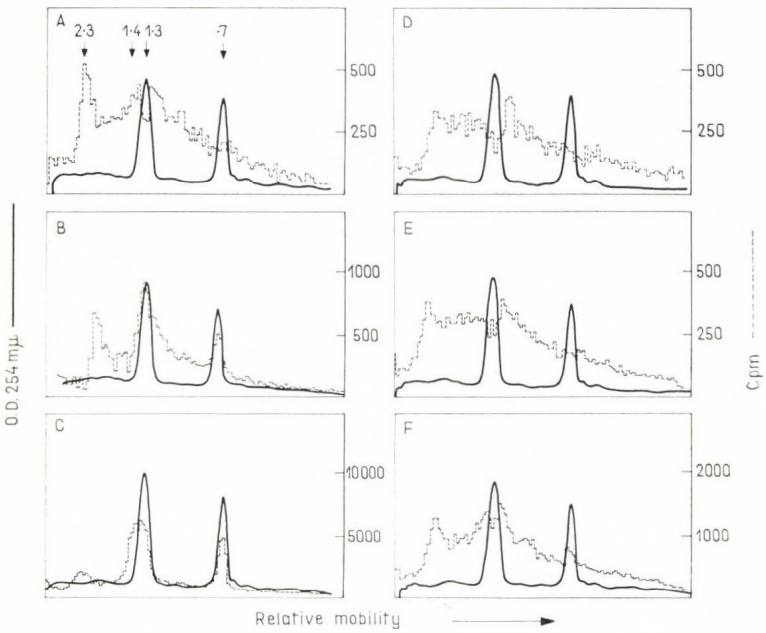

Fig. 2. Acrylamide gel fractionation of soybean nucleic acids. Total phenol detergent extracted nucleic acids from control and 5-fluorouracil-treated tissue incubated for 15 min, 30 min and 1 h in ^{32}P-orthophosphate were fractionated according to Loening (1967) on 2·4% gels at 6 ma/8 cm gel for 3 h (the 4 and 5S RNAs were electrophoresed off the gels under these conditions). The arrows denote the rRNA precursors (2·3 and 1·4 M molecular weight) and mature rRNA (1·3 and 0·7 M molecular weight or 25S and 18S, respectively)

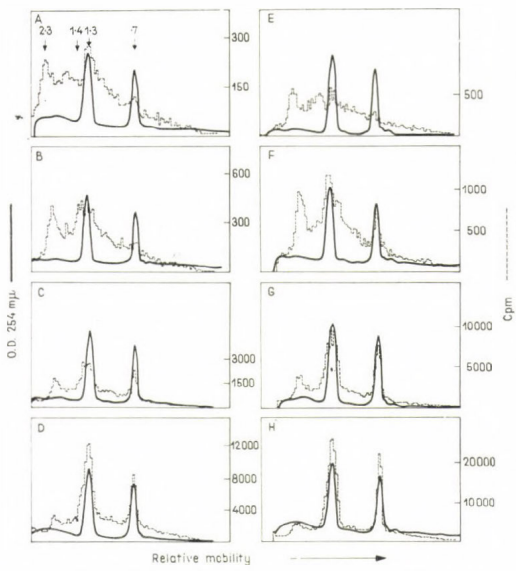

Fig. 3. Acrylamide gel fractionation of ^{32}P-RNA from control and auxin-treated soybean hypocotyl. Total RNA was fractionated as described in Fig. 2. Control tissue (A, B, C, D) and auxin-treated tissue (E, F, G, H) were labelled for 15, 30, 60 and 120 min, respectively

^{32}P-labelled rRNA precursors (Leaver and Key, 1970; Rogers et al., 1970). The bulk of the ^{32}P-labelled RNA in FU-treated tissue is of the polydisperse, AMP-rich type at all times. Auxin enhances the incorporation of ^{32}P into all RNA fractions relative to the control rate (Fig. 3). Thus the incorporation into rRNA precursors is increased as well as into the polydisperse RNAs. Although data are not presented here, in detail, auxin increases the proportion of ^{32}P-AMP-rich RNA present as D-RNA relative to the TB-RNA (compare total tissue "D- and TB-RNA" compositions of Table 6 with data of Table 5).

In the experiments which follow FU was used to depress rRNA synthesis resulting in the bulk of the ^{32}P-RNA being of the AMP-rich type. The FU treatment causes this pattern of RNA synthesis without impairing many biological functions which are dependent upon continued RNA synthesis in several plant systems which have been studied (Chrispeels and Varner, 1967; Key, 1966; Key and Ingle, 1964; Leaver and Key, 1967; Lin and Key, 1968).

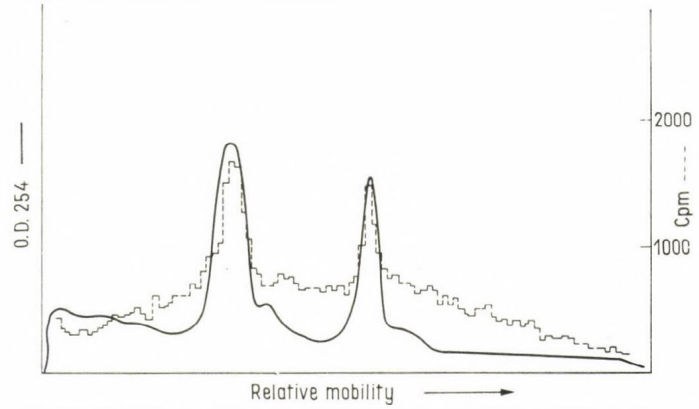

Fig. 4. Acrylamide gel fractionation of the ^{32}P-"TB-RNA". The ^{32}P-"TB-RNA" fraction from 2 h-labelled control tissue (Fig. 1) was fractionated with carrier rRNA under conditions described in Fig. 2

Data presented in Fig. 4 show that the "TB-RNA" fraction of 2 h-labelled tissue (control of Fig. 1) contains considerable 18 and 25S rRNAs in addition to the polydisperse AMP-rich RNA. About 15% of the rRNA remains bound to the MAK column under our experimental conditions. The depression by FU of total ^{32}P-labelled "TB-RNA" (Fig. 1) results primarily from the inhibition of rRNA accumulation in the fraction. So base composition analyses of "TB-RNA" from control tissue would be significantly affected by contaminating rRNA (which has a G/A ratio of about 1·25) until further purified.

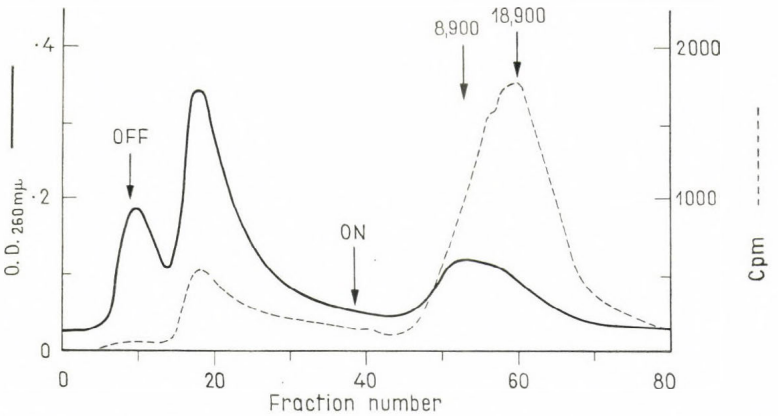

Fig. 5. MAK column fractionation of ^{32}P-"D-RNA". The "D-RNA" fraction from 5-fluorouracil-treated tissue (Fig. 1) was fractionated with carrier rRNA. After sample addition and washing of the column with 0·7 M NaCl, a linear 0·7 to 1·2 M (250 ml each) NaCl gradient was initiated. At the "off" position elution with the "mixing" chamber concentration of NaCl (about 0·9—0·95 M) was continued until the "on" position in the profile was reached. At that point a new gradient of 0·9 to 1·2 M NaCl (150 ml each) was initiated

The primary fractionation of the "D-RNA" region from the 25S rRNA was accomplished as illustrated in Fig. 5. As the 18S rRNA was eluting (arrow off) the salt gradient was maintained at the concentration (about 0·9—0·95 M NaCl) until the 25S rRNA was eluted (arrow on); then a new linear salt gradient was initiated. In this way the normal shoulder in the 25S region (Fig. 1), which represents primarily aggregated 18 and 25S rRNAs on an O. D. basis (Ingle and Key, 1968), and the ^{32}P-labelled "D-RNA" were separated from the majority of the 25S rRNA. Additionally, this fractionation shows that the "^{32}P-D-RNA" is not specifically associated with the major portion of the shoulder of aggregated rRNA as has been suggested by some investigators (Johri and Varner, 1970); rather the ^{32}P-labelled "D-RNA" appears to be associated with the trailing O. D. component of this fraction (note specific activity at O. D. peak and count peak). Normally when "D-RNA" was to be purified by refractionation on MAK columns, a 1·2 M NaCl wash was added at the "arrow on" to elute the crude "D-RNA" fraction in a small volume. The "TB-RNA" fraction was then eluted with 0·5% SLS.

The data of Table 1 show the redistribution of "D-RNA" and "TB-RNA" on subsequent refractionation on MAK columns. The "D-RNA" from column 1 ("D-RNA" No. 1) redistributed with about 75% eluting in the "D-RNA" region ("D-RNA" No. 2) with salt while about 25% remained as "TB-RNA" requiring SLS for elution. Of the 75%, about 80% eluted in the "D-RNA" region (D-RNA No. 3) and about 20% as "TB-RNA" on the third MAK cycle. The "TB-RNA" from column 1 redistributed with about 35% eluting as "D-RNA" and 65% as "TB-RNA" ("TB-RNA"

Table 1

Distribution of ^{32}P-RNA from the "D-RNA" and "TB-RNA" fractions following recycling on MAK columns

Fraction added to column	"D-RNA"	"TB-RNA"	Total recovery
"D-RNA" No.1	76·8	23·2	90
"D-RNA" No.2	79·3*	20·7	87
"TB-RNA" No.1	35·7	64·3	86
"TB-RNA" No.2	26·8	73·2**	91

* This fraction corresponds to D-RNA (No. 3) of Table 2.
** This fraction corresponds to TB-RNA (No. 3) of Table 2.
Data are averages of 3 experiments.

No. 2). Of the 65% eluting as "TB-RNA", about 25% eluted in the "D-RNA" region and 75% as "TB-RNA" on the third MAK cycle (TB-RNA No. 3). Thus the ^{32}P-RNA originally eluting as "D-RNA" continues to preferentially elute with salt in the "D-RNA" region while the "TB-RNA" fraction continues to preferentially remain bound to the column requiring SLS for elution; there appears to be no complete resolution of

Table 2

Base compositions of "D-RNA" and "TB-BNA" fractions of MAK columns after two and three cycles through the MAK column

RNA fraction	Mole %				G/A
	C	A	G	U	
"D-RNA" No.2	21·3	29·6	23·0	26·1	0·78
D-RNA No.3	20·3	29·3	23·4	26·0	0·80
"TB-RNA" No.2	17·9	39·1	19·5	23·5	0·50
TB-RNA No.3	17·2	40·7	19·0	23·1	0·47

The base compositions are based on the distribution of ^{32}P among the four nucleotides from KOH-hydrolyzed RNA which were separated by paper electrophoresis. Hydrolysis was done in 0·3 N KOH for 18 h at 37 °C. Electrophoresis was done at 1000 V for 2 h in 0·05 N ammonium acetate (pH 3·5).

these fractions on MAK columns, but the redistribution data of each fraction suggests that the major ^{32}P-RNA component of each fraction differs significantly from the other.

Base composition analyses of the "D-RNA" and "TB-RNA" fractions from the MAK columns, which gave the ^{32}P-RNA distributions reported in Table 1, are presented in Table 2. The "D-RNA" fraction reached a constant base composition after two cycles through the column with about 56 mole % A + U and a G/A ratio of about 0·8. The "TB-RNA"

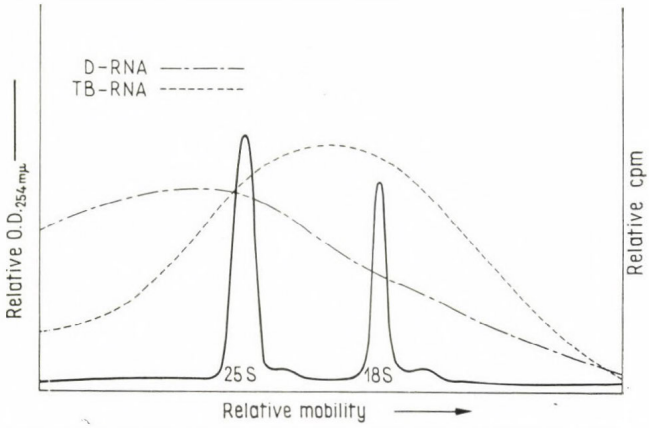

Fig. 6. Acrylamide gel fractionation of purified D-RNA and TB-RNA. Samples of D- and TB-RNA purified through 3 cycles of MAK column fractionation (samples corresponding to No. 3 from Table 2) were fractionated by acrylamide gel electrophoresis as described in Fig. 2. The count profiles are representative of a composite of three independent fractionations of 3 different preparations of purified D- and TB-RNA without considering minor slice to slice variation (100 slices of 0·8 mm each were counted per gel)

fraction appears to be approaching a constant base composition after three cycles through the MAK columns reaching a G/A ratio of about 0·45 to 0·50 for TB-RNA No. 3.

The purified ^{32}P-labelled D-RNA and TB-RNA samples (No. 3 from Table 2) were fractionated by polyacrylamide gel electrophoresis (Fig. 6) and sucrose gradient centrifugation (Fig. 7). Both samples gave a gel profile distribution of great heterogeneity relative to molecular size, but the D-RNA was of a larger average molecular size. As on gels, the purified D-RNA sedimented on sucrose gradients with S values ranging from about 10S to greater than 60S, with some ^{32}P-RNA being pelleted. On the other hand the purified TB-RNA sedimented over a much narrower range with a rather broad band peaking at about 16 to 18S. As with base composition analyses and MAK column fractionation, the purified D- and TB-RNAs (No. 3, Table 2) represent different populations of RNA molecules based on acrylamide gel and sucrose gradient fractionation.

Base composition analyses (Table 3) were made of sucrose gradient fractions of purified D- and TB-RNA corresponding to the letters shown in Fig. 7. The D-RNA fractions gave similar base compositions independent of the molecular size with values for AMP ranging from 29·5 to 30·7 giving G/A ratios of 0·74 to 0·79. The TB-RNA compositions indicate that the fractions collected from the gradient are somewhat different with a decreasing AMP content as the mean molecular weight increases. This result would be expected if the TB-RNA fraction is slightly contaminated with

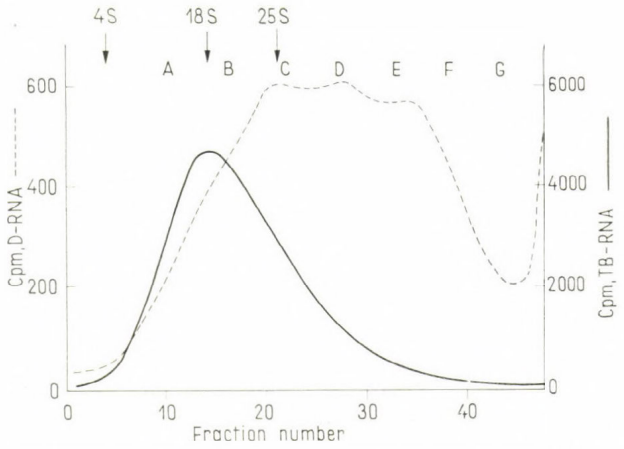

Fig. 7. Sucrose gradient fractionation of purified D-RNA and TB-RNA. Samples of D- and TB-RNA as described in Fig. 5 were fractionated on 5 to 20% linear sucrose gradients containing 0·01 N sodium acetate buffer (pH 6). Centrifugation was for 12 h at 23,000 rpm in a Spinco type SW25 rotor. 0·5 ml fractions were collected. Gradients using different buffers and various additives yielded results similar to those reported for the acetate-buffered gradients

D-RNA (i.e. RNA having a 30% AMP content and a G/A ratio of 0·75—0·80).

The data of Table 4 show that carrot D- and TB-RNAs purified by fractionation through 2 MAK column cycles are similar in base composition to purified D- and TB-RNA of soybean. The two fractions of carrot RNA are also clearly different based on their base composition analyses.

In order to evaluate the relative contribution of D-RNA and TB-RNA (defining these components as those fractions purified through three cycles of MAK columns and having G/A ratios of about 0·8 and 0·4, respectively, No. 3, Table 2) to the total AMP-rich RNA population, RNA was extracted from tissue labelled with ^{32}P-orthophosphate from 30 min to 6 h. The RNA was fractionated on MAK columns, and the base compositions of the "D- and TB-RNA" fractions were determined. The G/A ratios are reported in Table 5. There is a progressive increase in G/A ratio of each fraction with increasing label times. Based on the G/A ratios of purified D- and TB-RNA (Table 2, No. 3), the 30 min label yields a "D-

Table 3

Base composition of purified D-RNA and TB-RNA fractions from sucrose gradients

RNA fraction	Mole %				G/A
	C	A	G	U	
D-RNA No.3	21·2	29·9	22·3	26·6	0·75
A	20·1	29·5	23·2	27·2	0·79
B	20·2	30·2	23·3	26·3	0·77
C	21·2	30·0	23·5	25·3	0·78
D	20·3	29·9	23·7	26·1	0·79
E	21·2	30·6	23·0	25·2	0·75
F	20·6	30·7	22·9	25·9	0·74
G (Pellet)	21·6	29·9	22·3	26·2	0·75
TB-RNA No.3	17·8	41·7	17·7	22·8	0·42
A	16·1	44·5	17·9	21·8	0·40
B	16·9	41·3	18·2	22·2	0·44
C	18·4	38·0	19·8	23·5	0·52

D- and TB-RNA (No. 3, Table 2) were fractionated by sucrose gradient centrifugation and the letters correspond to those fractions noted in Fig. 6.

RNA" fraction which represents about a 1 : 1 mixture of each type while the "TB-RNA" fraction represents largely the TB-type. By 6 h, the "D-RNA" fraction represents ^{32}P-RNA primarily of the D-RNA type while the "TB-RNA" fraction represents close to a 1 : 1 ratio of each type. Using these values (i.e. the G/A ratio of each fraction) and the total amount of ^{32}P-RNA in each fraction, the percent D-RNA (and TB-RNA) at each time was estimated (Table 5). There is a progressive increase in the proportion of D-RNA (and a corresponding decrease in TB-RNA) with increasing time of ^{32}P-incorporation from 25 to 30% at 30 min up to about 75% at 6 h. These results imply that the TB-RNA has a shorter half-life than D-RNA.

Table 4

Base composition of purified ^{32}P-RNA fractions of carrot

RNA fraction	Mole %				G/A
	C	A	G	U	
rRNA (1·3 + 0·7 M)	22·9	25·3	31·2	20·6	1·23
D-RNA*	21·1	31·6	23·7	23·5	0·75
TB-RNA*	18·0	38·3	21·7	22·0	0·57

* The carrot D- and TB-RNA samples were purified through only two cycles of MAK fractionation. Carrot discs were excised and incubated as previously described (Leaver and Key, 1970).

Table 5

The time-course distribution of the total AMP-rich RNA between D-RNA and TB-RNA

Hr in ^{32}P	G/A		D-RNA	TB-RNA
	"D-RNA"	"TB-RNA"	as % total ^{32}P-AMP-rich RNA	
$^1/_2$	0·60	0·42	28	72
1	0·64	0·45	45	55
2	0·75	0·54	58	42
4	0·80	0·58	68	32
6	0·78	0·63	75	25

Total RNA was extracted from FU-treated excised soybean hypocotyl after the indicated times in ^{32}P-orthophosphate following a 2 h-preincubation. The samples of RNA from duplicate experiments were fractionated on MAK columns as described in Fig. 1. The base compositions and total ^{32}P-RNA in the "D-RNA" and "TB-RNA" fractions were determined. From the G/A ratios of each fraction, relative to the G/A ratios of purified D-RNA and TB-RNA (No. 3, Table 2), and the total cpm in each fraction, the percent of the total ^{32}P-AMP-rich RNA present as D-RNA (or TB-RNA) was calculated. In order to make this calculation, the assumption must be made that the total ^{32}P-AMP-rich RNA is composed of a mixed population of ^{32}P-RNA molecules either of the D-RNA type or of the TB-RNA type (No. 3, Table 2). There is some preliminary evidence that there are purine-rich clusters in the total AMP-rich RNA fraction which consist primarily of AMP, but this likely does not affect the above interpretation.

Although "chase experiments" are practically impossible to perform in tissues of this type (Ingle et al., 1965) attempts were made in this direction. The tissue was labelled with ^{32}P-orthophosphate for 30 min; the tissue was then surface washed and placed in a 10^{-3} M KPO_4 (pH 6·0)buffer containing 10 μg/ml actinomycin D. Under these conditions ^{32}P-RNA accumulates at a steadily decreasing rate for about 1 h followed by a decrease in total ^{32}P-RNA (Ingle et al., 1965). The amount of^{32}P-RNA eluting in the "D- and TB-RNA" regions from MAK columns and the respective base compositions were determined. The data were sufficient only to conclude that the TB-RNA has a half-life of 1 h or less while the D-RNA has a half-life of about 4 h. The values certainly are adequate to distinguish between D-RNA and TB-RNA. The composite value from these experiments for D- and TB-RNA is in agreement with the value previously reported for the total AMP-rich RNA of soybean (Ingle et al., 1965).

Preliminary results on the association of D- and TB-RNA with polyribosomes are presented in Table 6. It was previously shown that a relatively small amount of the AMP-rich RNA ("D-RNA") of soybean was polyribosome associated (Lin et al., 1966). The data of Table 6 show that the major proportion of the AMP-rich RNA associated with polyribosomes is of the D-RNA type. Certainly the polyribosome-associated ^{32}P-RNA is greatly enriched in D-RNA relative to TB-RNA compared to the total ^{32}P-AMP-rich RNA.

Table 6

Association of AMP-rich RNA with polyribosomes

Tissue	% of total AMP-rich RNA	Mole %				G/A
		C	A	G	U	
Soybean hypocotyl						
Total tissue						
"D-RNA"	63	19·2	32·3	23·3	25·1	0·72
"TB-RNA"	37	19·4	34·9	22·2	23·8	0·64
Polyribosomes						
"D-RNA"	75	20·6	30·3	24·4	24·8	0·81
"TB-RNA"	25	20·4	29·6	24·8	25·4	0·84
Carrot root						
Polyribosomes	—	19·1	30·3	24·6	25·3	0·81

Soybean hypocotyl was labelled for 45 min with ^{32}P-orthophosphate after a 6-h preincubation in the presence of 5×10^{-5} M Auxin and FU. Auxin was used in these experiments in order that a much larger percentage of the ribosomes would be present as polyribosomes. Auxin alters the distribution of ^{32}P-RNA present as D- or TB-RNA when compared to control tissue (see Table 5). ^{32}P-RNA was prepared from total tissue and from polyribosomes isolated from comparable tissue. The RNA was then fractionated on MAK columns; the total ^{32}P-RNA (cpm) and the base composition analysis of the "D-RNA" and "TB-RNA" fractions were determined. The "TB-RNA" fraction from polyribosomes clearly contains RNA representative of D-RNA (No. 3, Table 2) and not of TB-RNA (No. 3, Table 2). Further the MAK distribution of the polyribosome-associated AMP-rich RNA between the "D- and TB-RNA" fractions is that expected of purified D-RNA (see Table 1). The base composition is reported for polyribosome-associated ^{32}P-RNA of carrot discs following a 20 min exposure to ^{32}P-orthophosphate at which time all of the ^{32}P-RNA is of the polydisperse type (Leaver and Key, 1970), and clearly representative of D-RNA.

DISCUSSION

The data presented here show that there are at least two distinct types of rapidly-labelled, AMP-rich RNAs in soybean (and in carrot). Purified D-RNA has the following properties: 1) the base composition gives an A + U value of about 56 mole % and a G/A ratio of about 0·8, 2) the half-life is about 4 to 5 h, 3) the size distribution is very heterogeneous in sedimentation on sucrose gradients and acrylamide gel fractionation, 4) a few percent is associated with ribosomes in the polyribosome structure and 5) it is labelled with ^{32}p-orthophosphate at a greater rate than rRNA but slower than TB-RNA. Purified TB-RNA has the following properties: 1) the base composition gives an A + U value of about 63 to 65 mole % and a G/A ratio of about 0·4 to 0·45, 2) the half-life is shorter than that of D-RNA, being about 1 h (or less), 3) TB-RNA is heterogeneous in size but has a smaller mean size distribution than D-RNA, 4) it appears not to be polyribosome-associated in a significant amount and 5) it is labelled with precursor at about three times the rate of D-RNA labelling.

While being similar to corresponding fractions of short-time labelled ^{32}P-RNA from other plant tissues (Ewing and Cherry, 1967; Johri and Varner, 1970; Tester and Dure, 1967), there are some differences. These

differences may well be the result of the fact that what we are referring to as D-RNA and TB-RNA are those fractions of ^{32}P-RNA which are purified through three cycles of MAK column fractionation to or approaching homogeneity in base composition, while this was not done in most other studies.

The TB-RNA of soybean appears similar to that reported for pea by Ewing and Cherry (1967) where purification was extensive but different from that reported for pea by Johri and Varner (1970) except for their 1 h-label data and Tester and Dure for cotton (1967) where either little if any additional purification was attempted or where the labelling time was very long. In both cases some ^{32}P-rRNA and D-RNA would be present in the "TB-RNA" fraction and would significantly affect the base composition analyses. In addition, by 16 h based on our results, the proportion of D-RNA (No. 3) to TB-RNA (No. 3) would lead to a "TB-RNA" fraction similar to D-RNA (No. 3) as observed by Johri and Varner (1970). There is a large difference in the apparent half-life of D-RNA in pea and soybean. Johri and Varner (1970) report a "D-RNA" ("m-RNA" in their paper) half-life of about 15 min for pea while the value for soybean is certainly in the range of 4 to 5 h (these half-life values would be at best approximations because of the difficulty in doing chase experiments in plant systems). The only ^{32}P-RNA which is "chased" by ^{32}P-orthophosphate and actinomycin D in our soybean and carrot in this short a time are the precursors to rRNAs (Leaver and Key, 1970) which are processed into mature rRNA with a half-life of minutes (Leaver and Key, 1970 and unpublished observations). Since precursors to rRNA elute in the "D-RNA" region of MAK columns and since the column can be run under conditions which cause the bulk of D-RNA to elute as "TB-RNA", it may well be that these factors account for the apparent short half-life of "D-RNA" in the experiments of Johri and Varner (1970). These half-life differences might also represent species differences. Additionally, TB-RNA which has a shorter half-life in soybean than D-RNA, was reported not to chase by Johri and Varner (1970). Yet in their Fig. 8 the "TB-RNA" fraction appears to chase as fast or faster than their "m-RNA" fraction (corresponding to soybean D-RNA) while in their Fig. 9 the "TB-RNA" continues to accumulate label while the "m-RNA" chases. As pointed out by Johri and Varner (1970) the discrepancy between data of Figs 8 and 9 is not clear as is the case for the apparent loss of counts from rRNA in their studies. The apparent 4 to 5 hr half-life of soybean D-RNA, as reported here, a part of which is polyribosome-associated, is consistent with the rate of decay of protein synthesis (as measured by ^{14}C-amino acid incorporation by tissue slices) and the decay of polyribosomes which show a half-life of about 4 h in soybean following addition of actinomycin D (unpublished data).

Although only limited data are presented here on the influence of auxin on short-time labelled RNAs, it is evident that auxin treatment leads to an enhancement of the rate of ^{32}P-incorporation into polydisperse AMP-rich RNAs and into rRNA precursors. The latter is consistent with the large increase in ribosome level which occurs following auxin treatment. There may well be control mechanisms (e.g. at the level of processing and/or turnover of the precursors), however, other than rate of synthesis of rRNA precursors which lead to the large accumulation of ribosomes in auxin-

treated relative to control tissue. Based on base composition analyses and size distribution on acrylamide gels (unpublished data of Key), auxin increases the amount of D-RNA relative to TB-RNA in the total population of AMP-rich RNAs. This change in relative amounts of D- and TB-RNA supports and extends the results of Tester and Dure (1967) which showed a different base composition of "TB-RNA" from control and auxin-treated *Avena* coleoptile.

The apparent association of D-RNA (in our experiments) and of RNAs similar in base composition from other systems with polyribosome preparations has been observed in several studies of plant ribosomes (Johri and Varner, 1970; Lin et al., 1966; Loening, 1965). The specificity of this association has not been adequately assessed in these studies. While it appears highly probable that the polyribosome-associated D-RNA is in fact m-RNA, work with polyribosome-associated short-time labelled RNAs and ribonucleoprotein particles in animal systems (Baltimore et al., 1970; Henshaw, 1968, 1970; Infante and Nemer, 1968; Lee and Brawerman, 1971; Olsnes, 1970; Penman et al., 1968; Perry and Kelley, 1968) points out the difficulty in interpreting such data. In addition, our data show an enrichment of D-RNA relative to TB-RNA in polyribosome preparations relative to total tissue of both soybean and carrot. The data in fact are suggestive that TB-RNA may not normally be polyribosome-associated. Based on the extraction properties of this AMP-rich RNA (Ingle et al., 1965) and the unique nuclear RNAs of animal cells (Darnell, 1968; Shearer et al., 1967), it may well be that the TB-RNA is in the nuclear fraction in plants. There are several recent reports of adenine-rich clusters in RNAs of animal cells (Darnell et al., 1971; Edmonds et al., 1971; Hadjivassilion and Brawerman, 1967, Lim and Canellakis, 1970; Lim et al., 1970; Penman et al., 1968). Although the significance of this component is unknown, (Sussman, 1970) and others have similarly speculated about the function (Darnell et al., 1971; Edmonds et al., 1971; Penman et al., 1968). Experiments are underway to evaluate the possible presence of adenine-rich clusters in soybean AMP-rich RNAs by the methods of Lim and Canellakis (1970) and of Lee et al. (1971). Preliminary results indicate the presence of AMP-rich clusters in soybean polyribosomal RNA similar to those reported by Lim and Canellakis for reticulocytes (1970) and Lee et al. (1971) for Mouse Sarcoma ascites cells.

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RAPIDLY REASSOCIATING DNA IN JERUSALEM ARTICHOKE RHIZOMES AND AUXIN-TREATED EXPLANTS

by

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INTRODUCTION

The presence of repeated sequences of DNA in the genome of eucaryotes has been demonstrated by studying the reassociation kinetics of complementary strands that have been sheared to a suitable size. Such DNA, detected by its relatively rapid reassociation, has been found in almost all species higher than fungi examined so far. It can constitute an enormous portion (from 20 to 80%) of the total DNA. From an analysis of reassociation as a function of DNA concentration and time it has been deduced that families of repeated or closely related sequences occur in the genome of a cell and that within a particular family the frequency of repetition can vary from 50 to as much as 2,000 000 copies (Britten and Kohne, 1966; 1967; 1968; 1969*a*; Waring and Britten, 1966).

The function of this DNA is at present obscure. Though it has sometimes unjustifiably been referred to as redundant or even "nonsense" DNA, a certain evolutionary persistence of the large families clearly implies far more importance than hitherto presumed. Unlike the unique single-copy DNA which codes for proteins, repetitive DNA is apparently not translated, but there is ample evidence that it is transcribed into RNA (Britten, 1969). At least one recent hypothesis (Britten and Davidson, 1969) assigns a predominant role to these sequences in the complex regulatory mechanisms of higher organisms.

In what follows we shall describe some of our preliminary attempts to study the rapidly reassociating DNA of higher plant tissues during the course of growth and differentiation. We have chosen the rhizome of the Jerusalem artichoke as a typical resting tissue and compared its DNA with that of explants of the same tissue induced to develop callus growth *in vitro* by auxin.

MATERIALS AND METHODS

Rhizomes of Jerusalem artichoke (*Helianthus tuberosus* L. var. Violet de Rennes) were uprooted in October, at the beginning of winter rest, and stored in plastic bags at 4 °C for subsequent use. Explants were made on an agar medium containing Heller's inorganic salts, 4% glucose and 5×10^{-6} *M* α-naphthaleneacetic acid as auxin and left for two days in culture.

The required milligram quantities of highly purified DNA were extracted from the two tissues, resting rhizomes and auxin-stimulated explants, by a recent technique (Jeannin et al., 1971). It involves disruption in

sodium lauryl sulfate and diethyl pyrocarbonate, removal of polysaccharides by 2-methoxyethanol, selective precipitations with cetyl trimethyl ammonium bromide and with isopropanol, RNase treatment and final preparative column chromatography on hydroxyapatite.

Reassociation kinetics were determined following the standard procedure as recommended by Britten and Kohne (1966). DNA in dilute saline-citrate was sheared at 40,000 psi, concentrated by lyophilization and then transferred to 0·12 M phosphate (pH 6·8) by passing through Sephadex G-25 equilibrated in the same buffer. Suitable concentrations were measured by the absorbance at 260 nm, the samples sealed in ampules, boiled in a water-bath for 15 min and incubated at 60° for various periods of time. C_0t values as defined (Britten and Kohne, 1966; Britten, 1967) were used to plot logarithmically the complex function of concentration and time. For practical purposes we considered that 2 A_{260} units/ml incubated for 1 h gives a C_0t of 1. The extent of reassociation was measured by separating single-stranded from double-stranded DNA on hydroxyapatite columns (1·5 ×1·5 cm) equilibrated in 0·12 M phosphate at 60 °C. Single-stranded DNA is not retained, while double-stranded DNA is eluted by increasing the molarity to 0·4 M. The quantity of DNA in each of the two fractions was estimated at 260 nm and reassociation expressed in terms of the quantity of double-stranded DNA as a percentage of total DNA recovered from the column.

RESULTS AND DISCUSSION

The accompanying figure shows the comparative reassociation kinetics of the two DNA's determined under the same conditions. Both curves are very heterogeneous, showing the presence of several families of repeated

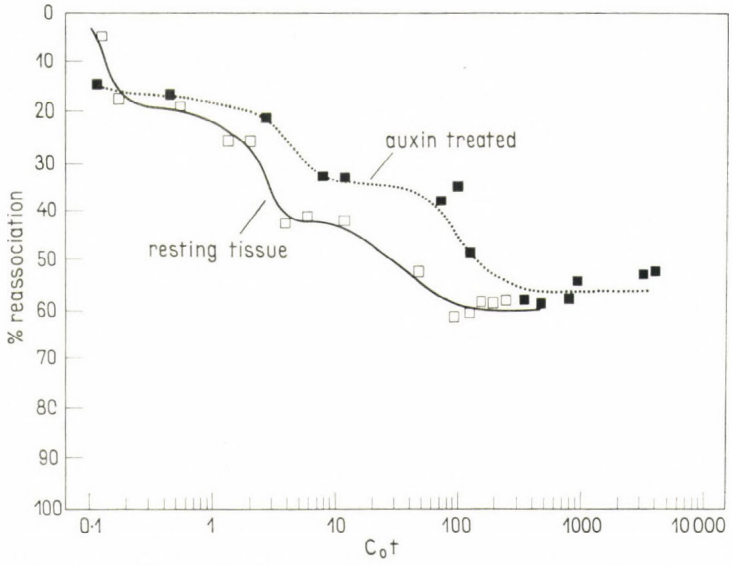

Fig. 1. Reassociation kinetics of DNA extracted from rhizomes and from auxin-treated explants (see under Methods)

DNA sequences as would be expected of any eucaryote (Fig. 1). However, significant differences in the DNA of tissues appear following the activation of growth by auxin.

We do not yet have adequate experimental data to state with any precision that particular families appear or disappear. Indeed, the fact that such modifications could arise is, by itself, quite surprising since families of repeated sequences are believed to arise by saltatory replication during the evolutionary history of a species (Britten and Kohne, 1969*b*). So far there has been no indication that the physiological state of a tissue or the onset of differentiation could be reflected in such a change, although it can be imagined that eventual polyploidy or gene amplification may perhaps be discerned if the sensitivity of the method permits it.

Repetitive DNA has been attributed a role in the regulation of higher organisms (Britten and Davidson, 1969) but the corresponding modulation is held to occur through transcription, since experimental evidence now available only shows that different repeated sequences are transcribed in different tissues of the same species. Modulation of the DNA itself is not usually mentioned in polite biological society; it would understandably involve the concept of a "metabolic" DNA that features a certain turnover. For instance, in the scheme proposed by Britten and Davidson one may entertain the idea that the sensor genes could be amplified under given circumstances. Would it therefore be so far-fetched since these specific sequences are supposed to be the binding sites of inducing agents and hormones of which auxin is the outstanding example in the system we have adopted?

SUMMARY

Growth induction by auxin in explants of Jerusalem artichoke tissue is accompanied by a modification in the reassociation kinetics of the DNA.

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RAPIDLY LABELLED DNA-RNA COMPLEX IN RADISH SEEDLINGS

by

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The occurrence of rapidly labelled DNA-RNA complexes in plants has already been reported (Cherry, 1964). Since the complex appears to be present in a wide variety of plant material, including storage tissues, hypocotyles, and unicellular algae (Cherry, 1964; Ingle et al., 1965; Galling and Richter, 1966), its occurrence does not seem to depend on cell division. Some authors have suggested that the occurrence of DNA-RNA complex in plant material might be due to bacterial contamination (Hock, 1967). Although it is difficult to rule out entirely this possibility, in most cases bacterial contamination does not seem to explain the experimental findings (Julien, 1971). In the root, the DNA-RNA complex appears in the phase of cell elongation and maturation, which follows the first cell divisions taking place during gemination. The physico-chemical and biological properties of the complex suggest that it probably plays a role in the initiation of RNA synthesis which is highly active in this period of development.

ISOLATION OF THE COMPLEX

By MAK chromatography of a total nucleic acid extract from 48-hour-old radish seedlings incubated for 2 hours in ^{32}P, one can observe that a radioactive nucleic acid is eluted somewhat before the DNA peak (Fig. 1). The DNA peak itself is made up not exclusively of DNA. Degradation tests (Table 1) show the presence of a radioactive complex containing both RNA and DNA. This complex appears to be a real hybrid molecule because the radioactivity contained by the RNA can be solubilized by RNase only after thermal denaturation of the complex.

Three fractions can be distinguished in the region of the complex: (a) a DNA fraction, (b) a DNA/RNA hybrid and (c) an RNA fraction which is not hybridized with DNA.

The relative amounts of the three fractions are not constant as shown by the kinetics of degradation by nucleolytic enzymes (Fig. 2).

In seedlings incubated for 30 minutes in a solution of ^{32}P the ratio of newly synthesized RNA/DNA was 1/1. Approximately half of the RNA and DNA was present in hybrid form. With longer incubation times the DNA/RNA ratio increased and the extent of DNA-RNA hybridization decreased markedly.

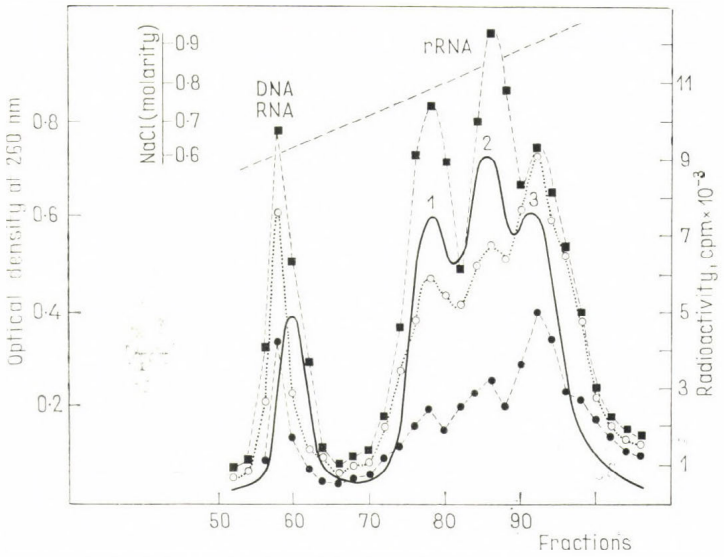

Fig. 1. Chromatographic separation of nucleic acids from 48-hour-old radish seedlings. 50 seedlings were incubated for various times in a solution of ^{32}P (100 μCi/ml). The nucleic acids extracted (approx. 4 mg) were analysed by MAK column chromatography. The nucleic acids were eluted by a NaCl gradient (0·3 M—1·3 M) at pH 8·5. 3·5 ml fractions were collected. Nucleic acids eluted at NaCl concentrations lower than 0·6 M are not shown in the figure. ● - - ● - - ●; 30 min incubation; ○ - - - ○ - - ○, 2 h incubation; ■ - - ■ - - ■, 2 h incubation and 2 h chase

Table 1

Enzymatic and alkaline hydrolysis of the ^{32}P labelled DNA-RNA complex isolated by MAK column chromatography (2 h incubation)

Treatment	Acid precipitable radioactivity in cpm/O. D. units	% degradation
Control	2894	—
RNase	2171	25
RNase + RNase T_1	2180	25
RNase, denaturation at 100 °C and new RNase treatment	1852	36
DNase	1042	64
KOH	1845	36

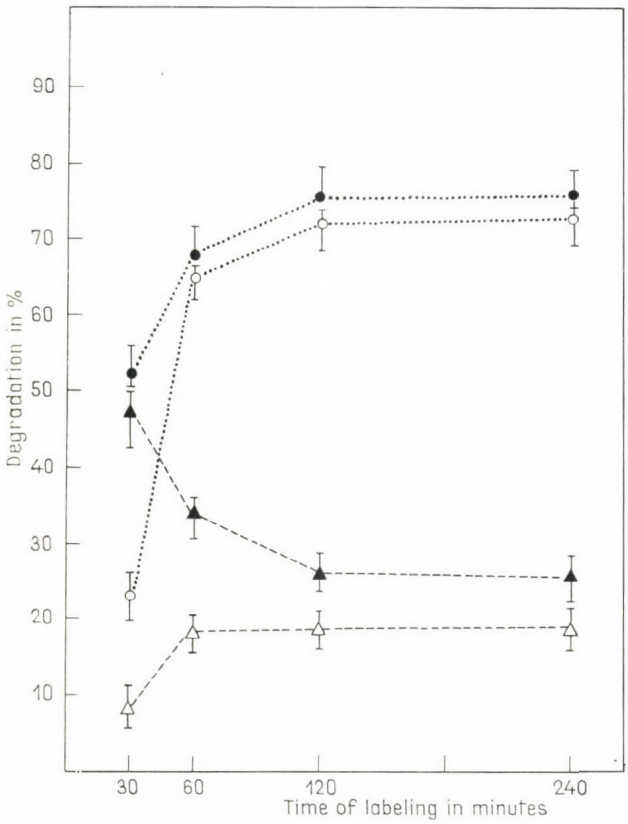

Fig. 2. The sensitivity of the DNA-RNA complex towards RNase and DNase as a function of time. The DNA-RNA complex of radish seedlings, isolated by MAK column chromatography, was exposed to the action of pancreatic RNase and DNase after thermal denaturation. The seedlings were incubated in a solution of ^{32}P (100 μCi/ml). ○ - - ○ - - ○, DNase; ● - - ● - - ● DNase after thermal denaturation; △ - - △ - - △, RNase; ▲ - - ▲ - - ▲, RNase after thermal denaturation

PHYSICO-CHEMICAL PROPERTIES OF THE RNA

When the RNA in the complex is hybridized to a large extent, it has a high guanine content. This means that the DNA of the hybrid is exceptionally rich in cytosine. By contrast, when the radioactivity is chased, the RNA of the complex becomes highly enriched in UMP (Table 2).

The DNA-RNA hybrids obtained "in vitro" as well as "in vivo" remain annealed at high ionic strength. Thus, the DNA-RNA hybrid was examined by density gradient centrifugation in Cs_2SO_4 (Fig. 3).

In a cesium sulfate gradient of a medium density of 1·535 the nucleic acids are separated into two bands: one consisting of RNA, located at

higher densities, and another consisting of DNA, located close to the top of the gradient. One can see (Fig. 3) that the radioactive fraction eluted from the MAK column at a NaCl concentration of 0·6 M separates in the gradient into two bands. The major part of the radioactivity remains associated with the DNA peak the position of which was determined by measuring UV absorption. When nucleic acids treated with RNase had been subjected to ultracentrifugation (Fig. 3b) the second band which sediments at a density higher than DNA disappeared. Furthermore, when the treatment with RNase had been carried out after thermal denaturation the specific activity of DNA decreased (Fig. 3c) indicating that RNA had been associated very strongly with DNA during centrifugation in cesium sulfate, i.e. the nucleic acid in question is DNA-RNA hybrid. The nucleotide composition of the hybrid fraction, as assayed by alkaline hydrolysis, is characterized by high guanine content, which is, however, lower than the guanine content of the free RNA (Table 3).

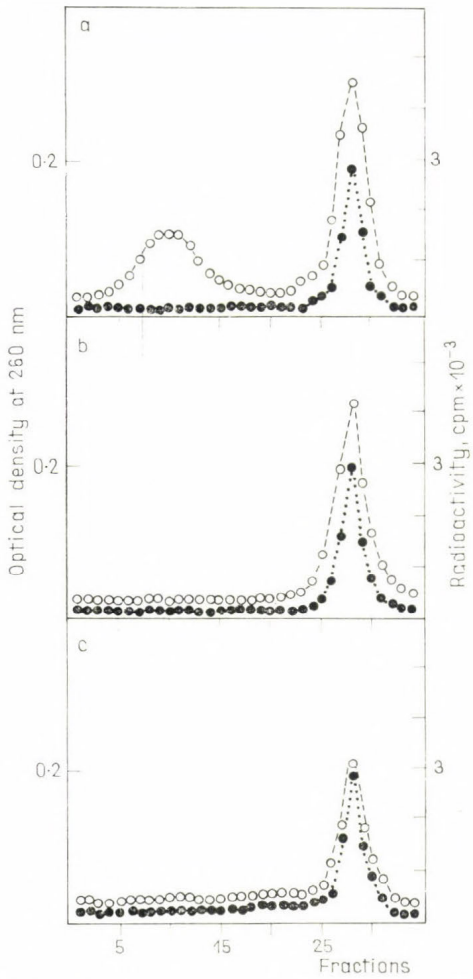

Fig. 3. Cs_2SO_4 density gradient centrifugation (average density 1·535) of the DNA-RNA complex isolated from a MAK column. 48-hour-old seedlings were incubated with ^{32}P (100 μCi/ml) for 2 h. The DNA-RNA complex was separated from the rest of nucleic acids by MAK column chromatography. Centrifugation for 60 h at 37,500 rpm, 20 °C, rotor No. 50 in a Spinco preparative ultracentrifuge. (a) Before treatment with RNase (b) after treatment with RNase. (c) After heat denaturation and treatment with RNase, ○ - - ○ - - ○, radioactivity; ● - - ● - - ● - - ●, absorbance

PHYSICO-CHEMICAL PROPERTIES OF THE DNA

The DNA of radish seedlings can be separated into two components by isopycnic centrifugation in a cesium chloride gradient. The major component has a buoyant density of 1·696, whereas the minor component has a density of 1·720. When after pulse labelling of the seedlings with ^{32}P the DNA of the complex and the rest of the DNA had been separated by MAK column chromatography and compared by cesium chloride density gradient centrifugation, we found that the radio-

activity cosedimented with the satellite DNA (1·720), whereas the rest of the DNA remained unlabelled (Fig. 4). This means that the synthesis of the DNA of the complex proceeds independently from that of the bulk of DNA. With other words, the synthesis of the complex is not coupled to cell division.

Table 2

Distribution of ^{32}P among the monoribonucleotides of the RNA of the DNA-RNA complex

Treatment	Labelling, h	Chase, h	AMP	CMP	GMP	UMP
Before RNase	1	—	10·4	9·1	68·3	12·2
	2	—	10·8	8·2	67·1	13·9
	2	1	17·3	21	37·6	24
	2	3	15·6	17·3	27·3	39·6
After RNase	2	—	13·8	10·4	47·3	28·5

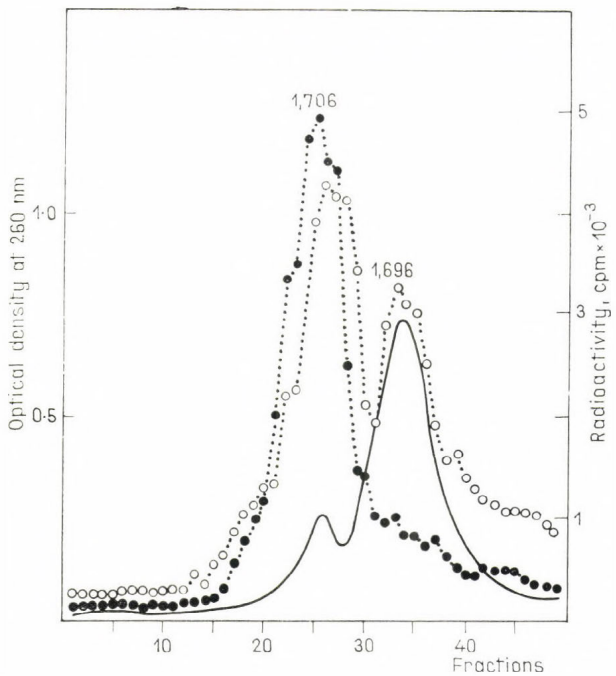

Fig. 4. Centrifugation in a preparative cesium chloride gradient of the DNA of tissues and the DNA of the complex. The DNA of the radish seedlings, after incubation in a solution of ^{32}P, was extracted according to Marmur (1961). Centrifugation at 25,000 rpm in the rotor No. 30 of the Spinco preparative ultracentrifuge at 25 °C for 96 h. ———, absorbance; ● - - ● - - ● - - ●, incubation for 2 h in ^{32}P (100 μCi/ml); ○ - - ○ - - ○, 14 h incubation in ^{32}P (100 μCi/ml)

Table 3

Distribution of ^{32}P among the monoribonucleotides of RNA after gradient centrifugation on Cs_2SO_4 gradient

	AMP	CMP	GMP	UMP
RNA associated with DNA	14·1	10·2	46·9	28·8
RNA not associated with DNA	13·5	8·0	76·9	1·5

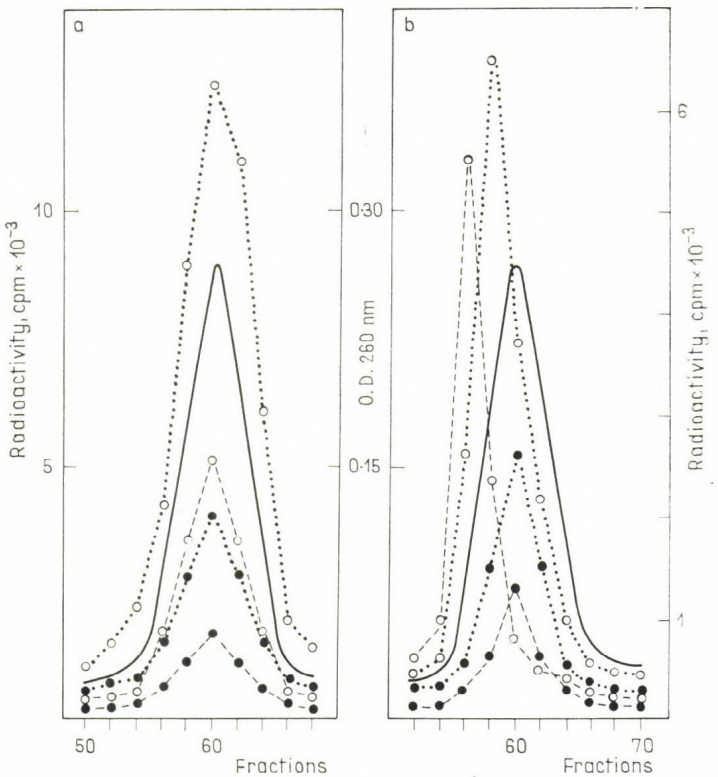

Fig. 5. Chromatographic separation of the bulk of the DNA of the tissues and of the DNA of the complex at various stages of germination. (a) Thymidine ^{3}H; only the chromosomal DNA incorporates this precursor. (b) Phosphate ^{32}P. First phase of germination (0—12 h): ● - - ● - - ● - - ●, 3 h imbibition in the presence of 50 μCi ^{3}H or 2 mCi ^{32}P; ● · · ● · · ● · · ●, 6 h imbibition in the presence of 50 μCi ^{3}H or 2 mCi ^{32}P. Second phase of germination (48 h): ○ - - ○ - - ○ - - ○, 2 h incubation in the presence of 1 μCi ^{3}H or 100 μCi ^{32}P; ○ . . ○ . . ○ . . ○, 14 h incubation in the presence of 1 μCi ^{3}H or 100 μCi ^{32}P; ————, absorbance. Gradient elution, 0·3 M—3·0 M NaCl, pH 8·5

Table 4

Base composition of the bulk of DNA of the tissues and that of the DNA of the complex (enzymatic hydrolysis)

	DNA of the tissues		DNA of the complex
	Deproteinization		
	Chloroform	Phenol	Chloroform
A	30·9	32·9	21·2
T	30·9	16·3	19·8
G	19·1	36·8	26·4
C	16·2	14·0	32·6
5MeC	2·9	—	—
GC	38·2	50·8	59·0
A/T	1	2·0	1
G/C	1	2·6	0·8

Number of experiments: 2.

The buoyant density of the DNA of the complex is significantly higher than that of the bulk of DNA. This is probably due to a higher G + C content. The determination of the base composition of the DNAs, indeed, revealed a markedly higher G+C content (59 %) for the DNA of the complex

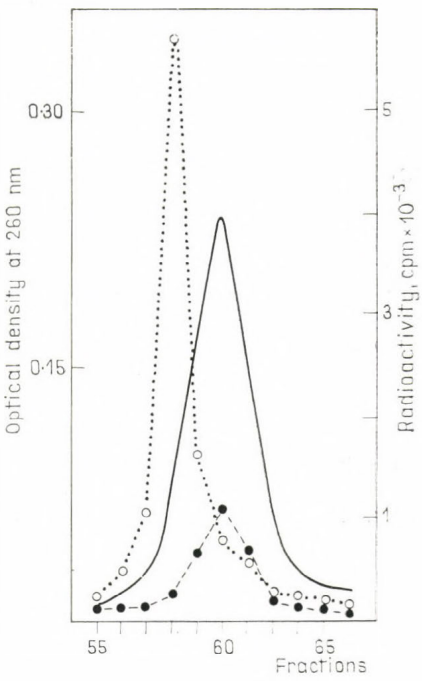

Fig. 6. MAK column chromatography of the DNA-RNA complex after double labelling for two hours with uridine-^{14}C and thymidine-^{3}H. Gradient elution, NaCl 0·3 M—1·3 M, pH 8·5. The optical density of each fraction was determined at 260 nm and the radioactivity of ^{3}H and ^{14}C was measured selectively in aliquot samples. ———, optical density at 260 nm; ○ - - - ○ - - - ○, radioactivity of ^{14}C, ● - - - ● - - - ●, radioactivity of ^{3}H

as compared to the bulk of the DNA of the tissues (38%). Also, the DNA of the complex was shown to contain less methylcytosine (Table 4).

The two different DNAs also differ in the nature of their precursors. Whereas the bulk of DNA, under normal conditions, incorporated thymidine (Fig. 5), the DNA of the complex utilized uridine as a precursor. The label appeared in the cytosine and thymine of the DNA molecule. Thymidine was not utilized as a precursor. By contrast, uridine was not a precursor of the bulk of DNA (Fig. 6).

It seems that the utilization of uridine as a precursor is closely associated with the polymerization of the DNA molecule of the complex. Otherwise, the bulk of DNA should have incorporated the ^{14}C label of the uridine as well.

The results described (presence of dC-rich zones in the DNA of the complex, the demonstration dC-rG hybrids, as well as the special type of metabolism of pyrimidine precursors) underline the importance of pyrimidine sequences in the DNA of the complex. This is confirmed by the investigation of the effect of phenol on the complex. In fact, this deproteinizing agent induces a selective loss of cytosine and thymine from the DNA of the complex. The new complementarity of the bases observed (Table 4) suggests that the pyrimidine bases of the DNA of the complex are organized in special chain sections. These results, which are in line with those obtained by Skinner and Triplett, (1967) on the satellite poly d(AT) of crab, suggest that the DNA of the DNA-RNA complex isolated from radish seedlings also exhibit a special structure.

CONCLUSIONS

In the cells of radish seedlings, during differentiation, two different kinds of DNA syntheses take place: (a) DNA synthesis in the cells, which corresponds to cell replication and precedes cell division, (b) DNA synthesis in the absence of cell division, a process which seems to correspond to the amplification of particular zones of the "normal" tissue DNA responsible for transcription. This particular DNA, called "metabolic DNA" by some authors, appears as a DNA-RNA complex in the cells of radish seedlings.

The synthesis of the DNA of the complex appears to depend on a DNA polymerase different from the enzyme synthesizing "normal" tissue DNA. Both the pool of precursors (particularly that of the pyrimidines) and the template utilized might be different. It has been shown (Spiegelman et al., 1970) that a dC-rG type hybrid is able to direct very effectively the synthesis of DNA. Under these conditions methylation and amination can play a role in the (qualitative and quantitative) regulation of information transfer. Apparently, the presence of DNA-RNA complexes is not restricted to the plant kingdom. DNA-RNA complexes of a similar nature have been described also from He La cells (Szapary-Winckelmans et al., 1968).

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TURNIP YELLOW MOSAIC VIRUS-RNA SYNTHESIS IN THE PLASTIDS: PARTIAL PURIFICATION OF A VIRUS-SPECIFIC, DNA-INDEPENDENT ENZYME-TEMPLATE COMPLEX

by

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PROPERTIES OF THE CELL-FREE PREPARATION "100 P_2 10,000" IN THE SYNTHESIS OF RNA *IN VITRO*, AND NATURE OF THE PRODUCTS FORMED

Chinese cabbage (*Brassica chinensis* L.) infected with turnip yellow mosaic virus (TYMV) has been one of the very first plant-systems with which viral RNA-synthesis could be studied *in vitro* (Bové et al., 1965; Bové, 1966; Ralph and Wojcik, 1966). In the last few years most of our work has been done with a cell-free preparation from Chinese cabbage leaves, 100 P_2 10,000, described previously (Bové et al., 1965). It is derived from a filtered leaf-homogenate and contains essentially the cell-constituents which sediment between 100 and 10,000 g. The filtrate is first centrifuged at 100 g; the supernatant is then submitted to 10,000 g for 15 min. The resuspended pellet is taken up with pH 9 buffer (Bové et al., 1965) and centrifuged at 10,000 g; the resuspended pellet, brought up with pH 9 buffer to one tenth of the volume of the initial filtrate, represents the cell-free preparation 100 P_2 10,000. We have carried out most of our work with such preparations because practically all the RNA-synthesizing activity present in the initial leaf-homogenate ends up in 100 P_2 10,000.

The RNA-synthesis is measured by incubating the cell-free preparation in the presence of a reaction mixture containing the four ribonucleoside-5'-triphosphates one of which, GTP or UTP, is labelled with ^{32}P in the alpha-phosphate, a phosphorylating system, PEP + pyruvate kinase, magnesium and potassium ions, beta-mercaptoethylamine, and Tris-HCl buffer (Bové et al., 1965; Bové et al., 1968). The reaction is stopped with trichloroacetic acid (TCA) at a final concentration of 10%, and the radioactivity of the washed acid-insoluble precipitate is determined. Prior to the addition of TCA, the reaction mixture can be diluted with SSC buffer (1·0 ×SSC is 0·15 M NaCl + 0·015 M Na-citrate, pH 7), and the ribonuclease (RNase) resistance of the synthesized RNA can be determined.

Two RNA synthesizing activities have been detected in the course of this work. The first can be inhibited to less than 10% by the presence of DNase I or actinomycin-D in the reaction mixture, and represents DNA-dependent RNA-polymerase activity; as expected, the product of the reaction is single-stranded RNA, entirely sensitive to pancreatic or T_1 ribonuclease even when the RNase test is carried out at high ionic strength (1·0 ×SSC). The RNA-polymerase activity is present as well in the cell-free preparation obtained from healthy Chinese cabbage leaves, 100 P_2 10,000 (H), as in that from TYMV-infected leaves, 100 P_2 10,000 (V). The DNA-template required for the RNA-polymerase activity is part of the cell-free preparation 100 P_2 10,000 (H) or (V); no increase in activity is obtained by the addition of DNA to the reaction mixture. The RNA-polymerase

molecules must thus be bound to chromatin, which can, indeed, be shown to be present in 100 P_2 10,000 after staining with the Feulgen or Brachet reagent (Bové, 1967*a*).

The second RNA-synthesizing activity which has been studied is only present in the cell-free preparation 100 P_2 10,000 (V), isolated from TYMV-infected Chinese cabbage leaves. It could be detected because, contrary to the RNA-polymerase activity, it is not inhibited by the presence of DNase I or actinomycin D in the reaction mixture. The RNA-product of this DNA-independent reaction has been studied extensively (Bové, 1967*a* and *b*; Bové et al., 1969; Guschlbauer et al. 1968; Dupont et al., 1971). After phenol treatment of the reaction mixture at the end of the incubation, the nucleotidyl residues incorporated by the DNA-independent enzyme-system into RNA can be shown to be part of the TYMV-specific double-stranded RNA (dsRNA). Specific dilution experiments have shown that at least 80% of the labelled product is RNA of the viral or "plus" type (Bové, 1967*a* and *b*). Optical properties of the dsRNA have been determined (Guschlbauer et al., 1968). The double-stranded structures have a length of 1·95 μ, a sedimentation coefficient of 17·0 S, a molecular weight of $4{\cdot}1 \times 10^6$ daltons, twice that of the single-stranded viral RNA and they have a helical structure.

It can be shown that the cell-free preparation 100 P_2 10,000 (V) contains a great proportion of the "minus", TYMV-RNA complementary, strands present in the whole leaves. Since the product of the *in vitro* DNA-independent reaction is RNA of the "plus" or viral type, and since for activity no "minus" strands need to be added to the reaction mixture, it can be assumed safely that the template-molecules on which the synthesis of this RNA occurs, are the "minus" strands present in, and firmly bound to, the cell-free preparation. Thus, in this system the synthesis of RNA of the viral type seems to represent addition of nucleotidyl residues to viral RNA-chains, the formation of which had already been initiated *in vivo* at the time when the leaves were used for homogenization. The DNA-independent enzyme-system present in 100 P_2 10,000 (V) is thus of the holo-enzyme type, composed of the enzyme associated with its template. It could be the viral RNA replicative complex, the enzyme itself being the viral RNA-replicase. If so, one should expect that besides double-stranded RNA-product, single-stranded viral RNA would also appear in the reaction mixture in the course of the reaction. However, so far the RNA-product of the reaction has been found to occur only as double-stranded RNA, even before phenol extraction, a procedure which is supposed to favour annealing of complementary RNA-strands, and thus the formation of dsRNA (Feix et al., 1968). Even though the cell-free system is certainly not entirely free of endogeneous ribonucleases, the absence of single-stranded RNA of the viral type does not seem to be due to such RNase activity since the single-stranded product of the RNA-polymerase system does accumulate in the course of the reaction. The inability of the DNA-independent enzyme-system, at least under the conditions used in our experiments, to turn out single-stranded RNA of the viral type, could eventually mean that the system is a repair-enzyme rather than a RNA-replicating one. This possibility is presently being considered.

Another property of the 100 P_2 10,000 (V)-system lies in the fact that in the course of the reaction the RNA-product of the DNA-independent system does not become free: it remains bound to the cell particles or particle-fragments of the cell-free preparation. This behaviour is in contrast to that of the single-stranded RNA-product of the RNA-polymerase system also present in 100 P_2 10,000 (V), as illustrated by the results of Table 1. In that experiment 100 P_2 10,000 (V) or 100 P_2 10,000 (H) were used for RNA-synthesis in the presence or in the absence of actinomycin D.

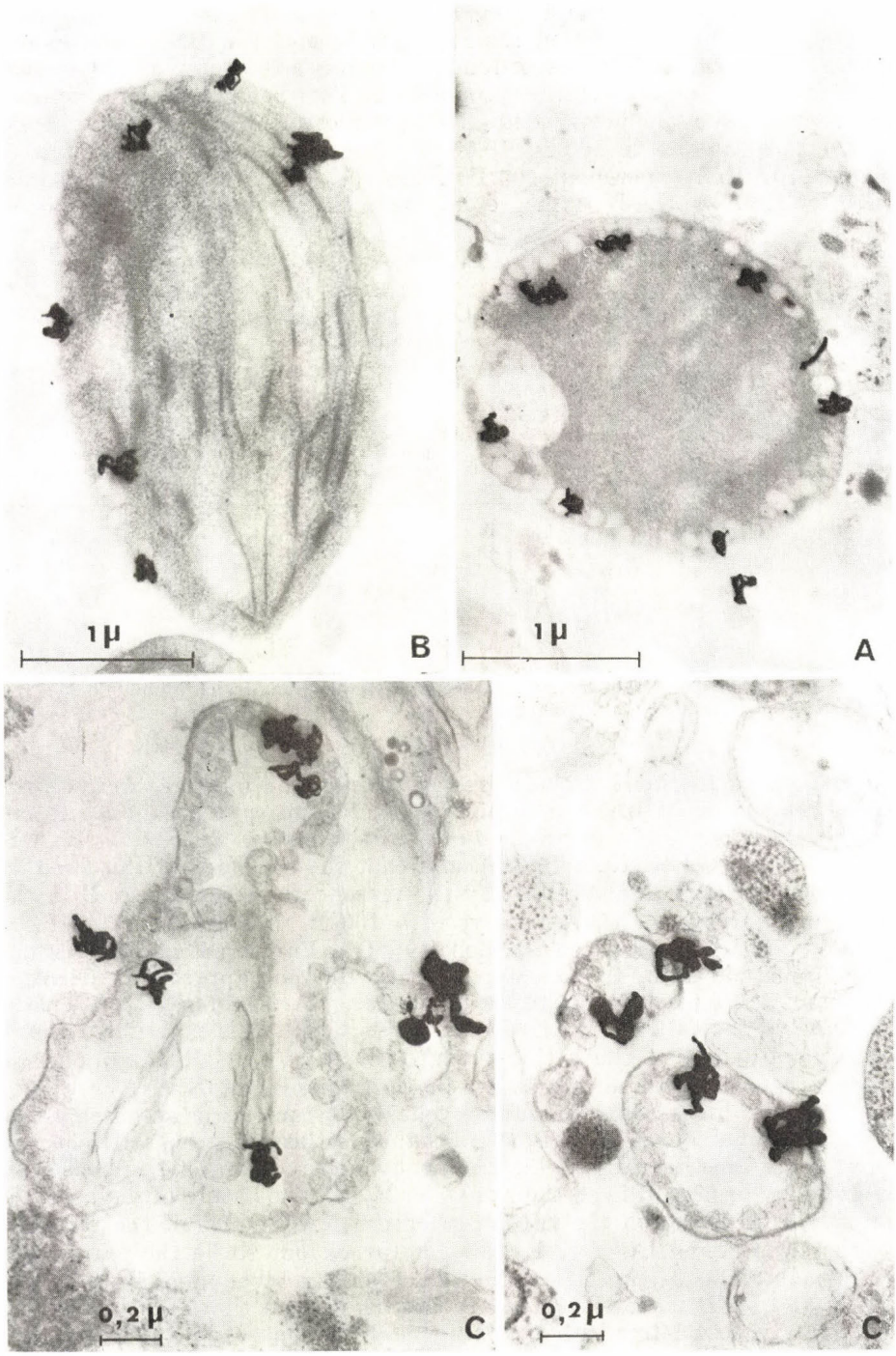
Table 1

RNA-synthesis with cell-free preparation 100 P_2 10,000 followed by centrifugation of reaction mixture; distribution of radioactive product between pellet (P_3) and supernatant (S_3)

Reaction mixture	100 P_2 10,000 (H) from healthy leaves		100 P_2 10,000 (V) from TYMV-infected leaves	
	Radioactivity (cpm) in pellet P_3 (H)	Radioactivity (cpm) in supernatant S_3 (H)	Radioactivity (cpm) in pellet P_3 (V)	Radioactivity (cpm) in supernatant S_3 (V)
Complete	89	7,226	8,210	11,829
	(1%)	(99%)	(41%)	(59%)
Complete +	115	560	7,949	1,151
Actinomycin D			(86%)	(14%)

After 30 min at 30 °C the reaction mixture (0·4 ml) was diluted with 7·6 ml of 0·05 × SSC and centrifuged for 10 min at 25,000 g.

At the end of the incubation the respective reaction mixtures were centrifuged for 10 min at 10,000 g to yield pellet P_3 and supernatant S_3. It can be seen that 99% of the product obtained with 100 P_2 10,000 (H) was present in the supernatant S_3 (H), and that the synthesis of this product was decreased to less than 10% by the presence of actinomycin D in the reaction mixture. On the contrary, with 100 P_2 10,000 (V), 41% of the product remained bound to the pellet P_3 (V). Furthermore, the presence of actinomycin D in the reaction mixture did not appreciably decrease the amount of product bound to the pellet, but it did result in a 90% decrease of the product in the supernatant S_3 (V). This experiment shows that the product of the actinomycin D-sensitive, host RNA-polymerase system becomes free and ends up in the supernatant S_3 (H) or S_3 (V), but that the product of the actinomycin-D-resistant, virus-specific system remains bound to the pellet P_3 (V). Because of this property, Lafleche and Bové were able recently to determine by autoradiography and electron microscopy of ultra-thin sections of pellet P_3 (V), the nature of the particles in the pellet to which the tritium-labelled RNA-product of the replicase system was bound. As Fig. 1 shows, it turned out to be the chloroplast outer-membrane system (Bové et al., 1969; Lafleche and Bové, 1970, 1971*a* and *b*). It should be noted in this connection that TYMV-infection is precisely associated with dramatic modifications of the plastids, and especially with the development of numerous double-membrane vesicles


1 µ
B
1 µ
A
0,2 µ
C
0,2 µ
C

connected to the plastidial envelope (Lafleche and Bové, 1969). Since the product of the replicase-system does not become free at any time during the reaction, but remains bound to a specific site in the plastids, it follows that the very site to which it is bound must also be the site where it is being synthesized and thus the site where the DNA-independent enzyme system is located, namely the chloroplast outer membrane-system. Autoradiography studies with uridine-^{3}H infiltrated leaf discs have strengthened the view that this plastidial membrane-system is involved in the replication of TYMV-RNA, not only with 100 P_2 10,000 (V) *in vitro*, but also *in vivo* (Lafleche and Bové, 1968; 1970; 1971*a* and *b*).

Knowing that within 100 P_2 10,000 (V) the virus-specific, DNA-independent system was bound to a cell-membrane, experiments were undertaken to solubilize and purify the enzyme complex. The system could indeed be purified approximately 100 times, and obtained free of RNA-polymerase activity, as shown in this communication.

ACTINOMYCIN D-RESISTANT RNA-SYNTHESIS WITH CELL-FREE PREPARATION 100 P_2 10,000 (V): EXPERIMENTS ON LOOSENING THE MEMBRANE-BOUND RNA-PRODUCT

Since after purification, the product of the actinomycin D-resistant RNA-synthesis with 100 P_2 10,000 (V) is always found to be associated with its template — the "minus" strand — within a double-stranded structure, it seemed likely that at the end of the reaction, before purification, the product should also be associated with its template, within the enzyme-template complex. It was hoped that conditions under which the product of the reaction could be detached from the membrane sites, would also liberate the enzyme-template complex itself. Therefore, the following experiments were undertaken.

Effect of various buffers

In the experiment of Table 2, after 30 min at 37 °C the reaction mixture was diluted with buffers of low (0·1 × SSC) or high (1·0 × SSC, 4·0 × SSC, 0·5 M K_2HPO_4) ionic strength. The presence of actinomycin D in the reaction mixture prevented the functioning of the RNA-polymerase system. The diluted reaction mixtures were centrifuged for 15 min at 25,000 g. The acid-insoluble radioactivity of the supernatant S_3 (V) and of the pellet P_3 (V), resuspended in 0·1 × SSC or 1·0 × SSC buffer, was determined

←

Fig. 1. Autoradiography and electron microscopy of ultrathin sections through pellet P_3 (V) obtained by centrifugation of reaction mixture after *in vitro* RNA synthesis with the cell-free preparations 100 P_2 10,000 (V) (A) or 0 P 1,000 (V) (B) incubated for 10 min at 30 °C in the presence of actinomycin D (tritiated nucleotide: UTP). Cell-free fraction 0 P 1,000 (V) was the resuspended pellet obtained by centrifuging the filtered leaf homogenate for 15 min at 1,000 g. Only chloroplasts (A and B) and detached chloroplast membranes (C) were found to be labelled.
Note the presence of numerous double membrane vesicles on the inner face of the chloroplast envelope (A and B). They remain bound to the plastidial envelope after its detachment (C) (from Lafleche and Bové, 1971*a*)

Table 2

RNA-synthesis with cell-free preparation 100 P_2 10,000 (V). Distribution of radioactive product between pellet P_3 (V) and supernatant S_3 (V): Influence of buffer used to dilute reaction mixture before centrifugation, and ribonuclease resistance of product in pellet and supernatant

Buffer used to dilute reaction mixture	Pellet P_3 (V)			Supernatant S_3 (V)		
	Radioactivity (cpm) after treatment with or without RNase			Radioactivity (cpm) after treatment with or without RNase		
	— RNase (in 1·0×SSC)	+ RNase (in 0·1×SSC)	+ RNase in 1·0×SSC)	— RNase	+ RNase (in 0·1×SSC)	+ RNase (at high ionic strength)
0·1×SSC	3,958 (100%)	2,408 (+61%)	3,773 (95%)	1,138 (22%) (100%)	329 (8%) (29%)	1,049* (22%)** (92%)**
1·0×SSC	3,961 (100%)		3,578 (90%)	1,140 (29%) (100%)		1,080 (27%) (95%)
4·0×SSC	3,677 (100%)		3,798 (≠ 100%)	1,363 (37%) (100%)		979 (27%) (72%)
0·5 M K_2HPO_4	3,610 (100%)		3,920 (≠ 100%)	1,512 (42%) (100%)		1,435 (40%) (95%)

After 30 min at 37 °C, the reaction mixture (0·4 ml) was diluted with 3·2 ml of the respective buffers and centrifuged for 15 min at 25,000 g. The pellets were resuspended in 0·1×SSC or in 1·0×SSC buffer as indicated.

All reaction mixtures contained actinomycin D (0·05 mg/0·4 ml).

* RNase resistance was tested after addition of 20×SSC to final concentration of 1·0×SSC.

** The % figures of a given line of the table must be compared to the 100% value indicated on the left of the same line.

Table 3

RNA-synthesis with cell-free preparation 100 P_2 10,000 (V). Resuspension of pellet P_3 (V) and centrifugation: Distribution of radioactive product between pellet P_4 (V) and supernatant S_4 (V)

Buffer used to resuspended pellet P_3 (V)	Radioactivity (cpm) in	
	Pellet P_4 (V)	Supernatant S_4 (V)
0·1×SSC	3,171	386
1·0×SSC	3,306	590
4·0×SSC	2,830	990
0·5 M K_2HPO_4	2,890	939

Pellet P_3(V) (see Table 2) was resuspended with the respective buffer and centrifuged for 15 min at 25,000 g.

after a 30 min incubation at 37 °C in the presence or in the absence of pancreatic ribonuclease (0·05 mg/ml).

As seen in Table 2, the use of 4·0 × SSC or 0·5 M K_2HPO_4 increased the amount of product in supernatant S_3, but it never reached much more than 40 % in the best case. Essentially the same results were obtained when the pellet P_3 was centrifuged again after resuspension with one of the previous buffers (Table 3).

Table 2, as well as Table 4, shows that the product in the supernatant S_3 was entirely RNase-resistant at high ionic strength, but largely RNase-sensitive at low ionic strength, in agreement with a double-stranded nature

Table 4

RNA-synthesis with cell-free preparation 100 P_2 10,000 (V) at various temperatures; distribution of radioactive product between pellet P_3 (V) and supernatant S_3 (V) and RNase resistance

Incubation of reaction mixture for 30 min at	Pellet P_3 (V)			Supernatant S_3 (V)		
	Radioactivity (cpm) after treatment with or without RNase			Radioactivity (cpm) after treatment with or without RNase		
	— RNase (in 0·05×SSC)	+ RNase (in 0·05×SSC)	+ RNase (in 2·0×SSC)	— RNase (in 0·05×SSC)	+ Rnase (in 0·05×SSC)	+ RNase (in 2·0×SSC)
10 °C	3,023 (100%) (97%)	1,791 (59%)	2,842 (94%)	83 (3%)	79	5
20 °C	5,979 (100%) (93%)	4,229 (66%)	6,001 (≠100%)	433 (7%)	218	430
30 °C	7,412 (100%) (86%)	4,851 (65%)	6,878 (93%)	1,239 (100%) (14%)	253 (20%)	1,091 (88%)
37 °C	8,459 (100%) (83%)	5,028 (59%)	8,172 (97%)	1,672 (100%) (17%)	0 (0%)	1,574 (94%)

At the end of incubation, the reaction mixtures (0·4 ml) were diluted with 7·6 ml of 0·05×SSC and centrifuged for 15 min at 25,000 g. The pellets were resuspended with 8 ml of 0·05×SSC or 2·0×SSC buffer as indicated. The concentration of SSC buffer in supernatant was left at 0·05×SSC or adjusted to 2·0×SSC with 20×SSC buffer.

of this product. The product in the pellet P_3 was also RNase-resistant at high ionic strength (1·0 × SSC) but at low ionic strength (0·1 ×SSC) it was still appreciably RNase-resistant (61 %). We have shown previously that this residual RNase-resistance could be abolished, and the product in the pellet rendered entirely RNase-sensitive at low, but not at high ionic strength, by freezing and thawing the resuspended pellet before carrying out the RNase-test (Bové et al., 1968). Also, this treatment rendered the product sensitive to RNase III, an enzyme which hydrolyses specifically dsRNA (Robertson et al., 1967), under conditions where pancreatic RNase was without effect (Bové et al., 1968). This establishes the double-stranded nature of the product in the pellet.

The residual RNase-resistance of approximately 60% which is observed at low ionic strength could be due to the association of the product with a membrane, and/or the protection offered by the membrane bound enzyme itself if it covers up parts of the newly synthesized RNA-product.

Effect of temperature of reaction

In the experiment of Table 2 the RNA-synthesis was carried out at 37 °C. In those of Table 4 the reaction was allowed to occur at 10, 20, 30 and 37 °C. The reaction mixture was diluted with 0·05 × SSC buffer prior to centrifugation. It can be seen that the amount of product that ended up in the supernatant S_3 increased with increasing temperatures of reaction. At the temperature of 30 °C at which most experiments have been done, the percentage of product in the supernatant S_3 varied approximately from 15 to 20%.

The results concerning the RNase-resistance of the product in the pellet or in the supernatant are the same in Table 4 and in Table 2: they were not influenced by the temperature of reaction within the limits studied.

EFFECT OF VARIOUS MEMBRANE-DISPERSING AGENTS ON RNA-SYNTHESIS WITH 100 P_2 10,000. SOLUBILIZATION OF THE TYMV-SPECIFIC, DNA-INDEPENDENT RNA-SYNTHESIZING SYSTEM

The previous experiments have shown that diluting the reaction mixture with various buffers, washing the pellet P_3, or carrying out the reaction at high rather than low temperatures, did not detach the bulk of the TYMV-specific product from the membrane system. Therefore, various membrane-dispersing agents were tried: sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC), digitonin, Triton X 100, Brij 35 and Lubrol W. Table 5 shows that the presence of these agents in the reaction mixture did not appreciably inhibit RNA-synthesis by 100 P_2 10,000 (V).

In the experiment of Table 6 the preparation 100 P_2 10,000 (V) was first treated for 5 min at 20 °C with various agents at two concentrations. The mixture was then centrifuged for 10 min at 30,000 g. The supernatant $S_{30,000}$ and the pellet, resuspended in pH 9 buffer, were tested for their ability to support RNA-synthesis. Table 6 shows that in the absence of a membrane-dispersing agent no RNA-synthesizing activity ended up in the supernatant: both the DNA-dependent and the DNA-independent activities remained associated with the pellet. After treatment of 100 P_2 10,000 (V) with one of the detergents, and especially when they were used at a concentration of 0·5%, large amounts of RNA-synthesizing activity were detached from the membranes and occured in the supernatant. In all cases, except with 0·5% DOC, the activity which ended up in the supernatant was found to be actinomycin D resistant.

The same results were obtained with the neutral detergent Lubrol W (Imperial Chemical Industries, Providence, Rhode Island), which was used most extensively in these studies, and which led to the following solubilization procedure.

Five percent Lubrol W was added to 100 P_2 10,000 to a final concentration of 0·5 %. After 10 min at 15 °C the mixture was centrifuged in the cold for 60 min at 60,000 g in a swinging bucket rotor. The following zones

Table 5

Effect of various membrane dispersing agents on RNA-synthesis with cell-free preparation 100 P_2 10,000 (V) in the presence or absence of actinomycin D

Reaction mixture	Radioactivity (cpm) of acid-insoluble precipitate	
	RNA-synthesis in the absence of actinomycin D	RNA synthesis in the presence of actinomycin D (0·05 mg/0·4 ml)
Complete	20,301	9,768
Complete + SDS (0·1%)	25,201	6,833
Complete + DOC (0·1%)	26,914	7,659
Complete + Digitonin (0·1%)	20,464	9,598
Complete + Triton×100 (0·1%)	17,198	8,487
Complete + Brij 35 (0·1%)	21,263	9,327
Complete	32,063	10,083
Complete + Lubrol W (0·5%)	30,648	13,445

After 15 min at 30 °C, the reaction was stopped with 3 ml of 15% TCA.

Table 6

Centrifugation of cell-free preparation 100 P_2 10,000 (V) after treatment with various membrane dispersing agents: Distribution of proteins and RNA-synthesizing activities between pellet and supernatant

Treatment of 100 P_2 10,000 (V) for 5 min at 20 °C with:	Actinomycin D in reaction mixture for RNA-synthesis	Protein content and RNA-synthesizing activities (radioactivity incorporated) of			
		Pellet		Supernatant	
		mg prot./ml	cpm	mg prot./ml	cpm
H_2O	−	17·0	18,325	1·8	564
	+		10,664		69
DOC (0·1%)	−	10·0	10,822	7·3	4,548
	+		3,955		4,449
DOC (0·5%)	−	10·0	4,670	12·0	22,496
	+		1,324		6,883
Triton×100 (0·1%)	−	12·5	15,287	3·8	730
	+		8,110		835
Triton×100 (0·5%)	−	12·3	7,731	6·2	4,620
	+		3,585		4,849
Brij 35 (0·1%)	−	11·4	14,201	4·3	1,655
	+		8,782		1,348
Brij 35 (0·5%)	−	12·2	7,228	9·1	5,777
	+		3,575		6,548

After treatment of 100 P_2 10,000 (V) with the respective agents, the mixtures were centrifuged for 10 min at 30,000 g. The supernatants, S 30,000, and the pellets resuspended in pH 9 buffer, were tested for protein content and RNA-synthesis in the presence or absence of actinomycin D.

Table 7

RNA-synthesis by various cell-free preparations derived from 100 P_2 10,000 by treatment with Lubrol W: Solubilisation of replicase activity

Exp.	Type of leaf material used	Radioactivity (cpm) of acid-insoluble precipitate after RNA-synthesis in the presence or absence of actinomycin D with:									
		100 P_2 10,000 — Lubrol W		100 P_2 10,000 + Lubrol W		S 60,000		C 200,000		S 200,000	
		— act.	+ act.	— act.	+ act.	— act.	+ act.	— act.	+ act.	— act.	+ act.
313	Healthy	17,875	1,421	13,823	1,384	600	361	580	550	535	560
313	TYMV-infected	32,063	10,083	30,648	13,445	10,649	11,415	5,560	5,470	2,390	2,640
321	Healthy	—	190	—	—	—	27	—	0	—	0
321	TYMV-infected	—	1,372	—	—	—	1,360	—	616	—	58
344	TYMV-infected	13,452	4,563	—	—	4,160	3,585	2,443	2,533	229	—
382	TYMV-infected	40,905	15,357	24,950	10,400	6,390	6,450	4,270	3,990	343	408
383	TYMV-infected	29,762	12,146	26,600	12,700	8,030	7,140	3,470	3,680	257	196
385	TYMV-infected	22,786	7,052	11,300	6,220	4,960	4,420	2,670	2,300	630	—
388	TYMV-infected	27,263	9,580	19,500	9,150	12,396	10,693	6,250	6,160	1,805	1,725
403	TYMV-infected	54,558	11,892	25,800	9,810	10,170	8,320	4,108	3,460	479	450

The cell-free preparations were obtained as follows: Lubrol W (5%) was added to 100 P_2 10,000 to a final concentration of 0·5%, and after 10 min at 15 °C the mixture ("100 P_2 10,000 + Lubrol W") was centrifuged at 60,000 g for 60 min; the supernatant, S 60,000, was centrifuged again for 120 min at 200,000 g to yield pellet C 200,000 and supernatant S 200,000. The sign "—" means that the experiment was not performed.

were obtained: a clear, pale-yellow, upper supernatant ($S_{60,000}$) amounting to 2/3 of the total volume, a dark-green lower supernatant (1/3 of the volume), and a white pellet covered by more or less well-packed green material.

Experiments 313 and 321 of Table 7 show that when this procedure was applied to the cell-free preparation isolated from healthy leaves, 100 P_2 10,000 (H), practically no RNA-synthesizing activity ended up in the clear supernatant, $S_{60,000}$ (H): the DNA-dependent RNA-polymerase system was not solubilized by the detergent. On the contrary, with the preparation from the TYMV-infected leaves, 100 P_2 10,000 (V), large amounts of activity, and more precisely DNA-independent RNA-synthesizing activity, appeared in supernatant $S_{60,000}$ (V): the DNA-independent system was solubilized by Lubrol W.

In the experiments of Table 7 all reactions were carried out with an amount of cell-free preparation corresponding to 0·15 ml of the initial 100 P_2 10,000 preparation. Thus, the figures for each experiment are directly comparable. It can be seen that from one experiment to the other, the specific activity of supernatant $S_{60,000}$ (V) varied from 50 to 100% of that of the initial 100 P_2 10,000 (V) preparation. It averaged around 70 to 80%.

Table 8 illustrates some of the properties of fraction $S_{60,000}$ (V). The amount of RNA synthesized was proportional to the volume of $S_{60,000}$ (V) used in the reaction mixture. The reaction was linear with time for the first ten minutes. Practically all of the activity was of the DNA-independent type. No added template was needed for activity. Table 9 shows that the product of the reaction was 100% RNase-resistant at high ionic strength, and partly RNase-sensitive at low salt concentration. The residual RNase-resistance at low ionic strength might be due to protection of the newly synthesized RNA by the enzyme.

Table 8

RNA-synthesis with the cell-free preparation $S_{60,000}$ (V)

Volume (ml) of S 60,000 in 0·4 ml of reaction mixture	Time of reaction at 30 °C (min)	Radioactivity (cpm) of acid-insoluble precipitate	
		RNA-synthesis in the absence of act. D	RNA-synthesis in the presence of act. D
0·05	0	292	—
0·05	15	4,226	4,935
0·10	0	222	—
0·10	15	9,467	9,161
0·20	0	227	—
0·20	15	17,416	17,056
0·15	0	246	—
0·15	1	1,001	733
0·15	2	2,134	2,325
0·15	4	4,878	5,337
0·15	8	10,317	10,370
0·15	15	14,101	13,456
0·15	30	16,326	14,994

"—": experiment not performed.

Table 9

RNA-synthesis with the cell-free preparation $S_{60,000}$: ribonuclease resistance of synthesized RNA

Medium for RNase-test	RNase (10 μg/ml)	Radioactivity (cpm) of acid-insoluble precipitate	
		RNA-synthesis in the absence of act. D	RNA-synthesis in the presence of act. D
0·1×SSC	−	13,043	13,360
0·1×SSC	+	8,786	5,741
1·0×SSC	−	12,845	13,147
1·0×SSC	+	13,238	13,129

After 15 min at 30 °C the reaction mixture (0·4 ml) was diluted with 7·6 ml of 0·1×SSC or 1·0×SSC buffer. After addition of water (− RNase) or of pancreatic ribonuclease (+ RNase) to the final concentration of 10 μg/ml and incubation for 30 min at 30 °C the reaction was stopped with 0·9 ml of 100% TCA.

Table 10

Partial purification of the TYMV specific, actinomycin D-independent RNA-synthesizing system

Cell-free fraction	Volume (ml)	Proteins (mg/ml)	Radioactivity (cpm) incorporated (actinomycin D independent)		Specific activity cpm/mg Prot.	Purification factor	Yield %
			per 0·15 ml	total			
Filtrate	500·0	8·3	232	772,000	186	1	100
100 P_2 10,000	46·5	15·0	1,783	554,000	794	4	72
$S_{60,000}$	37·0	3·0	1,861	460,000	4,150	22	60
Green zone	29·0	16·8	1,872	207,000	426		
$C_{200,000}$	3·7	8·0	17,200	424,000	14,350	77	55
$S_{200,000}$	36·5	1·7	136	33,000	535		
Pooled sucrose gradient fractions (Enzyme "R")	27·0	0·45	951	171,500	14,100	76	22
Sucrose gradient fraction n° 27	2·4	0·44	1,495	24,000	22,600	121	3

PARTIAL PURIFICATION OF THE DNA-INDEPENDENT RNA-SYNTHESIZING SYSTEM

The DNA-independent RNA-synthesizing system present in $S_{60,000}$ (V) could be concentrated by a two-hour centrifugation at 200,000 g. Table 7 shows that the activity was recovered in the resuspended $C_{200,000}$ (V) pellet, and that in most experiments very little remained in the $S_{200,000}$ (V) supernatant.

The resuspended $C_{200,000}$ (V) pellet was layered on a 5—20% sucrose gradient, and centrifuged at 23,000 rpm for 11 hours in a Spinco SW25·1 rotor. One ml fractions were collected and aliquots were used to test for the presence of the DNA-independent activity. Fig. 2 shows the distribution of the enzyme activity in the sucrose gradient and the recorded optical density of the gradient. Gradient fractions 7 to 13 were pooled and frozen or dialyzed overnight against pH 9 buffer. The pooled gradient fractions were designated as enzyme "R".

Table 10 summarizes the purification achieved from the initial filtered leaf-homogenate to enzyme "R". In that experiment a purification factor of 76 was obtained. The highest purification was shown by fraction 27, corresponding to the maximum of the sucrose gradient peak.

RNA-SYNTHESIS WITH THE PARTIALLY PURIFIED ENZYME-SYSTEM "R"

Table 11 illustrates some of the RNA-synthesizing properties of enzyme-system "R". The amount of RNA synthesized was proportional to the amount of enzyme "R" used. No reaction occurred in the absence of ATP,

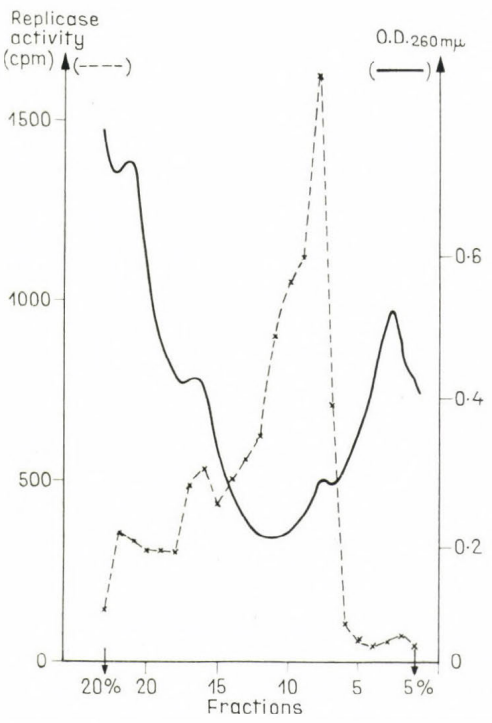


Fig. 2. Sucrose gradient centrifugation of resuspended pellet $C_{200,000}$ (V): recording of optical density at 260 mμ and replicase activity of the collected fractions

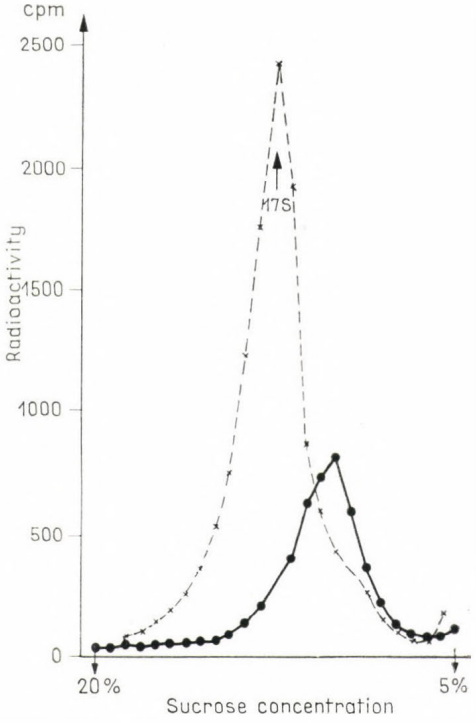


Fig. 3. Sucrose gradient centrifugation of the labelled dsRNA product of the 100 P_2 10,000 (V) reaction (- + - - + -) and that of the enzyme "R" reaction (——•——)

CTP and GTP. Actinomycin D had no effect. The synthesis was linear for the first 15 min. No added template was required for activity.

Table 12 indicates the RNase-resistance of the synthesized RNA. At high ionic strength (1·0 × SSC) the product of the reaction was 100% RNase-resistant, but if it had been heated for 10 min at 120 °C, followed by rapid cooling, only 6% RNase-resistance were left. At low ionic strength RNase-resistance was reduced to 10% or less in one experiment (Table 12, Exp. 370). In another (Table 12, Exp. 345) it remained at a level of about 50%. In this connection it might be relevant to note that in experiment 370 the sucrose gradient fractions containing enzyme "R" were frozen before being pooled and used.

The labelled RNA product of the enzyme "R" reaction was purified by two phenol extractions, chromatography on Sephadex G-50 and on cellulose-CF11 (Franklin, 1966), and precipitation with ethanol. The purified product was RNase-resistant in 1·0 × SSC, and RNase-sensitive in 0·1 × SSC buffer. For comparison, the labelled dsRNA-product of the 100 P_2 10,000 (V) reaction was purified in the same way. The two products were analysed by 5—20% sucrose gradient centrifugation at 23,000 rpm for 16 hrs in the Spinco SW25·1 rotor. Fig. 3 shows that the product of the enzyme "R" reaction sedimented slower than the 17S dsRNA product of the 100 P_2 10,000 (V) reaction. The estimated S value for the enzyme "R" product was of the order of 10 to 12. It thus seems that the product of the enzyme "R" reaction is definitely smaller than the 17S dsRNA from the 100 P_2 10,000 (V) reaction.

As mentioned before, enzyme "R" or $S_{60,000}$ (V), just as 100 P_2 10,000 (V) required no added template for activity. In the case of 100 P_2 10,000 (V) we have shown that the synthesized RNA belongs to the "plus" strand, and that the complementary "minus" strand needed as template, was present within preparation 100 P_2 10,000 (V) (Bové, 1967*a* and *b*). No added template was necessary because the enzyme was already associated with its template, the "minus" strand. The same situation must also be true with $S_{60,000}$ (V) and enzyme "R", since they originate from the preparation 100 P_2 10,000 (V) as a result of Lubrol W treatment. In other words, the enzyme-system in $S_{60,000}$ (V) or in enzyme "R" must represent the enzyme bound to its template. If so, the enzyme activity peak (enzyme "R" fractions) obtained by sucrose gradient centrifugation of pellet $C_{200,000}$ (V) (Fig. 2) should coincide with the template-RNA distribution peak. This could be shown to be true indeed in the following way: instead of submitting 100 P_2 10,000 (V) to the Lubrol W treatment immediately, the cell-free preparation was first used for a DNA-independent RNA-synthesis, and only then treated with the detergent to prepare $S_{60,000}$ (V) and $C_{200,000}$ (V). During these steps the radioactivity, initially bound to 100 P_2 10,000 (V) as a result of the RNA-synthesis prior to the Lubrol W treatment, ended up precisely in the same fractions as the DNA-independent RNA-synthesizing activity, determined at the same time in a parallel experiment. Finally, the radioactive peak obtained upon sucrose gradient centrifugation of the labelled $C_{200,000}$ (V) pellet, was located at the same position as the enzyme activity peak, namely in the 10—12S region. This is also the position at which the dsRNA product of the enzyme "R" reaction travels (Fig. 3).

Table 11

RNA-synthesis with the partially purified, TYMV-specific, actinomycin D-independent RNA synthesizing system (enzyme "R")

Volume (ml) of enzyme "R" in 0·8 ml of reaction mixture	Reaction mixture	Time of reaction at 30 °C (min)	Radioactivity (cpm) of acid-insoluble precipitate
0·1	complete	10	202
0·2	complete	10	365
0·4	complete	10	724
0·4	−ATP, −CTP, −GTP	10	63
0·4	+ act. D	10	726
0·4	complete	0	72
0·4	complete	5	469
0·4	complete	10	724
0·4	complete	20	1,224
0·4	complete	60	1,785

Table 12

RNA-synthesis with enzyme "R": Ribonuclease resistance of synthesized RNA

Exp.	Time of RNA-synthesis at 30 °C (min)	Buffer for dilution of reaction mixture after RNA-sythesis	Radioactivity (cpm) of acid-insoluble precipitate after incubation of diluted reaction mixture with or without RNase	
			− RNase	+ RNase
345	10	0·1×SSC	507 (100%)	284 (57%)
345	60	0·1×SSC	1,182 (100%)	525 (45%)
345	10	1·0×SSC	464 (100%)	578 (100%)
345	60	1·0×SSC	1,334 (100%)	1,379 (100%)
345	10	10·0×SSC	442* (100%)	18* (4%)
370**A	30	0·1×SSC	—	92 (10%)***
370**B	30	0·1×SSC	—	54 (6%)***
370**A	30	1·0×SSC	975 (100%)	1,160 (100%)
370**B	30	1·0×SSC	949 (100%)	919 (97%)

After RNA-synthesis, the reaction mixture was diluted 10-fold (Exp. 345) or 20-fold (Exp. 370) with 0·1 or 1·0×SSC buffer. After addition of water (−RNase) or pancreatic ribonuclease (+ RNase) to the final concentration of 10 μg/ml (Exp. 345) or 50 μg/ml (Exp. 370), and incubation at 30 °C for 15 min (Exp. 345) or 30 min (Exp. 370), the reaction was stopped with TCA to the final concentration of 10%.

* The two reaction mixtures, adjusted to 1·0×SSC with 10·0×SSC buffer, were heated in sealed tubes for 10 min at 120 °C and rapidly cooled, prior to addition of water (− RNase) or RNase (+ RNase).

** In Exp. 370, the sucrose gradient fractions containing enzyme R were kept frozen before being pooled and used.

*** Percentage based on the value of 975 taken as 100% (Exp. 370 A, 1·0×SSC).

CONCLUSION

It was concluded from previous experiments that the DNA-independent, TYMV-specific RNA-synthesizing system present in 100 P_2 10,000 (V), and more precisely in the plastidial elements of this preparation, as we have found recently, had to be a complex in which the enzyme was already associated with its template, the "minus" strand. This conclusion arose from the fact that no template needed to be added to the reaction mixture, even though the product of the reaction was a very specific RNA, namely RNA of the "plus" or viral type. The experiments presented here strongly suggest that it is this enzyme-template complex which could be detached from the plastidial elements of 100 P_2 10,000 (V) by the use of a detergent, and which is responsible for the activity of preparation $S_{60,000}$ (V), $C_{200,000}$ (V) and enzyme "R".

The fact that the solubilized enzyme activity could be sedimented into pellet $C_{200,000}$ (V) and further centrifuged on sucrose gradients, is very probably due to the presence of a rather large RNA template in the enzyme complex. However, in comparison with the RNA template present in 100 P_2 10,000 (V) and from which a 17S dsRNA is usually isolated, the RNA template of the sucrose gradient enzyme, enzyme "R", turns out to be smaller, as judged for instance by the 10—12S dsRNA which can be isolated from it. This is probably the result of some break-down during the steps following the detergent treatment.

The 17S dsRNA has a molecular weight of $4{\cdot}1 \times 10^6$ daltons, just twice that of intact single-stranded TYMV-RNA. This property suggests that each of these dsRNA structures contains at least one full-length RNA strand, probably the "minus" strand. The 10—12S dsRNA from the solubilized enzyme complex, is too small to contain a full-length RNA chain. Since the solubilized enzyme system is found to be active, full-length, intact strands do not seem to be a requirement of the system. Intact strands would not be required if only elongation (or repair !) of pre-existing chains, but not initiation of new RNA chains, did occur.

In comparison with the rather crude 100 P_2 10,000 (V) preparation, the more purified fractions do not exhibit new properties except that they are free of DNA-bound RNA-polymerase. In particular, neither with 100 P_2 10,000 (V) nor with the solubilized fractions, was it possible, under the conditions used, to obtain single-stranded RNA-product. Unfitness of the RNA-template in the cell-free preparations could explain this inability. We have not entirely proved, but it is rather probable, that in our cell-free preparations the RNA-template occurs essentially as a double-stranded structure. There are indications from the bacteriophage work that *in vivo* the "minus" strand template occurs predominantly as a single-stranded, but not double-stranded, form. It could thus be visualized that with our cell-free preparations, the enzyme would stop functioning as soon as it would encounter double-strandedness along the template; only the few single-stranded regions, probably at the enzyme-attachment sites, would be used as active template.

Finally, at this stage, it cannot be excluded that the enzyme system that we are studying is involved in repairing rather than in replicating RNA.

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NUCLEIC ACID AND PROTEIN SYNTHESIS IN GERMINATING SEEDS

by

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It has been emphasised (Koller et al., 1962) that the macroscopic changes accompanying the germination of seeds represents the final stages in a process which may well begin as early as fertilization. It is proposed to discuss the sequence of events occurring during germination, which, for this purpose, will be regarded as beginning with imbibition of the seed.

Germination, by its nature, implies a sudden onset of growth and differentiation, and a corresponding burst of metabolic acivity. Protein synthesis and other related anabolic processes feature largely, but catabolic processes

Table 1

Incorporation of [^{14}C] leucine into soluble protein of cotyledons of Pisum sativum during imbibition

Period of imbibition (h)	[^{14}C]leucine incorporated (counts/min/cotyledon pair)
2—4	4,445
22—24	5,730

are also involved. In our experiments with germinating peas, it is not possible to discern a phase, from the inception of imbibition, in which protein synthesis, as measured by incorporation of [^{14}C] leucine, is not operative (Table 1). During the early stages of germination, a number of enzymic activities develop. Some of these are synthesized *de novo*, including α-amylase in barley (Filner and Varner, 1967), isocitrate lyase in peanuts (Gientka-Rychter and Cherry, 1968) and acid phosphatase in peas (Barker et al., 1971*a*). The relationship of the initiation of nucleic acid and protein synthesis is therefore important in the biochemistry of germination.

Marcus and Feeley (1964) have shown that cell-free preparations from imbibed peanuts incorporate [^{14}C] leucine into protein whereas similar preparations from dry seed are inactive. The inactivity was shown to be due to inadequacy of the particulate component of the system because supernatant fractions from imbibed and unimbibed seed were equally active. Imbibition was accompanied by the formation of polyribosomes by association of monoribosomes with either pre-existing or newly synthe-

sized m-RNA (Marcus and Feeley, 1965). Similar results were obtained with peanuts by Jachymczyk and Cherry (1968) and in our own laboratory we have found that polyribosomes appear in pea cotyledons during imbibition (Barker and Rieber, 1967). A number of claims have been made that protein synthesis in germinating seed involves the participation of pre-existing m-RNA but much of the evidence has been shown to be inconclusive (Barker et al., 1971*b*). We have shown that incorporation of [^{3}H] uridine into RNA can be detected at an early stage in the imbibition of pea seed (Table 2), and it is therefore impossible to decide whether synthesis of RNA precedes that of protein in this system.

Ihle and Dure (1969) have recorded some elegant experiments in which immature cotton embryos were germinated before completion of ripening of the seed. Such embryos develop a protease activity when germinated and it was found that development of enzyme was inhibited by actinomycin

Table 2

Incorporation of [^{3}H] uridine into RNA of cotyledons of Pisum sativum during imbibition

Period of imbibition (h)	[^{3}H]uridine incorporated (counts/min/cotyledon pair)
0 – 1	819
0 – 2	1,472
2 – 4	21,780

D in embryos taken at a very early stage. The drug was without effect in embryos taken at a later stage and it was concluded that, at the later stage, m-RNA necessary for protease synthesis was already present, although the ripening of the seed was not complete. However, it cannot be concluded that m-RNA synthesised in the immature seed is the same material which controls protein synthesis on germination of ripe seed. Our experiments lead us to the view that the question regarding long-lived m-RNA in seed cannot be properly answered and that germination should be regarded as initiating the acceleration of metabolic processes which are not completely abolished during the resting stage.

In view of the difficulty of establishing the sequence of events at the very earliest stage of germination, we have attempted to investigate the relationship of protein and nucleic acid synthesis by studies of the events leading to loss of viability of seed.

Seed of *Pisum arvense* retain 96% germination in normal storage for three years, after which this drops to 86% and 6% in the fourth and fifth years respectively. We have carried out some experiments with naturally aged seed. Owing to the relative inaccessibility of such material, some experiments have been performed with seed artificially aged using the method of Roberts and Abdalla (1968), although the possibility must be borne in mind that such material may not be equivalent in all ways to naturally aged seed.

During germination, pea seed exhibit increases in ribonuclease (RNase) and acid phosphatase activities. The former activity shows a biphasic development (Fig. 1) and we have shown that the early RNase does not arise by *de novo* synthesis since the enzyme produced in seed germinated in 80% D_2O exhibits normal buoyant density on centrifugation in caesium chloride (Figs 2a and b). Autolysis of homogenates of cotyledons of peas imbibed for 17 h produces an increase in RNase activity. Acid phosphatase shows a single phase of development (Fig. 3) and is formed by *de novo* synthesis since an increase in buoyant density is produced by germinating the seed in D_2O (Figs 4a and b). It has proved difficult to maintain ger-

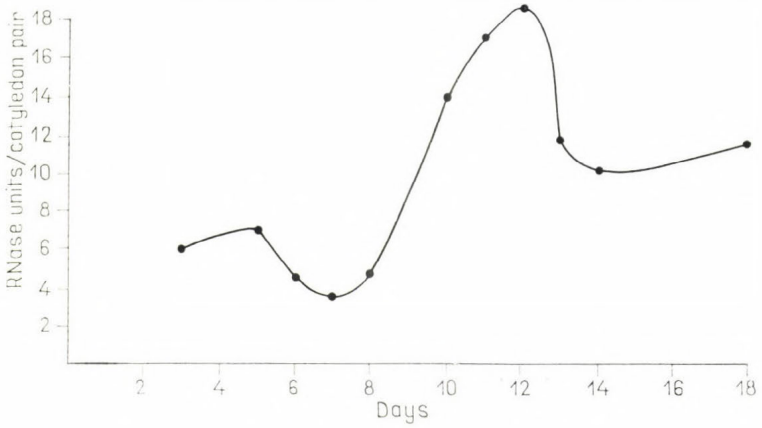

Fig. 1. Development of ribonuclease activity in the cotyledons of *Pisum arvense* during germination at 20 °C

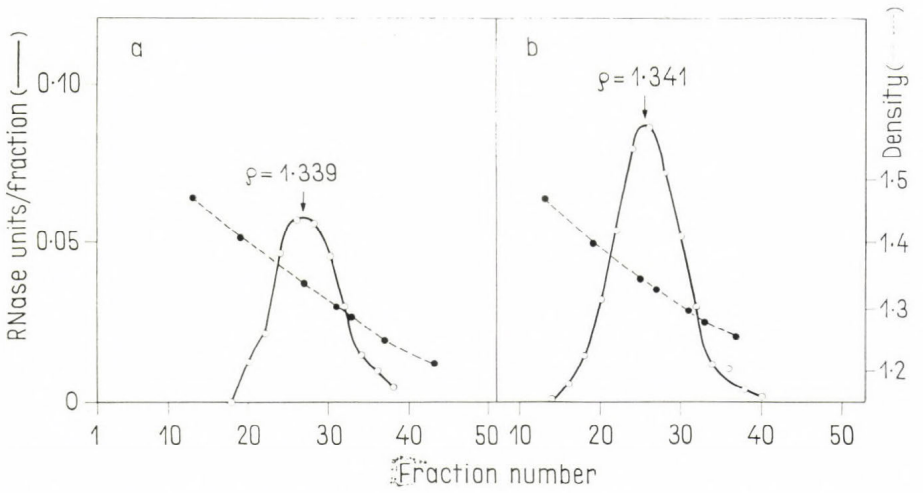

Fig. 2. Equilibrium density gradient centrifugation in caesium chloride of ribonuclease from 4-day old cotyledons of *Pisum arvense* (a) grown in H_2O, (b) grown in 80% D_2O

minating peas in D_2O long enough to allow a decision to be reached as to whether the late phase of RNase development arises by *de novo* synthesis, but the balance of evidence favours this. Thus the late phase of RNase development is abolished by cycloheximide (Fig. 5). This is in contrast to the early enzyme which is unaffected, but similar to the case of acid phosphatase development (Fig. 6). Although the two phases of RNase development differ in their origins, they appear to be subject to the same

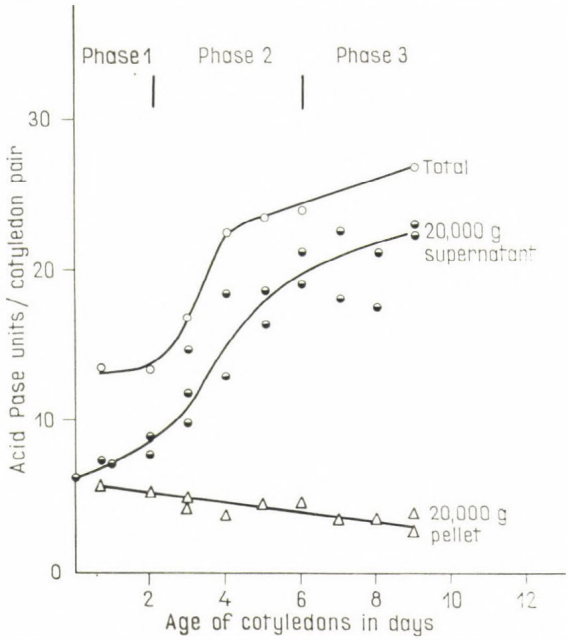

Fig. 3. Development of acid phosphatase activity in the cotyledons of *Pisum arvense* during germination

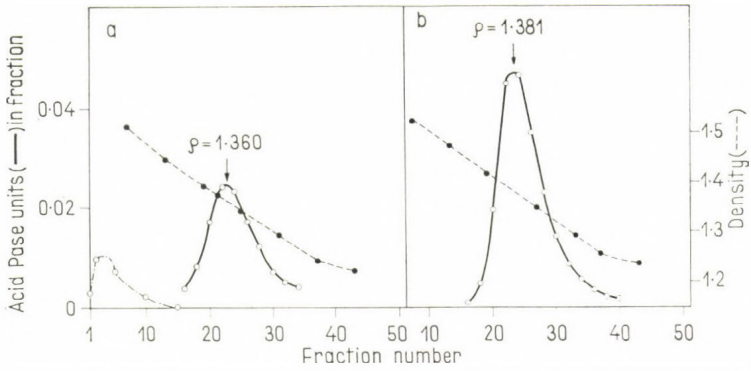

Fig. 4. Equilibrium density gradient centrifugation in caesium chloride of acid phosphatase from cotyledons of *Pisum arvense* grown (a) in H_2O for 4 days, (b) in 80% D_2O for 7 days

controls linked to the development of the plantlet: seed germinated at 2—4 °C show a biphasic development of RNase, which, although operating on a different time-scale, is related to the development of the plantlet in a

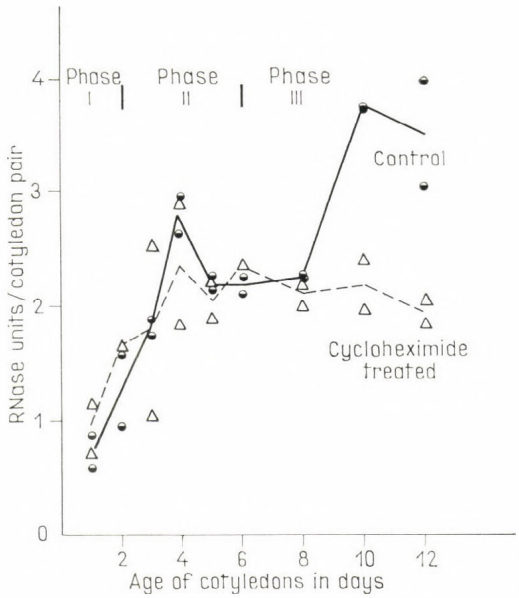

Fig. 5. The effect of cycloheximide on ribonuclease development in the cotyledons of germinating *Pisum arvense*

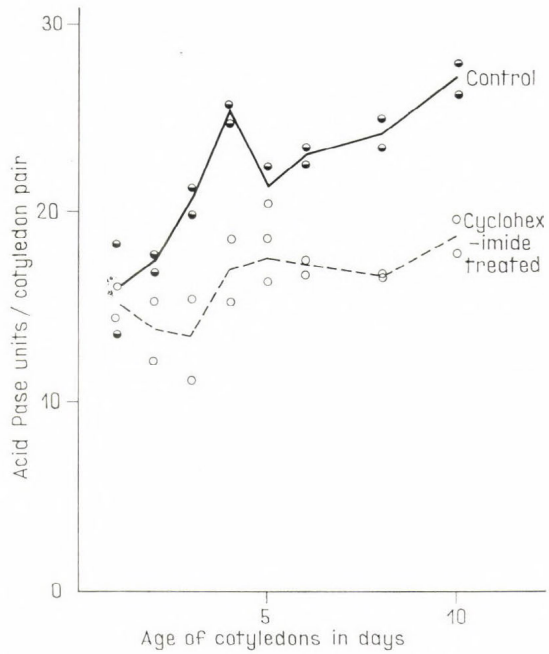

Fig. 6. The effect of cycloheximide on acid phosphatase development in the cotyledons of germinating *Pisum arvense*

similar way to that obtaining under normal conditions of germination (Fig. 7).

When pea seeds exhibiting not more than 6 % germination are maintained under normal germinating conditions, it is found that the imbibed seeds

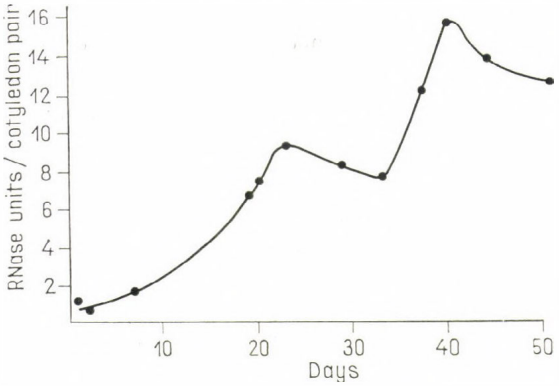

Fig. 7. Development of ribonuclease activity in the cotyledons of *Pisum arvense* during germination at 2—4 °C

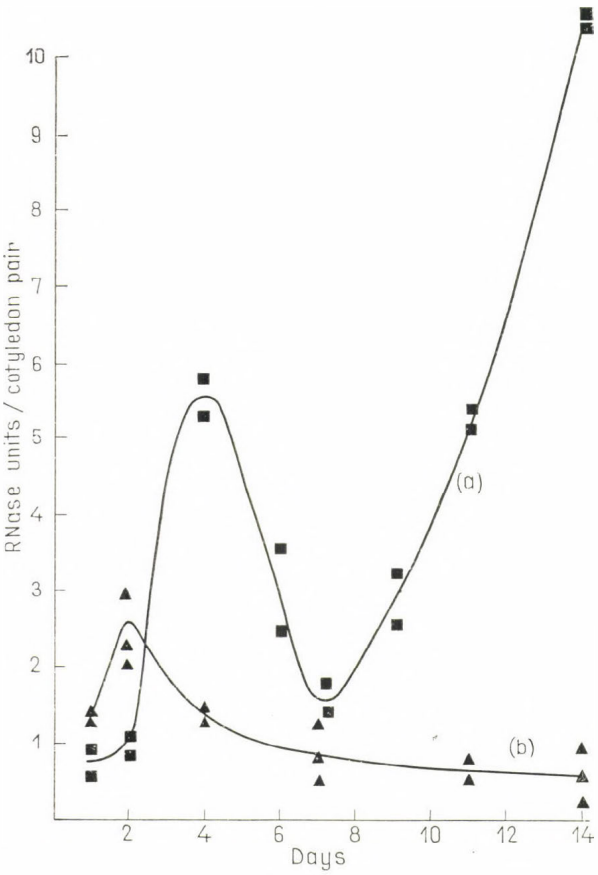

Fig. 8. Development of ribonuclease activity in the cotyledons of (a) n ormal seed maintained at 20 °C, (b) naturally a ged seed under the same c onditions

have approximately twice the normal activity of RNase and a further rise to a maximum is observed after two days (Fig. 8). However, no late phase of activity is observed. It thus appears that developmental control

Table 3

Incorporation of [^{14}C] leucine into protein of cotyledons of artificially aged seed of Pisum sativum

Percentage germination	[^{14}C]leucine incorporated (percent of control)	
	Total homogenate	12,000 g supernatant
88	100	100
(Control)		
49	25	24
0	0·2	0·1

Table 4

Incorporation of [^{3}H] uridine into RNA of artificially aged seed of Pisum sativum

Percentage germination	[^{3}H]uridine incorporated (percent of control)	
	Cotyledons	Embryo
79	100	100
(Control)		
0	22	22

of the release of RNase from inactive protein is not obligatory and that production of the second phase of activity is abolished. Incubation of artificially aged pea seed with [^{14}C] leucine results in virtually no incorporation of label into protein (Table 3) which is in accord with the assumption that late-phase RNase arises by *de novo* synthesis. In contrast to the results with [^{14}C] leucine, incorporation of [^{3}H] uridine into RNA of artificially non-viable seed is maintained at 22% of that in untreated seed (Table 4). If the reverse had been the case, it would have provided presumptive evidence for the presence of active species of RNA in the absence of synthesis, but, on the evidence available, no conclusion can be reached regarding protein synthesis in absence of RNA synthesis in pea seed either during germination or during loss of viability. It has not yet been established what species of RNA become labelled in non-viable seed, but it appears that translation is abolished earlier than transcription during loss of viability.

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METHYLATION OF NUCLEIC ACIDS BY HIGHER PLANTS

by

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INTRODUCTION

Methylation of nucleic acids occurs principally with tRNA and DNA. It consists of an enzymatic recognition of a special site and incorporation of a methyl radical into a preformed polymer. The enzymes are specific for tRNA or DNA, specific for the nucleotide methylated and for the site of nucleotide within the polynucleotide chain. The donor of the methyl radical is S-adenosylmethionine.

Till now most of the research on methylation was carried out on bacteria like *E. coli*. Biological significance of the methylation of nucleic acids is well known only for the methylation of DNA in *E. coli* and some bacteriophages, where it is in direct relation with host determined modification-specificity (Arber and Dussoix, 1962; De Waard, 1971). A bacterial host strain can be successfully infected only with a bacteriophage carrying the DNA modified by that particular strain, a property acquired through prior growth on the same strain. Infection of other hosts, leading to inappropriate or no modification of DNA leads to restriction, i.e. breakdown of the invading DNA. Some work has recently been done on animal cells: tRNA methylation by *Tenebrio molitor* (Baliga et al., 1965), by embryos of mice (Rennert, 1970), modification of the methylation level of tRNA after Marek disease (Mandel et al., 1969), and by human cells during carcinogenesis (Srinivasan and Borek, 1964) have been studied.

The present work is a summary of our studies on the methylation and other modification of tRNA and DNA by higher plants.

MATERIALS AND METHODS

Materials:

DEP, diethyl pyrocarbonate (Baycovin from Bayer); PVS, polyvinylsulphate; IPA, 6-($\gamma\gamma$-dimethylallylamino)-purine; YPHC^{14}, Yeast protein hydrolysate, C^{14} labelled, from Schwarz or CEA; (Methyl C^{14})-S-adenosyl-L-methionine 50 mC/mM, from Schwarz or ICN; L-cystine-S^{35} from CEA; *E. coli* K12/161 RC^{rel} met$^-$ resistant to phage T$_1$.; *E. coli B* and *E. coli* K12 58/161 RC^{rel} met$^-$ Cys$^-$.; *Nicotiana tabacum* L., *Brassica oleracea* L., *Corylus avellana* L. and *Valerianella olitoria* Poll.

DNA extraction: from bacteria, following the technique of Marmur, (1960); from plants, following the method of Guille et al., (1967), Quetier et al., (1968).

tRNA extraction: from bacteria with phenol or isoamyl alcohol (Rammler et al., 1965); from plants, using DEP (Solymosy et al., 1968).

Tracer techniques: tRNA methylation: 10 μmoles of Tris buffer, pH 8·0; 4 μmoles of $MgCl_2$; 2 μmoles of 2-mercaptoethanol; 1·2 μg of PVS; 0·5 μC of C^{14}-CH_3-SAM; 10 μOD_{260} of tRNA; enzymes; total in 0·25 ml.

DNA methylation: tRNA replaced by DNA; total in 0·25 ml.

tRNA aminoacylation (Burkard, 1970): cacodylate buffer, pH 7·0, 50 μM; KCl 5 μM; $MgCl_2$ 5 μM; 2-mercaptoethanol 2 μM; ATP 2 μM; PVS 0·0007 mg; tRNA 10 μOD_{260}; labelled amino acids and enzymes; total in 0·25 ml.

tRNA thiolation: Tris buffer, pH 7·5 20 μM; ATP 2 μM; $MgCl_2$ 2 μM; pyridoxal phosphate 0·002 μM; cystine S^{35} 0·2 μM; tRNA 10 μOD_{260}; enzymes; total in 0·25 ml.

RESULTS

Methylating enzymes are easily extracted and partially purified from bacteria following the method of Svenson (1963). The same method is not applicable for plants, because the DNA methylating enzymes from plants are easily sedimented at low speed centrifugation following the homogenization of plant material. The extracts from bacteria methylated both DNA and tRNA but with plants to obtain active systems we had to apply two different specific techniques.

tRNA methylation

Our method of purification of tRNA methylases from plants was based on the work of Hall and Tao (1970), but we included some important modifications: 10 g of fresh material was homogenized at 4 °C with 30 ml HEPES buffer, pH 7·6, 0·05 M, dithiothreitol, 0·004 M, and potassium metabisulfite, 0·1%. The homogenate was filtered through cheese-cloth and centrifuged at 105,000 g. Proteins were precipitated from the supernatant, at pH 5·0 and the enzyme-containing fraction was centrifuged at 12,000 g.

Table 1 shows an example of testing the enzyme activity. Table 2 shows the importance of HEPES buffer, and Table 3 the influence of the concentration of enzymes.

Table 1

Methylation of tRNA of E. coli with enzymes from tobacco

tRNA, *E. coli* 58/161	Enzymes, tobacco	cpm	$\mu\mu$M $^{14}CH_3$
0·5 mg	1·6 mg	251	98
		280	110
0·5 mg after incubation	1·6 mg	103	40
		117	44

Extent of methylation of tRNA: 156 cpm or 62 $\mu\mu$M.

Table 2

Effect of HEPES buffer on the methylation of tRNA

tRNA, *E. coli* 58/161	Enzymes, tobacco	cpm	$\mu\mu$M $^{14}CH_3$
0·5 mg	1·5 mg	406	158
		431	167
0·5 mg after incubation	1·5 mg	158	62
		168	65

Extent of methylation of tRNA: 256 cpm or 100 $\mu\mu$M.

Table 3

Effect of enzyme concentration in the methylation of tRNA

tRNA, *E. coli* 58/161	Enzymes, tobacco	cpm	$\mu\mu$M $^{14}CH_3$
0·5 mg	7·5 mg	658	256
0·5 mg after incubation	7·5 mg	283	110

Extent of methylation of tRNA: 375 cpm or 146 $\mu\mu$M.

Table 4

Inhibition of the methylation of tRNA by pH 5 supernatant

tRNA, *E. coli* 0·5 mg	Precipitated proteins	Supernatant proteins	cpm	$\mu\mu$M
Incubated	2·1	—	358	139
			292	113
Added after incubation	2·1	—	157	61
			167	65
Incubated	2·1	2·8	173	67
			149	59
Added after incubation	2·1	2·8	146	56
			145	56

Extent of methylation of tRNA with the precipitate: 163 cpm; with the precipitate plus supernatant: 16 cpm.

The temperature optimum was 30 °C. During 10 hours of incubation the incorporation continued. It was possible to inhibit the methylation with some products contained in the pH 5 supernatant (Table 4).

DNA methylation

Because of the rapid sedimentation of DNA methylases from plants after low speed centrifugation, it is difficult to study the properties of these

enzymes. However, experiments carried out with crude extracts demonstrate the existence of DNA methylases in higher plants.

Our attempts to purify these enzymes failed.

tRNA thiolation

Methylation is not the only enzymatic modification process of nucleic acids. Thiolation of tRNA is known with bacteria, and specific enzymes catalysing this process are present in cell-free extracts (Hayward and Weiss, 1966; Lipsett and Peterkofsky, 1966). Harris et al. (1969) using a special mutant of *E. coli*, K 12 58/161 met^- cys^-, have been able to incorporate radioactive sulphur into tRNA extracted from the same strain. The enzymes responsible were present in the same cell-free extract.

For plants, we demonstrated the existence of enzymes thiolating tRNA from plants and tRNA from an *E. coli* strain deficient for cystine. Our technique employed for the extraction of enzymes will be published elsewhere (Janssens de Varebeke et al.).

Table 5

In vitro methylation of tRNA with IPA

	− IPA	+ IPA
tRNA from *E. coli* and enzymes from	355	830
E. coli	428	894
Controls with IPA	178	
	199	
tRNA from tobacco and enzymes	174	215
from *E. coli*	167	327
Controls with IPA	88	
	136	
tRNA from tobacco and enzymes	171	218
from tobacco	179	203
Controls with IPA	115	
	127	

Table 6

Methylation in vitro of tRNA with IPA. Comparison of values calculated for 1 mg of tRNA

	− IPA	+ IPA
tRNA from *E. coli* and enzymes from *E. coli*	710	1660
	856	1968
tRNA from tobacco and enzymes from *E. coli*	696	1308
	668	860
tRNA from tobacco and enzymes from tobacco	1368	1744
	1432	1624

All values are in cpm.

Influences of cytokinins on the methylation and aminoacylation of tRNA

The cytokinins, more particularly 6-($\gamma\gamma$-dimethylallylamino)purine, a natural one, play an important role in cell differentiation. In vitro action of IPA on methylation and aminoacylation is presented in Tables 5 to 10.

IPA increased the extent of methylation for all tRNA-enzyme combinations tested. The efficiency of IPA was, however, different in different systems (Tables 5 and 6). In contrast, IPA decreased the rate of aminoacylation.

Some effect of IPA on the hydrolysis of tRNA in the incubation mixture has also been observed (Abeels and Montasser Kouhsari, 1971).

Table 7

Aminoacylation of tRNA from E. coli with enzymes from E. coli in the presence and absence of IPA

Treatment	cpm	mμg YPH ^{14}C
With IPA	111	2,264
	136	2,774
Without IPA	496	10,123
	339	7,015
Controls	101	2,060
	110	2,244

Table 8

Aminoacylation of tRNA from E. coli with enzymes from plants in the presence and absence of IPA

Treatment	cpm	mμg YPH ^{14}C
With IPA	458	1·78
	433	1·68
Without IPA	6601	25·61
	6835	26·52
Controls	70	0·27

Table 9

Aminocylation of tRNA from plants with enzymes from plants in the presence and absence of IPA

Treatment	cpm	mμg YPH ^{14}C
With IPA	322	1·25
	372	1·44
Without IPA	1185	4·59
	900	3·48
Controls	70	0·27

Table 10

Aminoacylation of tRNA from plants with enzymes from E. coli in the presence and absence of IPA

Treatment	cpm	mμg YPH ^{14}C
With IPA	986	3·82
	1022	3·96
Without IPA	571	2·20
	655	2·54
Controls	559	2·16

CONCLUSIONS

The presence of tRNA- and DNA-methylases as well as tRNA thiolases was demonstrated in plants. Our results show that species homologous and development heterologous modifications are possible in higher plants. Previous results indicated variations in the methylation pattern during development. It appears that the modification of tRNA and DNA is in relation to cell differentiation and development. Research along these lines is continued in our laboratory to throw some light on the role of nucleic acid modification in cell differentiation.

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USE OF BENTONITE IN THE ISOLATION OF tRNA FROM PLANT LEAVES

by

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For chemical studies we were interested in isolating comparatively large amounts of tRNA from plant leaves. Isolation of total RNA with phenol followed by fractionation by salt precipitation had been used to isolate tRNA from wheat germ (Glitz and Dekker, 1963) but in our experience, application of this method to tissues such as leaves which contain comparatively large amounts of ribonuclease can result in tRNA markedly contaminated with degradation products of ribosomal RNA. To avoid this it is preferable to remove ribosomes intact and inhibit ribonuclease at the earliest possible stage. During work on the purification of plant viruses (Dunn and Hitchborn, 1965) it was observed that bentonite, largely in the magnesium form, would adsorb ribosomes and most proteins from leaf sap (Fig. 1). Under similar conditions RNA was not adsorbed. As bentonite is a potent inhibitor of ribonuclease (Brownhill et al., 1959; Fraenkel-Conrat et al., 1961; Huppert and Pelmont, 1962) it seemed possible it might prove useful in preparing undegraded tRNA from leaf tissue.

Initial experiments with Chinese cabbage indicated that RNA could be isolated from bentonite treated sap following treatment with phenol and chromatography on DEAE with similar conditions to those used by Holley et al., (1961). However, it was necessary to have EDTA present in the solution used to dissolve the ethanol precipitate, as this contained a large amount of otherwise insoluble magnesium phosphate. The resulting white product was found to contain RNA with similar proportions of minor nucleotides to RNA isolated from the 105,000 g supernatant of sap from similar leaves (Table 1) (Dunn, 1965). Application of this and slightly modified methods to leaves from other species (Fig. 2), particularly tobacco and French beans, resulted in products (Fraction IV) highly contaminated by polyphenols. The addition of KCN (10^{-3} M), avoiding contact with iron and homogenising the preparation below 5 °C, all reduced polyphenol formation, but did not markedly reduce the contamination of the tRNA. Attempts to remove the polyphenols from the RNA using "Polyclar AT" (Loomis and Battaile, 1966), differential adsorption onto charcoal (Dutta et al., 1953), extraction with methoxyethanol (Kirby, 1956), or chromatography on DEAE using urea (Cherayil and Bock, 1965), were all unsuccessful and emphasized the similar properties of the polyphenol-containing material to the tRNA. While the affinity of tRNA for DEAE does not vary very markedly over the pH range 7—4, it was found that the polyphenol-containing material lost its affinity as the pH was reduced. This indicated that the polyphenols were not adsorbed onto the RNA but onto polymers

containing carboxylic acid groups, probably acidic polysaccharides. This observation was the basis of a method for removing the contamination.

Cherayil and Bock (1965) had chromatographed tRNA on DEAE at pH 4 and their observations were useful in devising a suitable separation

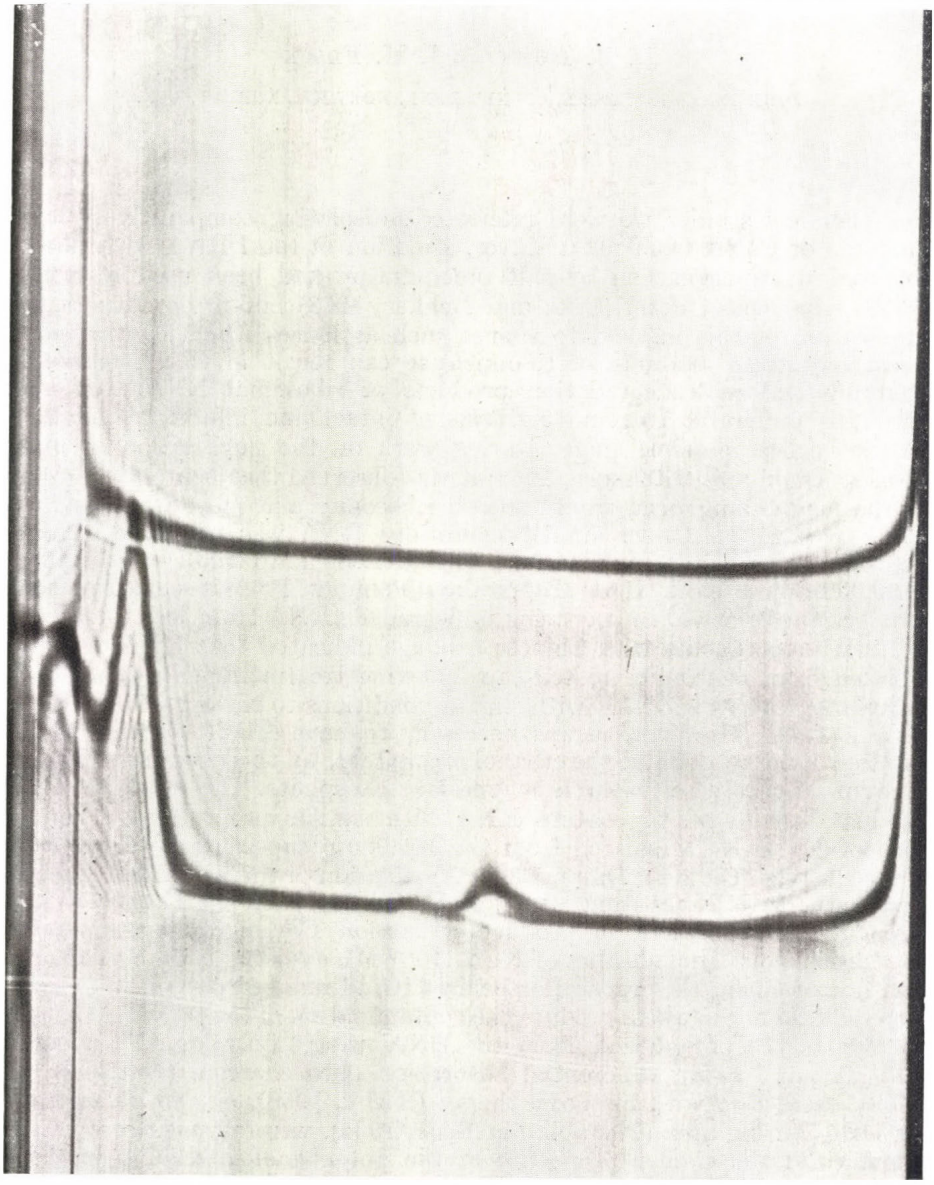

Fig. 1. Effect of magnesium bentonite on leaf sap from Chinese cabbage. Schlieren ultracentrifuge pattern at 35,600 rpm; bottom: sap expressed from Chinese cabbage leaves; top: the same sap after treatment with 25 mg/ml magnesium bentonite in presence of 5 mM $MgSO_4$

Table 1

Chinese cabbage leaf tRNA
Minor nucleosides in Tris-DEAE Fraction IV.
Separate batches of leaves were used for the 3 preparations.
Values are mole/100 mole uridine

Nucleoside	Centrifugation prepared	Bentonite prepared (2 samples)	
Pseudouridine	15·3	15·0	14·9
5-Methyluridine	4·2	6·3	3·2
5-Methylcytidine	4·2	4·3	4·0
1-Methyladenosine	3·4	3·3	2·9
1-Methylguanosine	2·0	2·0	2·2
N^2-Methylguanosine	1·1	1·2	0·6
N^2, N^2-Dimethylguanosine	2·6	3·3	2·1
7-Methylguanosine	1·5	2·3	1·4
Terminal groups			
Guanosine diphosphate	4·7	4·7	4·6
Adenosine	2·6	3·8	6·9

technique. This was finally achieved using formate buffer pH 3 and 0·3 M NaCl to elute the brown material without any of the RNA (Fig. 3). While the major part was eluted in this way, some remained on the DEAE and in order to reduce contamination of the tRNA to a minimum, the latter was eluted at pH 5 with the minimal salt concentration (0·6 M NaCl) needed

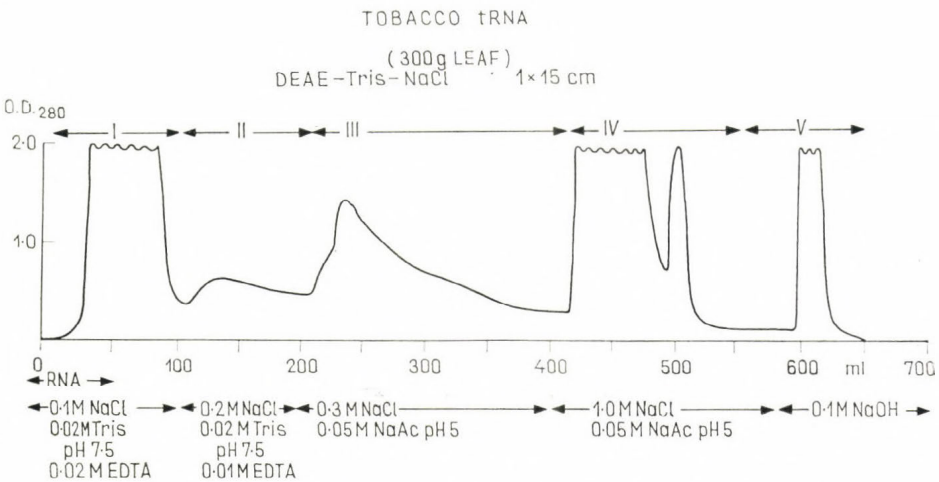

Fig. 2. Tris-DEAE chromatography of tRNA preparation from tobacco leaves. Ethanol precipitate made after successive treatments with bentonite and phenol used. Recording of extinction at 280 mμ of effluent from column. The tRNA is contained in Fraction IV

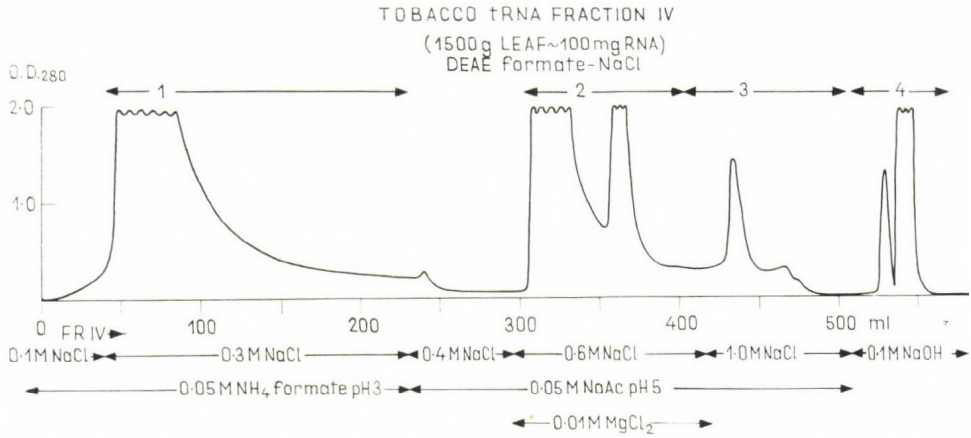

Fig. 3. Formate-DEAE chromatography of tRNA preparation from tobacco leaves. Tris-DEAE Fraction IV used. Recording of extinction at 280 mμ of effluent from column. Polyphenols elute in fraction 1, tRNA in fraction 2

when 0·01 M $MgCl_2$ was also present. Some 10% of the tRNA remains on the DEAE and can be eluted with M NaCl only after the Mg^{++} is removed by treatment with EDTA in Tris buffer, pH 7·5. When refractionated on a DEAE formate column this RNA elutes in fraction 2 and analysis (Table 3, fraction 3) shows that it has a similar composition to the bulk of the tRNA. It is possible that this behaviour results from aggregation of the tRNA induced by the low pH and presence of Mg^{++} (Zachau, 1968).

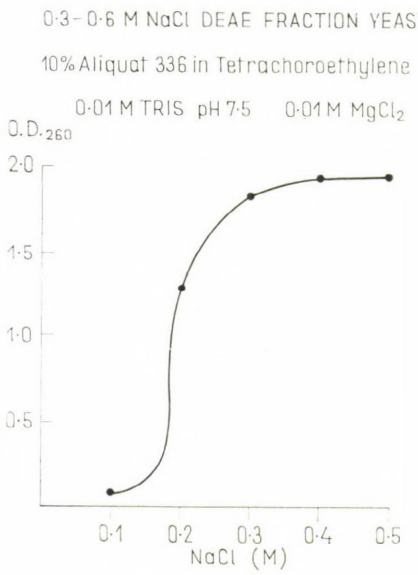

Fig. 4. Absorption of tRNA-like fraction of yeast RNA by Aliquat 336 in tetrachloroethylene. Extinction at 260 mμ of aqueous phase with variation of NaCl concentration

Even after this step the tRNA still contains traces of the brown material. During attempts to remove these from the Tris-DEAE Fraction IV we tested separation on an Aliquat 336 (tricaprylylmethyl ammonium chloride) — Freon — Chromosorb W column of Weiss and Kelmers (1967). The low capacity of the column resulted in saturation with our impure preparation but it was noted that the brown material was retained in preference to the RNA. It has proved possible to use Aliquat 336 dissolved in tetrachloroethylene in a two phase system to achieve the final purification of the tRNA. As shown in Fig. 4, tRNA is eluted from the organic phase by 0·3 M NaCl, while the

residual polyphenol-containing polysaccharide requires 0·5—0·6 M NaCl. The effect on the purification of the tRNA is illustrated by the spectra in Fig. 5. As the aqueous phase acquires some ultraviolet absorption due to the organic solvent, the correct spectrum of the RNA is only seen after it has been precipitated with ethanol and redissolved in aqueous solution (Fig. 5, curve 5).

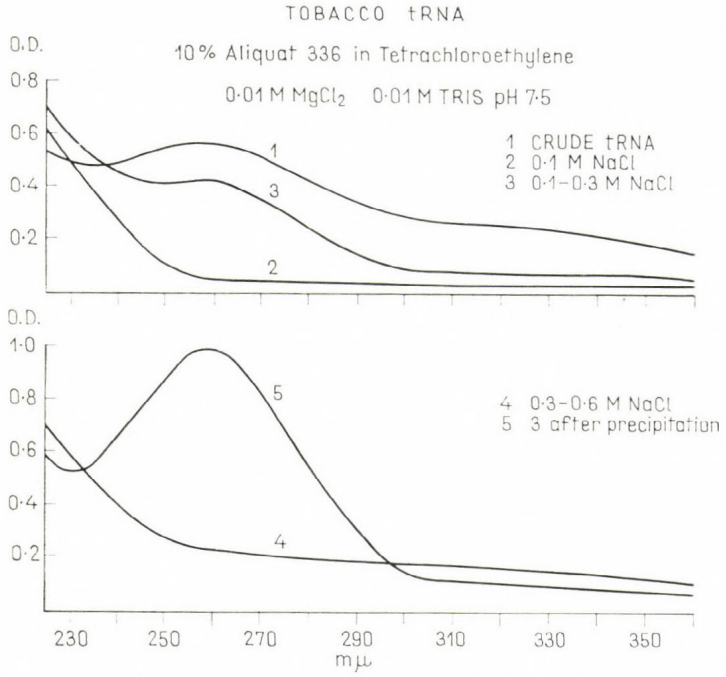

Fig. 5. Spectra of aqueous solution showing purification of tobacco tRNA by Aliquat 336-tetrachloroethylene. To emphasise the absorption due to the polyphenols a Tris-DEAE Fraction IV sample was used rather than the formate-DEAE fraction 2 normally used
1. Initial RNA in 0·1 M NaCl-Tris-$MgCl_2$
2. 1 after treatment with Aliquat-tetrachloroethylene
3. 0·3 M NaCl-Tris-$MgCl_2$ extract from organic phase of 2
4. 0·6 M NaCl-Tris-$MgCl_2$ extract from organic phase of 3
5. 3 after ethanol precipitation, redissolved in Tris buffer

In order to compare the yield and composition of the bentonite prepared tRNA with that isolated from the 105,000 g supernatant, leaves from tobacco were employed. Half of each leaf was used for the two methods and similar purification with phenol and DEAE chromatography to isolate Fraction IV was used. There was no significant difference in the yields obtained by the two methods and as shown in Table 2 the proportions of minor nucleotides in the RNA samples were very similar. Examination of the terminal nucleosides, however, indicated that whereas the sample isolated by centrifugation had both adenosine and cytidine terminal groups, only adenosine was found in the bentonite prepared sample. We

conclude that *in vivo* all the tRNA is terminated with adenosine but that during the isolation procedure using centrifugation enzymic removal of some terminal adenosine groups occurs.

Table 2

Tobacco leaf tRNA
Minor nucleosides in Tris-DEAE Fraction IV.
The same batch of leaves was used for the two preparations.
Values are mole/100 mole uridine

Nucleoside	Centrifugation prepared	Bentonite prepared
Pseudouridine	13·3	12·6
5-Methyluridine	4·4	4·0
5-Methylcytidine	3·7	3·6
1-Methyladenosine	3·8	3·8
1-Methylguanosine	1·9	1·9
N^2-Methylguanosine	1·2	1·1
N^2, N^2-Dimethylguanosine	2·7	2·3
7-Methylguanosine	1·1	1·3
Inosine	0·5	0·6
Terminal groups		
Guanosine diphosphate	7·3	5·9
Adenosine	4·8	6·3
Cytidine	1·4	—

Table 3

Analyses of DEAE fractions, tobacco leaf tRNA; values are mole/100 mole uridine

Nucleoside	Tris DEAE IV	Formate DEAE 2	2 After Aliquat 336 -tetrachloro-ethylene	Formate DEAE 3	Formate DEAE 4
Pseudouridine	14·9	14·6	16·2	15·8	10·3
5-Methyluridine	4·0	5·3	6·2	6·4	2·7
1-Methyladenosine	3·9	4·1	3·4	3·7	1·8
1-Methylguanosine	2·7	2·5	2·5	2·5	1·0
N^2-Methylguanosine	1·3	1·2	0·6	1·0	0·3
N^2, N^2-Dimethylguanosine	2·1	2·2	2·3	2·9	0·8
7-Methylguanosine	1·5	1·6	1·7	1·0	0·5
Inosine	0·6	0·6	0·7		0·2

Formate-DEAE 3 represents RNA eluted with M NaCl after Tris-EDTA treatment of the DEAE column.

Formate-DEAE 4 represents RNA eluted with 0·1 N NaOH from a formate-DEAE column not treated with Tris-EDTA, so will include some RNA normally in fraction 3.

The proportion of minor nucleotides in various fractions from the bentonite method are shown in Table 3. Fraction 4 which is eluted with 0·1 M NaOH probably represents high molecular weight RNA together with the additional 10% of tRNA which was not removed from the DEAE by Tris-EDTA treatment before this particular sample was eluted. The origin of the high molecular weight RNA which is eluted from both the Tris-DEAE and formate-DEAE columns is still obscure. A possibility that it originates from ribosomes cannot be entirely discounted as we have observed that when purified ribosomes are adsorbed onto bentonite, RNA

Table 4

Release of RNA from bean leaf ribosomes

Purified ribosomes (0·5 mg/ml) in 5 mM $MgSO_4$, 10 mM Na/K phosphate, pH 7·4 were treated with 5 mg/ml of magnesium bentonite. Immediately or after the incubation shown, the bentonite was removed by centrifuging at 15,000 rpm. for 10 min at 0°. Values represent the extinction at 260 mμ of supernatants expressed as percentages of that of a sample which contained no bentonite. Ribosomes prepared by method of Dunn and Hitchborn (1965)

Treatment	Temperature	
	0°	20°
Centrifuged immediately	5	15
Incubated without bentonite 3·5 h, then bentonite added and centrifuged immediately	6	24
Incubated 3—5 h with bentonite before centrifugation	24	46

Table 5

Incorporation of ^{14}C-leucine into tRNA using reticulocyte enzymes

tRNA added	Cpm	Cpm/mg RNA	mμ mole Leucine/mg RNA
None	36		
20 μg Yeast	258	11,120	0·82
20 μg Rabbit reticulocyte	443	20,370	1·50
20 μg Tobacco leaf 1	425	19,450	1·43
20 μg Tobacco leaf 2	351	15,790	1·16
20 μg Tobacco leaf 3	261	11,260	0·83
20 μg French bean leaf	305	13,490	0·99
20 μg Tobacco 1 + NaOH incubation	10		

Incubation mixtures (0·2 ml) contained 0·1 ml enzyme and 0·1 μC ^{14}C-leucine, 10 μM each 19 amino acids, 5 mM ATP, 0·1 mM CTP, 3 mM mercaptoethanol, 8 mM $MgAc_2$, 100 mM KCl, 20 mM Tris, pH 7·8. Incubation at 36° for 25 min. RNA precipitated with CTAB at pH 5 with addition of 1 mg yeast RNA.

is slowly released even at low temperature (Table 4). This release is dependent on salt concentration and is slower at pH 6·5 (the pH of bentonite-treated sap) than at pH 7·4 shown in Table 4. In order to test whether this bentonite-induced degradation of ribosomes was occurring in our tRNA preparation method, we incubated the homogenate for different lengths of time at 2 °C prior to removing the bentonite by centrifugation. The amount of RNA in the supernatant was not found to increase with time, indicating that our preparations are probably not contaminated with ribosomal RNA.

The yield of tRNA made from leaves of tobacco and beans by the bentonite method amounted to 2—4 mg/100 g leaf tissue. It was found to have a $S_{20,w}$ of 4·0 and was tested for amino acid acylation using a crude enzyme preparation from rabbit reticulocytes (Table 5). Incorporation in the absence of added RNA was due to a small amount of tRNA in the enzyme preparation. More leucine was incorporated into the leaf tRNA preparations than into a yeast tRNA preparation and the best tobacco preparation exhibited a similar incorporation to that of reticulocyte tRNA. Controls incubated with alkali were included to ensure that all the radioactivity was due to aminoacylation rather than protein synthesis.

As much of the isolation procedure is carried out at room temperature, and some of the aminoacylation experiments were performed on preparations stored for over 12 months, it seems certain that the preparations are completely free of ribonuclease.

As the minor nucleotide Y recently identified as a guanine derivative (Nakanishi et al., 1970) occurs in wheat germ $tRNA^{Phe}$ (Dudock et al., 1968) as well as in yeast $tRNA^{Phe}$ (RajBhandary et al., 1967) it seems probable that it is present in tRNA from other plants. As the free base of this nucleotide is released from RNA by incubation at pH 2·9 (Thiebe and Zachau, 1968) it seems likely that one defect of our method will be the inactivation of $tRNA^{Phe}$ by the pH 3 treatment.

A brief account of the bentonite method has previously been published (Dunn and Flack, 1970). The aminoacylation experiments were carried out in the Department of Biochemistry of the University of Cambridge. We would like to thank Dr. R. J. Jackson for his advice on these experiments and Professor F. G. Young for allowing one of us (D.B.D.) to spend a period in his laboratory.

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NEW ASPECTS OF THE APPLICATION OF DIETHYL PYROCARBONATE IN NUCLEIC ACID RESEARCH

by

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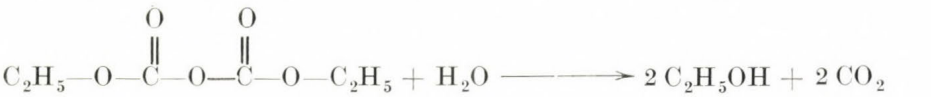
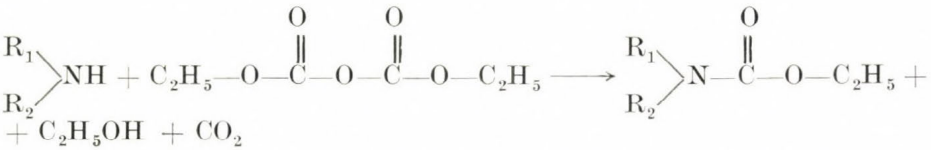
Diethyl pyrocarbonate (DEP; Mwt. 162, sp. gr. 1·12) is a colourless clear liquid with a very low solubility in water. A saturated DEP solution is 40 mM with respect to DEP.

DEP decomposes rapidly in water ($t_{1/2} \simeq 1$ hour at 20 °C and $\simeq 10$ min at 37 °C) with the formation of ethanol and carbon dioxide:



In the presence of reactive groups, such as primary or secondary amines, SH or OH groups, it gives rise to carbethoxylated products, e.g.



Since in proteins all the above reactive groups occur, it is not surprising that DEP reacts with proteins. The reaction leads to denaturation of the protein. It was suggested some years ago (Fedorcsák and Ehrenberg, 1966) that the reaction between proteins and DEP should be exploited by using DEP as a nuclease inhibitor in the extraction of undegraded nucleic acids. The idea was correct, and we succeeded in developing a method based on the use of DEP as a nuclease inhibitor for the extraction of undegraded nucleic acids from plant tissues (Solymosy et al., 1968; Solymosy et al., 1970; Bagi et al., 1970). The method has since been applied by a number of investigators for the extraction of undegraded nucleic acids from bacteria (Summers, 1970), fungi (Chet and Rusch, 1970*a*, 1970*b*; Melera et al., 1970), animal (Zsindely et al., 1970) and human (Abadom and Elson, 1970) tissues. With mitochondrial ribosomes the DEP method seems to be the only procedure by which undegraded rRNA can be extracted (Forrester et al., 1970; Anonym, 1971; Lizardi and Luck, 1971).

With respect to both the yields and the extent of degradation of the nucleic acids isolated the DEP method proved to be superior to the conventional phenol method. All nucleic acids extracted by the DEP method from different sources were analytically indistinguishable from nucleic acid preparations obtained by the phenol method (sedimentation properties,

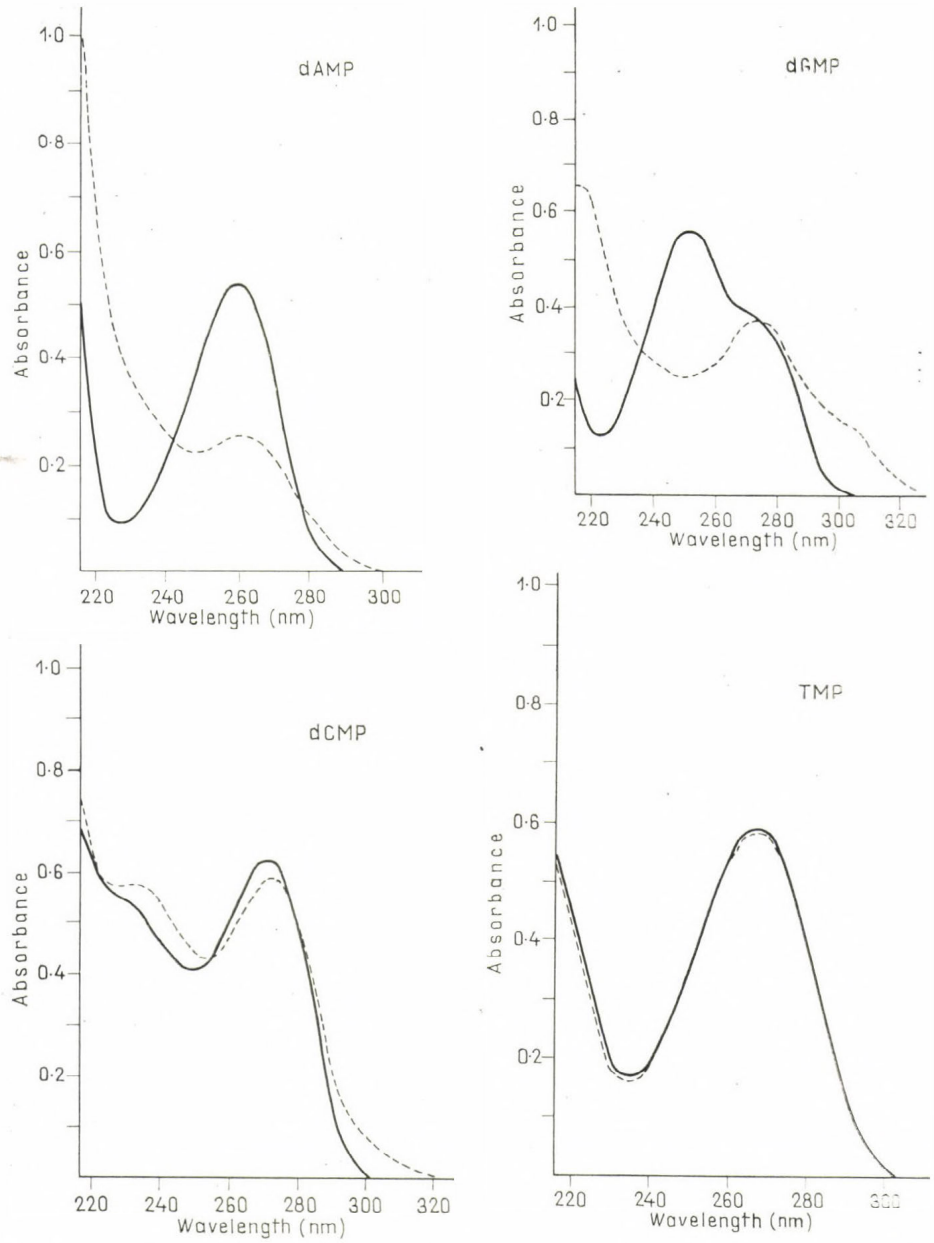


Fig. 1. Effect of treatment with DEP on the UV absorption spectra of deoxyribonucleoside 5' phosphates. To 0·6 ml of a 7×10^{-4} M solution of each deoxyribonucleoside 5' phosphate in 5 mM phosphate buffer, pH 7, 35 μl DEP (- - - - -) or 28 μl abs. ethanol (———) was added and the mixtures were shaken for 6 h at 37 °C. After incubation the samples were diluted with 5 mM phosphate buffer, pH 7, to give a reading of about 0·5 at 260 nm in cells of 1 cm light path. The UV absorption spectra were recorded in a Cary 15 recording spectrophotometer

electrophoretic mobility, behaviour on MAK columns, hybridization with DNA).

With respect to the biological activity of the RNAs extracted by the DEP method it should be emphasized that *DEP as used under the conditions of the extraction* procedure does not influence essentially the biological activity of RNAs (Fedorcsák et al., 1969; Abadom and Elson, 1970). On the other hand, *exhaustive treatment with DEP of isolated biologically active RNA* does lead to the loss of the biological activity of RNA as shown for TMV-RNA by Gulyás and Solymosy (1970) as well as by Oxelfelt and Årstrand (1970), and for single stranded poliovirus RNA by Öberg (1970).

The underlying mechanism of the inactivation by DEP of infectious RNA is most probably the carbethoxylation by DEP of the bases, as shown for adenine by Leonard et al. (1970). Carbethoxylation of nucleic acid components, whether separately or in a polynucleotide chain, is reflected by characteristic changes in the UV absorption spectra of DEP-treated bases, nucleosides, nucleotides (Fig. 1) and nucleic acids (Fig. 2), respectively.

An interesting aspect of the carbethoxylation of polynucleotides is that DEP appears to react only with single stranded nucleic acids but not with double stranded ones. This is shown by the following observations:

1. Neither the transforming activity of bacterial DNA (Fedorcsák and Turtóczky, 1966), nor the transfectivity of double stranded phage DNA (Kondorosi et al., 1971) is influenced by DEP-treatment.

2. DEP inactivates the infectivity of single stranded poliovirus RNA but not that of the double stranded replicative form (Öberg, 1970).

Since (a) DEP reacts only with single stranded nucleic acids (Öberg, 1970) and most probably only with the single stranded regions of a random coil structure (Öberg, 1971) and (b) this reaction is reflected by a characteristic change in the UV absorption spectrum, it should be possible to determine the extent of single-strandedness of a nucleic acid by reacting it with DEP and taking the UV spectrum of the reaction product. A quantitative parameter of the observed spectral change is provided by the decrease of given absorbance ratios ($A_{260\ nm}/A_{230\ nm}$; $A_{260\ nm}/A_{270\ nm}$ and $A_{260\ nm}/A_{280\ nm}$) of DEP-treated nucleic acids as compared to the controls (cf. Fig. 2). If the above correlation in fact exists, the UV spectra of nucleic acids with a more ordered structure are expected to undergo less change (i.e. there should be smaller decrease of a given absorbance ratio) upon exhaustive DEP treatment than those of nucleic acids with a less ordered structure.

Indeed, nucleic acids with a more ordered structure were found to be less reactive towards DEP than those with a less ordered structure (commercial yeast RNA > highly polymerized RNA > heavy rRNA from barley > tRNA (Fig. 3). Additional proof for the dependence of the reaction on the secondary structure of nucleic acids was obtained by carrying out the treatment of one particular nucleic acid with DEP in solutions of different ionic strengths (Fig. 4). It may be seen that increase in ionic strength, resulting in a more ordered structure of nucleic acids, was associated with a reduced reactivity of the nucleic acid preparations. This was shown even more conclusively by comparing the reactivity towards DEP of single stranded DNA with that of double stranded DNA (Fig. 5).

In further experiments it was tested whether there is a *quantitative* relation between the extent of single strandedness of a given nucleic acid preparation and the decrease of absorbance ratios calculated from the spectra of the nucleic acid subjected to exhaustive treatment with DEP. For this purpose DNA was isolated from *E. coli* according to Thomas et al. (1966), and half amount of the DNA preparation (2·5 $A_{260\ nm}$ units/ml) was heat denatured for 10 min at 100 °C and rapidly cooled in ice. This sample was designated denatured (D) DNA. The other half of the DNA preparation was kept in the native state (N). Then D and N were mixed in different proportions as shown in Fig. 6, and their UV spectra taken. As seen in Fig. 6 increasing amounts of D in the mixtures led to increased hyperchromicity at 260 nm.

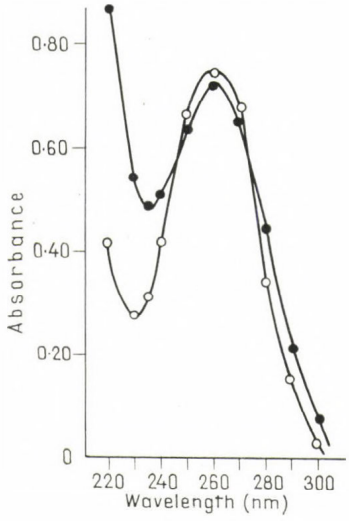


Fig. 2. Effect of treatment with DEP on the ultraviolet absorption spectrum of commercial yeast RNA. Commercial yeast RNA (9 μmoles of nucleotide residue/ml) was dissolved in 4 ml 0·15 M NaCl and shaken without the addition (○ — ○) or with 250 μl DEP (about 11-fold excess over saturation level) added (● — ●) on a wrist action shaker for 8 h at room temperature. The samples were then diluted 100-fold and their ultraviolet absorption spectra taken

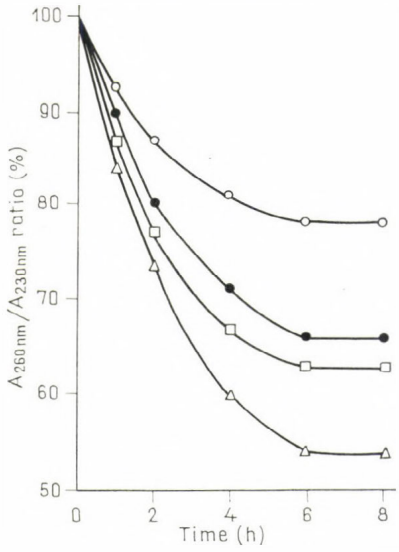


Fig. 3. Time-course of spectral changes in nucleic acids upon treatment with DEP. Rate of decrease of the $A_{260\ nm}/A_{230\ nm}$ ratio. Nucleic acids (9 μmoles of nucleotide residue/ml) were dissolved in 4 ml 0·15 M NaCl and were shaken with or without the addition of 250 μl DEP (about 11-fold excess over saturation level) on a wrist action shaker for 8 h at room temperature. At the time intervals indicated aliquots were withdrawn, diluted 100-fold and their ultraviolet absorption spectra taken. The $A_{260\ nm}/A_{230\ nm}$ ratios are expressed in percentage of the $A_{260nm}/A_{230\ nm}$ ratio of the respective control sample shaken under the same conditions in the absence of DEP. ○ — ○, tRNA; ● — ●, barley heavy tRNA; □ — □, highly polymerized RNA; △ — △, commercial yeast RNA

The samples were then divided into three aliquots. One aliquot served for the determination of the amount of native DNA in the mixtures by a spectrophotometric method described by Hirschman and Felsenfeld (1966). This was necessary to be able to estimate quantitatively the amount of double stranded structures in D due to renaturation. To the other aliquot (0·6 ml) DEP was added at a rate of 58 μl/ml (10-fold saturation). To the third aliquot (0·6 ml) abs. ethanol was added at a rate of 47 μl/ml (concentration of ethanol arising from the decomposition of DEP at a concentration of 58 μl/ml).

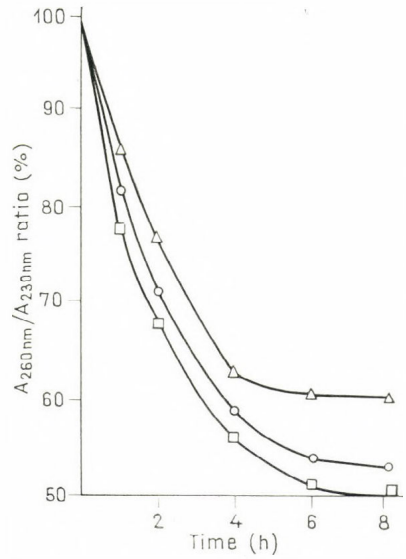


Fig. 4. Dependence of the reaction of commercial yeast RNA with DEP on the ionic strength of the milieu as revealed by changes in the ultraviolet spectrum. Extent of decrease of the $A_{260\,nm}/A_{230\,nm}$ ratio. Commercial yeast RNA (9 μmoles of nucleotide residue/ml) was dissolved in 4 ml of 0·03 M NaCl (□ — □), 0·15 M NaCl (○ — ○) or 0·05 M $MgCl_2$ (△ — △) and shaken on a wrist action shaker with or without the addition of 250 μl DEP (about 11-fold excess over saturation level) for 8 h at room temperature. Thereafter the samples were diluted 100-fold and their ultraviolet spectra taken. The $A_{260\,nm}/A_{230\,nm}$ ratios are expressed in percentage of the respective control sample shaken under the same conditions in the absence of DEP

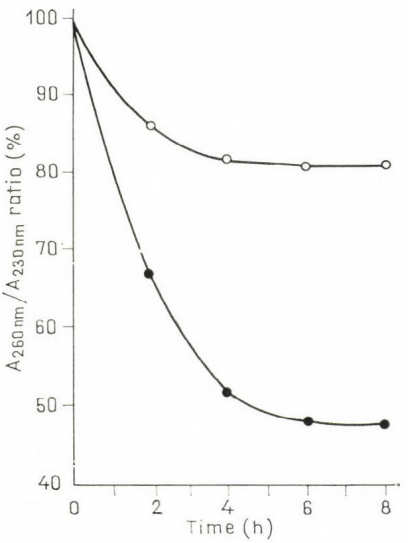


Fig. 5. Reaction of native and denatured DNA with DEP as revealed by changes in the ultraviolet spectrum. Rate of decrease of the $A_{260\,nm}/A_{230\,nm}$ ratio. Double-stranded (○ — ○) or single-stranded (● — ●) DNA in 4 ml of 15 mM sodium citrate (0·17 μmole nucleotide residue/ml) was shaken with or without the addition of 250 μl DEP (about 11-fold excess over saturation level) for 8 h at room temperature. The reaction between DNA and DEP was followed as described in the legend to Fig. 3. Single-stranded DNA was prepared by heat denaturation of native DNA

Treatment with DEP of the DNA samples containing different amounts of D was carried out under continuous shaking for 6 hours at 37 °C. Under these conditions the reagent added is practically completely decomposed. The ethanolic controls were treated under the same conditions. The UV spectra of both the DEP-treated samples (Fig. 7) and the ethanolic controls were recorded immediately after treatment and several absorbance ratios ($A_{260\ nm}/A_{230\ nm}$; $A_{260\ nm}/A_{270\ nm}$ and $A_{260\ nm}/A_{280\ nm}$) were calculated from the spectra. The above absorbance ratios obtained from the spectra of the ethanolic controls did not differ from those calculated from the spectra of the individual mixtures before treatment. The absorbance ratios of the DEP-treated samples were expressed in percentage of the corresponding absorbance ratios obtained with the respective controls. These percentage

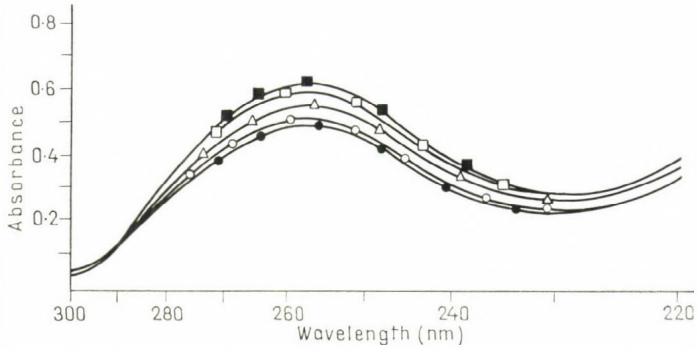


Fig. 6. UV absorption spectra of E. coli DNA samples prepared by mixing native *E. coli* DNA (N) with heat denaturated *E. coli* DNA (D) in different proportions ■ - ■ - ■ -, only D; □ - □ - □ -, 3 parts D + 1 part N; △ - △ - △ -, 1 part D + 1 part N; ○ - ○ - ○, 1 part D + 3 parts N; ● - ● - ●, only N

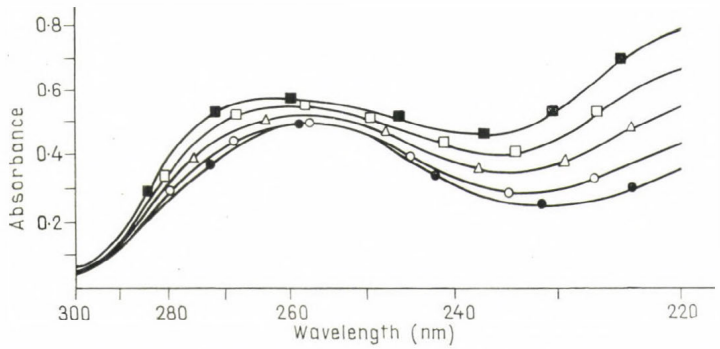


Fig. 7. UV absorption spectra of DEP-treated DNA samples prepared by mixing native E. coli DNA (N) with heat denaturated E. coli DNA (D) in different proportions. Treatment with DEP was done as described in the Text. For further explanation see caption to Fig. 6

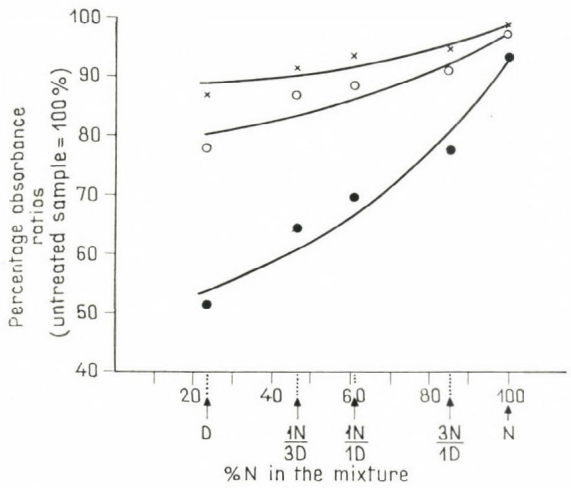


Fig. 8. Dependence of the decrease of some absorbance ratios calculated from the UV spectra of DEP-treated E. coli DNA and expressed in percentage of the corresponding absorbance ratios obtained with the respective controls, on the increase in the amount of denaturated DNA in the mixtures. The amount of denaturated DNA in the mixtures was experimentally determined according to Hirschman and Felsenfeld (1966). N, native DNA; D, denaturated DNA; ● - ● - ●, $A_{260\ nm}/A_{230\ nm}$; × - × - ×, $A_{260\ nm}/A_{270\ nm}$; ○ - ○ - ○ - ○, $A_{260\ nm}/A_{280\ nm}$

values are plotted in Fig. 8 versus the amount of native DNA in the mixtures, as determined by the method of Hirschman and Felsenfeld (1966). It can be seen that the extent of decrease of all three absorbance ratios tested was correlated with the amount of single stranded DNA (D) in the mixture. This suggests that DEP may be a useful tool in the estimation of the single strandedness of DNA samples. Experiments are in course to test the method with DNAs of different base compositions.

Part of the results presented here have been described elsewhere (Solymosy et al., 1971).

SUMMARY

The diethyl pyrocarbonate (DEP) method, developed in our laboratory for the extraction of undegraded nucleic acids, is used in a number of laboratories and, as a rule, yields biologically active nucleic acid preparations. Under specific conditions (exhaustive treatment of isolated nucleic acids), however, DEP reacts with single stranded nucleic acids. The reaction is reflected in well pronounced spectral changes of the treated nucleic acid, and this can be useful in the estimation of single strandedness of nucleic acid preparations as shown here for DNA samples containing different amounts of single stranded structures.

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FACTORS THAT CONTROL RNA POLYMERASE FROM PLANT CELLS

by

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RNA polymerases (I and II) and several protein fractions (A, B and C) from the chromosomal acidic proteins of coconut endosperm nuclei have been isolated. RNA polymerase I purified through the QAE-sephadex step gives a single band in polyacrylamide gel electrophoresis. In gels containing SDS, this enzyme shows multiple bands indicating its subunit nature, pH optima for both RNA polymerases are at 8·0. RNA polymerase I is maximally activated by Mn^{2+}; while II, by Mg^{2+}. The activities of both the enzymes are stimulated by fraction B. All the fractions except A are substantially free from nucleases. Both RNA polymerases require an addition of DNA for activity. Fraction B is ineffective either with denatured coconut DNA or native λ DNA.

The role of protein Factor B and C on RNA synthesis by RNA polymerase I isolated from chromosomal non-histone proteins of coconut nuclei has been studied further. Factor B has been implicated as the initiation factor on the experimental evidences that *a)* in its absence, RNA polymerase I shows only minimal activity; *b)* it can bind with RNA polymerase and the enzyme-factor B complex then binds to DNA, but Factor B alone cannot bind to DNA; c) it promotes the incorporation of β,γ—^{32}P—ATP into RNA and this stimulation reaches a plateau rather quickly while the incorporation of ^{14}C—ATP in the interior of RNA chain continues; *d)* it is active with native homologous DNA as template, but not with denatured or λ DNA; *e)* RNA molecules synthesized in its presence are of higher sedimentation value (10—20S) than that synthesized in its absence (4S); *f)* it can completely counteract the inhibitory effect of rifampicin, which is known to inhibit RNA synthesis at the initiation step.

Factor C seems to facilitate the release of synthesized RNA from the DNA template since (i) it stimulates RNA synthesis by polymerase I when added on top of Factor B, but in absence of Factor B, C alone is inactive (ii) it can reinitiate RNA synthesis after the reaction has reached a plateau in a system where DNA is limiting, an effect similar to that obtained at higher ionic strength. Factor C, however, does not influence the molecular size of RNA synthesized.

INTRODUCTION

The existence of ribonucleic acid polymerase (E.C. 2.7.7.6) has been recognised first by Weiss (1960) from mammalian tissue. The enzyme either in soluble or aggregate form has been reported later from bacteria (Hurwitz et al., 1960; Stevens, 1961; Ochoa et al., 1961), plants (Huang et al., 1960) and animal systems (Biswas and Abrams, 1962). It was thought previously that only the aggregate form was prevalent in eucaryotic organism, but the soluble form has also been isolated from higher organisms (Ramúz et al., 1965; Furth and Loh, 1963). It seems that both the soluble and aggregate forms exist in the eucaryotes, their concentration varies with the physiological condition of the cell (Ishihama, 1967). Specific RNA polymerases have been demonstrated in chloroplasts (Surzycki, 1969; Tewari

and Wildmann, 1969) as well as in mitochondria (Shmerling, 1969). RNA polymerase from these organelles are sensitive to rifampicin while that from the nucleus are insensitive. There are two or three types of RNA polymerase in eucaryotic cells (Widnell and Tata, 1964; Pogo et al., 1967; Maul and Hamilton, 1967; Roeder and Rutter, 1969; Kedinger et al., 1969). They differ in metal ion requirement for their activity (Widnell and Tata, 1964; Pogo et al., 1967; Maul and Hamilton, 1967; Roeder and Rutter, 1969) and also in sensitivity to antibiotics (Kedinger et al., 1969; Mondal et al., 1970). From our laboratory RNA polymerases from the chromatin of coconut differing in metal ion requirement and in susceptibility to rifampicin have recently been reported (Mondal et al., 1970). With the discovery of initiation factor (σ-factor) by Burgess et al. (1969) and termination factor (ρ-factor) by Roberts (1969), a new facet of research on the transcriptional processes has been stimulated. Before these discoveries, Hara and Mitsui (1967), Davidson et al. (1969) and Khesin et al. (1969), also isolated factors that stimulate RNA synthesis. But it is not known whether this is similar to the σ-factor, later discovered by Burgess et al. (1969). A factor that stimulates RNA polymerase from coconut nuclei has been found to have a definite role in initiation (Mondal et al., 1970) Another factor isolated from the acidic protein of chromatin seems to influence the release of RNA synthesized as well as termination (Mondal et al., in press). Some of these aspects are discussed in this lecture.

MATERIALS AND METHODS

Green coconuts *(Cocos nucifera)* 4—5 months old were obtained from the local market. ATP, GTP, CTP, UTP and ion-exchange resins were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Highly polymerized calf thymus DNA was obtained from Worthington Biochemical Corporation; rifampicin (Pitman-Moore, Division of the Dow Chemical Company, Indianapolis, U.S.A.) and DNA were obtained as a gift from Dr. S. Adhya, Bose Institute, Calcutta; ^{14}C-ATP (160 mCi/m mole), ^{14}C-GTP (17 mCi/m mole) and ^{3}H-UTP (2Ci/m mole) were obtained from Radiochemical Centre, Amersham, England, and ^{32}P-orthophosphoric acid was supplied by the Department of Atomic Energy, Trombay, India. ^{32}P-pyrophosphate was prepared from ^{32}P-orthophosphoric acid by the method of Bergman et al. (1961). Nuclei were isolated from the coconut endosperm by the method of Mondal et al. (1970). The crude chromatin was isolated from the nuclei by the method of Bonner and Huang (1963). RNA polymerases and factors were isolated as described earlier (Mondal et al., 1972). Polyacrylamide gel electrophoresis at pH 8·0 followed the general method of Davis (1964). Gels with added 8 M urea as described by Jovin et al. (1964) and with 0·1% SDS as described by Weber and Osborn (1969), were prepared whenever necessary. The pyrophosphate exchange method was followed for RNA synthesis for preliminary assay (Krakaw and Fronk, 1969). Electrophoresis of RNA was carried out in 2·5% polyacrylamide gel according to Bishop et al. (1967). β,γ-^{32}P-ATP was prepared by the method (Bergmann et al., 1961) of $^{32}PP_i$ exchange into β,γ positions of ATP in presence of amino acids and crude activating enzymes from *Azotobacter vinelandii*.

Isolation and purification of RNA polymerase

The acidic proteins were extracted from the chromatin as previously described and this fraction was passed through CM cellulose and the effluent was saturated with 30—55% ammonium sulphate. The precipitate was dissolved and dialyzed against TME buffer (Tris 0·01 M, pH 8·0, mercaptoethanol, 0·001 M and EDTA, 0·0001 M). The preparation was charged on

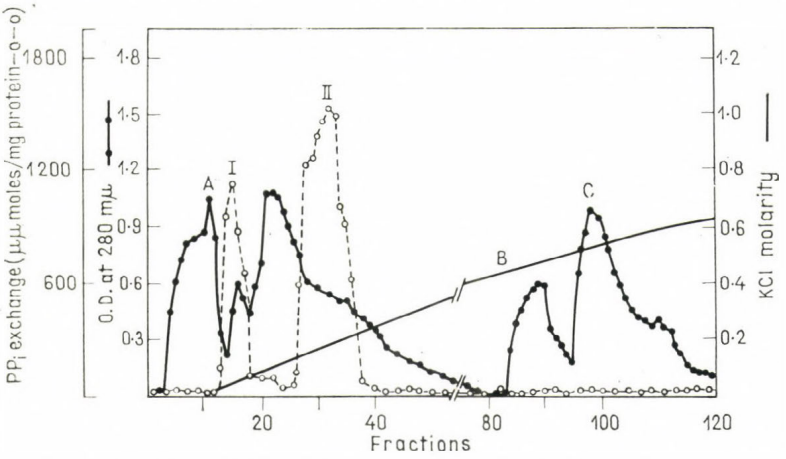

Fig. 1a. Elution profile of RNA polymerases and factors from DEAE cellulose column. The 30—55% ammonium sulphate fraction (27·5 mg) was charged on a (25×0·9 cm) DEAE cellulose column. 1 ml fractions of the KCl eluate (flow rate 1 ml/5 min) were collected and O.D. 280 mμ measured (- ● - ● - ● -). The polymerase activity was assayed with each fraction (- ○ - ○ - ○ -) by the pyrophosphate exchange method (Krakaw and Frank, 1969)

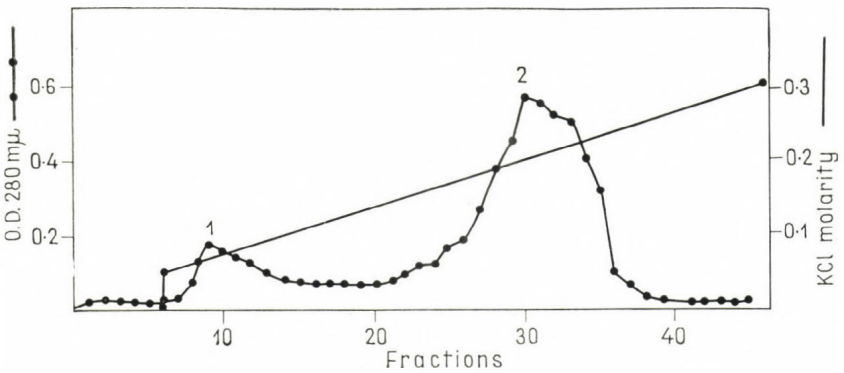

Fig. 1b. Elution profile of RNA polymerase I from QAE Sephadex (A_{50}). 2 ml containing 2 mg of RNA polymerase I obtained from cellulose column chromatography was loaded onto a QAE Sephadex column (25×0·9 cm), 1 ml fractions were collected at the rate of 1 ml/5 min and O.D. 280 mμ was measured

DEAE cellulose column equilibrated with the same TME buffer and eluted with a linear gradient of KCl in the same buffer. The RNA polymerase activities eluted at 0·1 M KCl and 0·2 M KCl were designated as RNA polymerase I and II, respectively. Along with the polymerases three other fractions of proteins (A, B, C) were eluted at 0·05 M, 0·55 M and 0·62 M KCl, respectively (Fig. 1*a*). RNA polymerase I was rechromatographed on a QAE Sephadex column and eluted with a linear gradient of KCl (Fig. 1*b*). RNA polymerase activity was detected in the first peak. Summary of the purification of enzymes is given in Table 1. RNA polymerase I and II are purified 170 and 70 fold, respectively, starting from the soluble chromatin with a recovery of 7% in the former case and 83% in the latter case.

Table 1

Summary of the purification of RNA-polymerases

Step	Total Protein (mg)	Specific activity (Units/mg)[a]	Total Units[b]
1. 2M NaCl extracted soluble chromatin	502	14	7,028
2. Acidic protein	143	44	6,292
3. CM cellulose chromatography at pH 6·4	140	43	6,020
4. 30—55% $(NH_4)_2SO_4$ fractionation	27·5	256	7,040
5. DEAE cellulose[c]			
A. First activity peak (R_I)	2·0	740	1,480
B. Second activity peak (R_{II})	6·0	972	5,832
6. QAE Sephadex (R_I)	0·213	2,340	514

[a] Enzyme activity was assayed by the incorporation of ^{14}C-ATP into acid-insoluble products.

[b] One unit is defined as that amount of enzyme which can incorporate 1 $\mu\mu$mole of ^{14}C-ATP in 15 min under the assay conditions.

[c] After this step of purification factor B (the protein fraction in Fig. 1) was added to the assay system.

Requirements for RNA polymerases

RNA polymerase I displays maximum activity with Mn^{2+} at a concentration of 2 mM. Partial activity with Mn^{2+} is maintained at a broader range (5—10 mM). In case of RNA polymerase II maximum activity is recorded with Mg^{2+} at a concentration of 10 mM but partial activity is obtained with 2 mM of Mn^{2+}. Both polymerases exhibit an absolute requirement for DNA; denatured DNA is slightly effective. If fraction B is omitted the activity decreases to 7—10% of that with fraction B. However, with denatured DNA or λ DNA this decrease is not noted suggesting that fraction B acts only with double stranded DNA (Table 2). When fraction C is added at 0 min, an inhibition in RNA synthesis is exhibited. However, no nuclease has been detected in fraction B and C. But fraction C added after 10 min

promotes RNA synthesis. This aspect will be discussed later. The enzyme activities are influenced by the ionic strength as is usually observed with other RNA polymerases. In the presence of 0·2 M KCl an increase of 40—50% in enzyme activity is recorded. The activity of both polymerases is maximal at pH 8·0.

Table 2

Requirements for RNA polymerases

The incubation system was the same as described under materials and methods (Mondal et al., 1972); 250 μg of RNA polymerase I, 250 μg of RNA polymerase II and 30 μg each of factor B and C obtained from the DEAE column (Fig. 1) were used in appropriate cases

Conditions of the experiment	^{14}C-ATP incorporated $\mu\mu$moles/mg of RNA polymerase I (DEAE fractions)	^{14}C-ATP incorporated $\mu\mu$moles/mg of RNA polymerase II (DEAE fractions)
1 Complete system*	740	972
— Coconut DNA	0	0
— Factor B	80	132
— Coconut DNA, — Factor B, + denatured coconut DNA	51	90
— Coconut DNA, + denatured coconut DNA	50	137
— Coconut DNA, + λ DNA, — Factor B	145	—
— Coconut DNA, + λ DNA,	180	—
— Coconut DNA, + Calf thymus DNA	632	901
+ Factor C	213	300
— Coconut DNA, + denatured DNA, + Factor C	110	130
— Mn^{2+}, + Mg^{2+}	382	—
— Mg^{2+}, + Mn^{2+}	—	448
+ 0·2 M KCl	1,072	1,436

* Complete system contains Factor B also.

Subunits of RNA polymerase I

Polymerase I after purification with QAE Sephadex (A_{50}), on polyacrylamide gel electrophoresis exhibits a single band (Fig. 2*a*) nearer to the cathode end. The same preparation, when electrophoresed in gels with added 8 M urea tends to be dissociated in more than one band (Fig. 2*c*), whereas in 0·1% SDS gels the subunit structure is the most clear cut. The enzyme resolved in at least 4 bands (Fig. 2*b*).

Role of Factor B

Of the five protein peaks obtained from DEAE cellulose column, the one eluted at 0·55 M KCl (fraction B) had a stimulatory activity on the RNA polymerases. The extent fo stimulation depended on the amount of Factor B added, when the amount of RNA polymerase I was kept constant.

Saturation was reached when the ratio of RNA polymerase I to Factor B was 10 : 1. The enzyme RNA polymerase I (and also RNA polymerase II) was almost incapable of *in vitro* RNA synthesis without the addition of Factor B (Fig. 3).

The mode of action of Factor B on RNA polymerase was further elucidated. For this purpose, the incorporation of β,γ-^{32}P-ATP (which is a measure of RNA chain initiation) and of ^{14}C-ATP (which is incorporated into the internucleotide linkages as well) into acid-insoluble products was

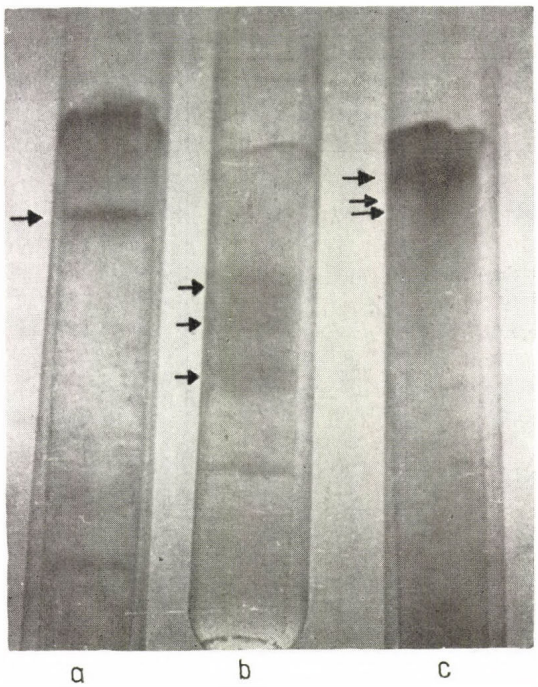

Fig. 2. Polyacrylamide gel electrophoresis of RNA polymerase I. (a) Polyacrylamide gels, pH 8·3, containing 5% acrylamide, and 0·133% methylene bisacrylamide, were electrophoresed at 4 mA per tube for 2½ h. RNA polymerase I (QAE fraction), 10 μg in 50 μl was applied. (b) SDS gels, containing 0·1% SDS, 0·1 M sodium phosphate, pH 7·2, 5% acrylamide, and 0·135% methylene bisacrylamide, were electrophoresed at 8 mA per tube for 4 h. Polymerase I (QAE fraction), 20 μg in 0·1 ml of application buffer containing 0·1% SDS, 1% 2-mercaptoethanol, 0·01 M sodium phosphate, pH 7·2 10% glycerol, and 0·002% bromophenol blue, and applied directly to the gels. (c) 8 M urea gels, pH 8·3, containing 5% acrylamide in the running gel and 2·5% acrylamide in the stacking gel, were electrophoresed at 3 mA per tube for 2 h. Polymerase I (QAE fraction), 20 μg in 50 μl of 8 M urea containing 1% mercaptoethanol and 0·0002% bromophenol blue was applied

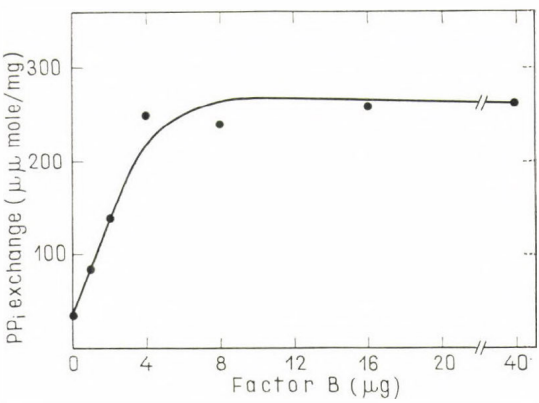

Fig. 3. Optimal concentration of Factor B for RNA polymerase I. The conditions of the experiment were the same as in Table 3. The enzyme activity was assayed by the pyrophosphate exchange method as described in previous paper (Mondal et al., 1972) varying concentrations of Factor B and 50 μg of polymerase I (DEAE fraction) were used in each set of this experiment

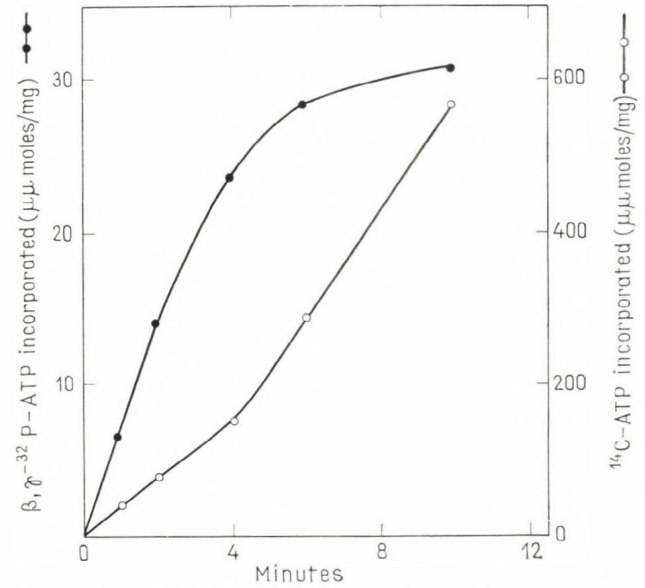

Fig. 4. Kinetics of initiation and total synthesis of RNA. The conditions of the experiment were the same as in Table 1. β,γ-^{32}P-ATP (2×10^8 cpm/μmole) was used for detection of the initiation and ^{14}C-ATP (1×10^7 cpm/μmole) was used for determining the total synthesis. Temperature was adjusted at 20 °C to follow the initial incorporation; 100 μg of polymerase I (DEAE fraction) and 10 μg of Factor B were used

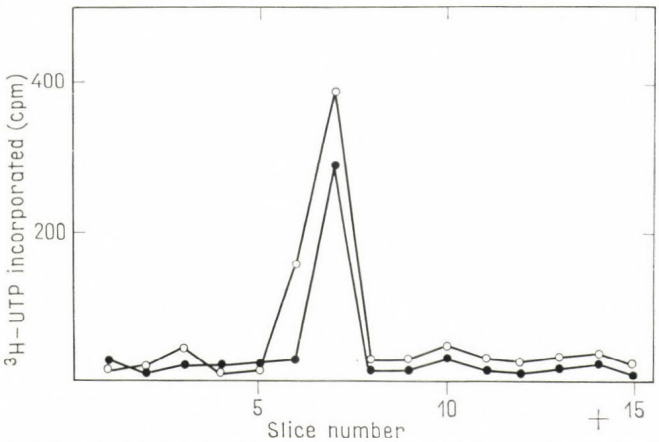

Fig. 5. Activity of Factor B with polymerase I and II. 200 μg of Factor B was separated by 5% polyacrylamide gel electrophoresis as described in the text (Mondal et al., 1972) each 2 mm slice of the gel was extracted overnight with 0·2 ml TMEG buffer. Then, each extracted fraction was assayed separately with 50 μg polymerase I (- ● - ● - ● -) and 50 μg polymerase II (- ○ - ○ - ○ -). The assay mixture was the same as in Table 1

studied. When assayed at 37 °C, it was found that the incorporation of β,γ-^{32}-P-ATP was very fast and reached a plateau within two minutes (results not given). To slow down this rate, the incubation was then carried out at 20 °C. In this case, the incorporation of β,γ-^{32}P-ATP was linear from the beginning and reached a plateau around 6 min. The incorporation of ^{14}C-ATP, on the other hand, was much slower at the beginning and then increased linearly from 5—10 min under the assay conditions (Fig. 4). It appeared, therefore, that Factor B might act as an initiation factor. Further evidence that B acts as the initiation factor came from the experiments to be described later.

As the fraction B from DEAE cellulose column was a mixture of proteins, as revealed by polyacrylamide gel electrophoresis, we wanted to see if the same protein was responsible for stimulating the activity of both RNA polymerases (I and II) or there were two different factors. We, therefore, subjected fraction B to polyacrylamide gel electrophoresis, sliced the gel and extracts of each gel slice were assayed for the stimulatory activity for both RNA polymerase I and II. Results presented in Fig. 5 may indicate that either the same factor acts on both polymerases or, at least, the factors,

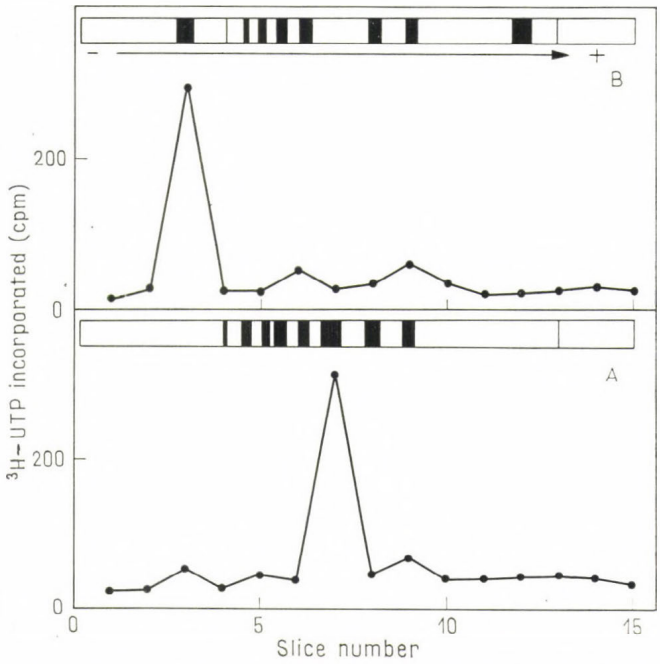

Fig. 6. Binding of Factor B with polymerase I. A. 50 μg Factor B alone; B. 50 μg Factor B with 100 μg polymerase I were electrophoresed separately on 5% polyacrylamide gel as described in the text (Mondal et al., in press). The gels were sliced, extracted and assayed as described. The activity of Factor B in Fig. A was assayed with 50 μg polymerase I added extraneously

if different, are closely similar and cannot be separated by this method. Later Factor B was purified through QAE Sephadex chromatography and resolved to a single protein.

Complex formation of Factor B with RNA polymerase and DNA

It is known in the case of *E. coli* that the σ factor binds with the core RNA polymerase and the enzyme-factor complex again binds with the template DNA (Burgess et al., 1969). A similar situation exists in the present case with the coconut RNA polymerase and Factor B. When the crude Factor B preparation was separated by electrophoresis on polyacrylamide gel and the slices were assayed for stimulatory activity with added RNA polymerase I, the factor activity was located at slice No. 7 (Fig. 6A). But when a mixture of RNA polymerase I and factor B was run and similarly assayed without any external addition of enzyme or factor, the activity could be located in slice No. 3 (Fig. 6B). It was observed in separate experiments that the activity of the enzyme, when run alone in electrophoresis, was also located in slice No. 3. It is clear from these results that under the

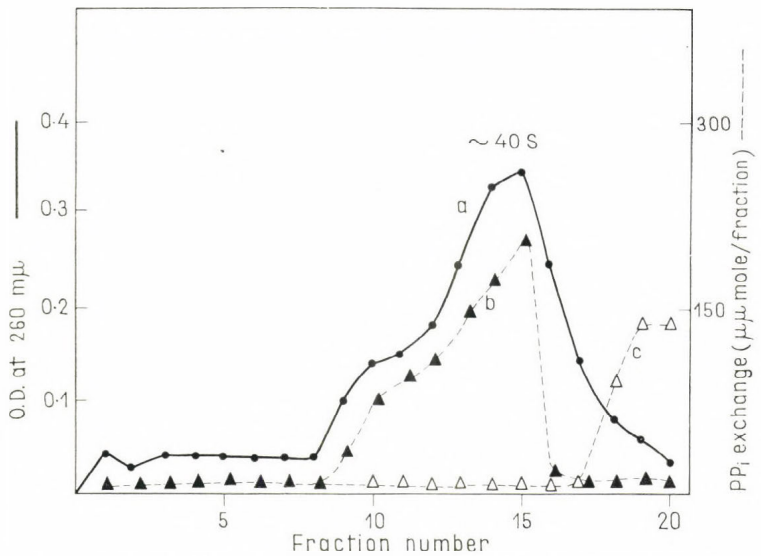

Fig. 7. Binding of RNA polymerase I and factor B with DNA. A mixture of 100 μg of RNA polymerase I (DEAE fraction) and 10 μg of factor B was centrifuged in the presence or absence of 2·0 O. $D._{260}$ units of coconut endosperm DNA on a 5—20% linear sucrose gradient in 0·01 M Tris, 0·1 M NaCl, pH 8·0 for 2 h at 37,000 rpm in the SW 39 rotor of Spinco model L-2 ultracentrifuge. After the run, fractions of 15 drops (*ca.* 0·25 ml) were collected from the bottom of the tubes. The fractions were assayed for RNA polymerase activity by $^{32}PP_i$ exchange. 1) O. D. at 260 mμ of DNA only (- ● - ● - ● -). 2) Activity of RNA polymerase run with DNA and Factor B (- ▲ - ▲ - ▲ -). 3) The activity of the same run with FB only (- △ - △ - △ -). The DNA was added extraneously in the 3rd set for detection of the activity

incubation conditions, Factor B associates with RNA polymerase I. The factor being presumably of much smaller molecular weight than the enzyme, its association with the enzyme does not appreciably change the size or charge of the latter.

When a mixture of RNA polymerase I and Factor B was centrifuged along with DNA in a sucrose gradient, both the enzyme and factor activities sedimented at the same region as DNA (Fig. 7). However, when RNA polymerase I and Factor B were separately centrifuged along with DNA, it was found that the enzyme was bound to DNA while the factor remained at the top of the gradient (results not presented). These experiments clearly show that the enzyme-factor complex can bind to DNA, though the free factor cannot.

Inhibition of RNA polymerase I by rifampicin and its reversal by Factor B

Nuclear RNA polymerase systems from eucaryotic cells have generally been found to be insensitive (Hartmann et al., 1967) to the drug rifampicin which inhibits RNA polymerase from procaryotes at the initiation step (Wehrli et al., 1968; Mauro et al., 1969). However, we found that of the two enzymes from coconut nuclei, RNA polymerase I was inhibited by the drug whereas RNA polymerase II was not (Mondal et al., 1970). Figure 8 shows that maximum inhibition of RNA polymerase I in the presence of saturating amounts of Factor B (5 μg) is obtained at a rifampicin concentration of

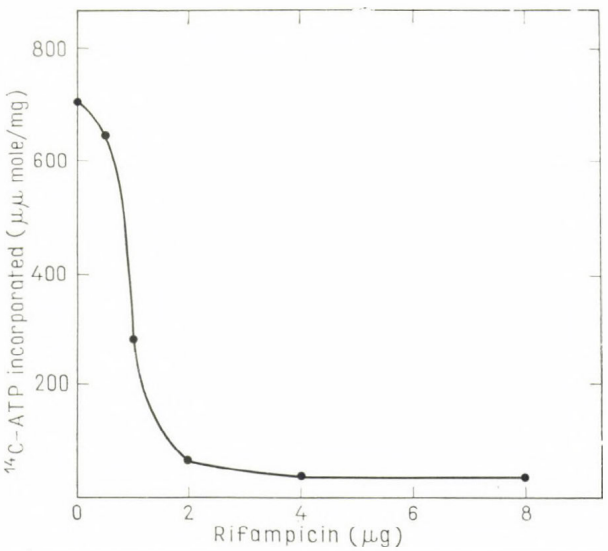

Fig. 8. Minimal concentration of rifampicin to inhibit the RNA synthesis by RNA polymerase I. The conditions of the experiment were same as in Table 3. Rifampicin in varying concentration was added to the incubation mixtures before Factor B was added (5 μg) to the RNA polymerase I (50 μg) in each experiment

Table 3

The effect of rifampicin on RNA polymerase I

The incubation system (either 0·5 ml or 0·25 ml) containing Tris-HCl, pH 8·0, 0·04 M; EDTA 0·2 mM; 2-mercaptoethanol 5 mM; $MnCl_2$, 0·002 M; K_2HPO_4 0·4 mM; KCl 0·16 M; the four triphosphates each 0·15 mM, of which one was labelled (^{14}C-ATP, specific activity 1×10^7 cpm/μmole, or ^{3}H-UTP, specific activity 4×10^6 cpm/μmole); coconut endosperm DNA 40 μg/ml. 50 μg polymerase I, 5 μg Factor B and rifampicin 4 μg/ml were used in each set of this experiment. The components were added in specific order as described below

Order of addition	^{14}C-UTP ($\mu\mu$moles/mg)
1. NTP*, DNA, enz. I, rifam., Factor B	32
2. NTP, enz. I, Factor B, rifam., DNA	790
3. NTP, enz. I, Factor B, rifam., DNA	750
4. NTP, DNA, Factor B, rifam., enz I	52
5. DNA, NTP, enz. I, Factor B, rifam.	800
6. DNA, enz. I, Factor B, NTP, rifam.	740

* NTP means 4 nucleoside triphosphates including labelled ATP, before addition of NTP the other components were added as mentioned above.

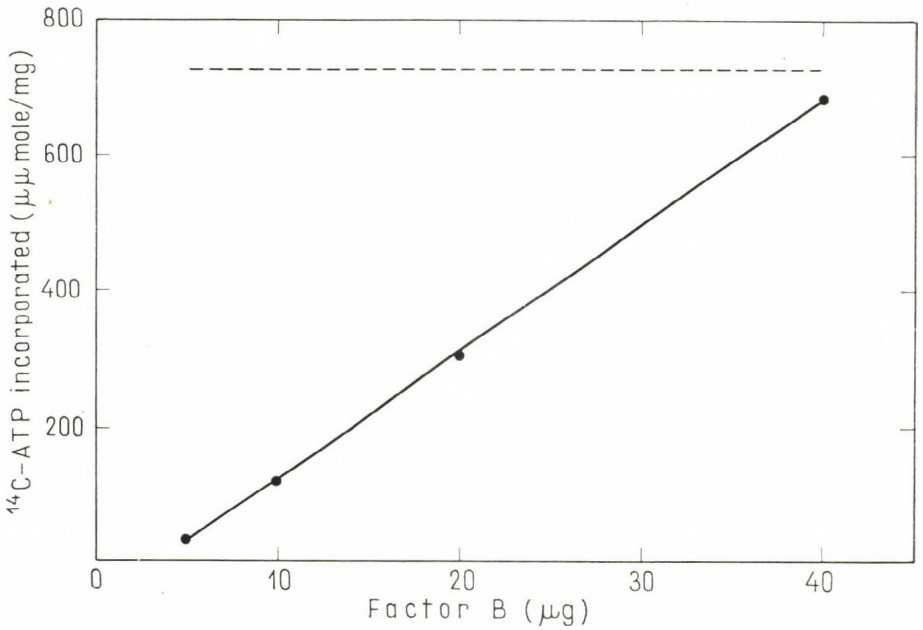

Fig. 9. Competitive effects of rifampicin and Factor B on the activity of RNA polymerase I. The conditions of the experiments were the same as in Table 3. In one set of experiments, 2 μg of rifampicin was added to 50 μg of RNA polymerase I followed by Factor B in varying concentration (- ● - ● - ●). The dotted line (— — — —) indicates the control activity obtained with 50 μg of polymerase I and 5 μg of Factor B in the absence of rifampicin

Table 4

Effect of Factor C on initiation and polymerization

The incubation mixture (0·25 ml) contained the components as described in Table 1. Both β, γ-^{32}P-ATP (2×10^8 cpm/μmole; 0·15 mM) and ^{3}H-UTP (1×10^7 cpm/μmole; 0·15 mM) were used in these experiments. In each incubation, 50 μg of RNA polymerase I (QAE fraction) and 5 μg of Factor B were added at zero time. Then, 5 μg of crude Factor C (dialysed DEAE fraction) or purified Factor C (eluted from gel after electrophoresis and corresponding to 5 μg of crude C) was added at the specified period. Aliquots from the incubation mixture were soaked in Whatman 3MM filter paper discs and dried. The discs were treated with 10% TCA, washed with 5% TCA containing 0·25 M sodium pyrophosphate, ethanol and finally with ether. The discs were then dried and counted

Incubation system	Time of addition of Factor C	Total incubation period	β,γ-^{32}P-ATP incorporated	^{3}H-UTP incorporated
	Minute	Minute	$\mu\mu$mole/mg	mμmole/mg
Polymerase I + Factor B	0	20	10	3·89
Polymerase I + factor B	0	40	13	4·09
Polymerase I + B + crude C	0	20	10	2·88
Polymerase I + B + purified C	0	20	16	6·12
Polymerase I + B + purified C	20	40	23	8·96

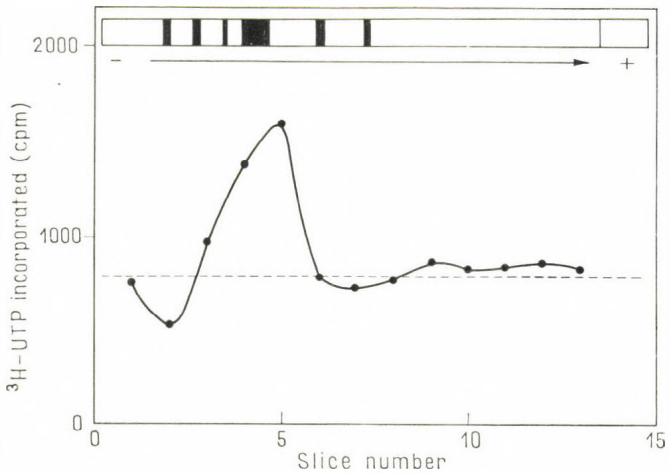

Fig. 10. Scanning of Factor C activity after gel electrophoresis. 40 μg of Factor C were subjected to 5% polyacrylamide gel electrophoresis as described in the text. 2 mm gel slices were extracted with TMEC buffer and each fraction was assayed for activity with 50 μg RNA polymerase I (QAE fraction) along with 5 μg Factor B added at zero minute. The assay mixture was the same as described in the legend to Fig. 5. The control (— — — —) contained all the ingredients minus Factor C

2 μg/incubation volume of 0·5 ml. Furthermore, it was interesting to note that rifampicin was effective only when it was added to the polymerase before the addition of Factor B. The effects of the different orders of additions of the components in the incubation mixture are presented in Table 3. This led us to believe that rifampicin binds with RNA polymerase I probably at the same site where Factor B also can bind. This was borne out by the fact that the inhibitory effect of rifampicin could be completely reversed by higher concentrations of Factor B. Thus, the effect of 2 μg of rifampicin could be completely nullified by the addition of 40 μg of Factor B. The RNA polymerase activity in this case was equal to the control value obtained with saturating amounts of factor B (5 μg) in the absence of rifampicin (Fig. 9).

Role of Factor C

The protein peak eluted from DEAE-cellulose column at 0·62 M KCl (after exhaustive dialysis, fraction C) had an inhibitory activity on RNA synthesis (Mondal et al., 1970). However no RNase or DNase could be detected in this fraction (Mondal et al., 1972). Later, it was found that the fraction was heterogeneous and contained material absorbing UV at 260 mμ. When this UV-absorbing material (presumably nucleic acid) was isolated by deproteinization of fraction C and added to the incubation mixture, there was no inhibition of RNA synthesis (results not presented). The inhibition by the crude fraction C may be due to several reasons. One obvious possibility is that the fraction C had some component which might inhibit initiation of RNA synthesis. Results presented in Table 4 show that fraction C does not inhibit the initiation of RNA synthesis as measured by incorporation of β,γ-^{32}P-ATP. However, the total synthesis of RNA (as measured by the incorporation of ^{3}H-UTP) is inhibited. This indicates that C has a component that inhibits RNA synthesis at some point beyond initiation.

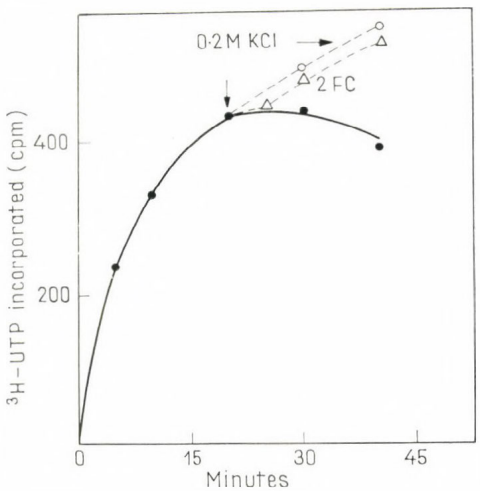

Fig. 11. Role of Factor C on RNA synthesis by RNA polymerase I. The ingredients of the experiments were the same as in Table 3. After 20 min incubation of 50 μg of RNA polymerase I (QAE fraction with 5 μg of Factor B, 5 μg of Factor C (fraction C from DEAE column exhaustively dialyzed and purified through gel electrophoresis) was added and the reactions were terminated at various time intervals (- △ - △ - △). One set (- ○ - ○ - ○) was incubated exactly as above with 0·2 M KCl minus Factor C and (- ● - ● - ● -), the control one was incubated without Factor C and KCl

Next we attempted to purify the Factor C by polyacrylamide gel electrophoresis and the extract of gel slices was assayed for any inhibitory or stimulatory activity during RNA synthesis by polymerase I and Factor B (Fig. 10). Curiously enough, in addition to the inhibitory band a stimulatory band of protein was also found. Our attention was focussed on this band

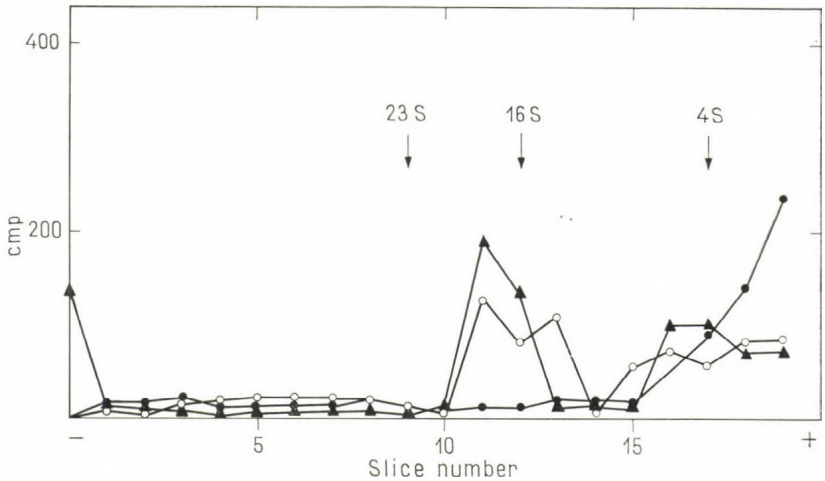

Fig. 12. Polyacrylamide gel electrophoresis of *in vitro* synthesized RNA. The incubation-mixture contained all the components as described in Table 3, except that both ^{14}C-GTP (spec. act. 17 mC/mmole) and ^{3}H-UTP (spec. act. 2 C/mmole) were used to increase incorporated counts. 200 μg RNA polymerase I; 30 μg Factor B and 30 μg purified Factor C were used in the appropriate cases. 1) (- ○ - ○ - ○ -) with both Factor B and C; 2) (- ▲ - ▲ - ▲ -) with Factor B only; 3) (- ● - ● - ● -) without any added factor. Labelled RNA was isolated from the incubation mixture by hot phenol-SDS and precipitated by ethanol in the presence of 500 μg of *E. coli* RNA as carrier. Precipitated RNA was dissolved in 0·3 ml of electrophoresis buffer containing 10% glycerol. Samples containing 8×10^3 acid-insoluble counts were run in 2·5% polyacrylamide gel for 1 h at a current of 4 mA/tube. Details of the electrophoresis, gel slicing and counting are given under Materials and Methods (Mondal et al., in press). Arrows mark the stained *E. coli* RNA bands

which stimulates the RNA synthesis by a combination of polymerase I and Factor B. However, in absence of Factor B, this protein had no stimulatory effect. It occurred to us that C might act on RNA chain termination and release from the DNA template so that fresh initiation would be reflected in the stimulation. To test this idea, we allowed RNA synthesis by RNA polymerase I and Facotr B in presence of limiting amount of DNA to proceed up to the plateau point when all the DNA is held in the enzyme-DNA-nascent RNA complex. If at this point, either fraction C eluted from the gel after electrophoresis or 0·2 M KCl was added, a new spurt of RNA synthesis begins (Fig. 11). The reinitiation of RNA chain by Factor C is also indicated in Table 4. From the similarity of action of high ionic strength and Factor C, the ability of Factor C to release the synthesized RNA from the complex is indicated.

Analysis of the RNA product

The RNA synthesized by RNA polymerase I alone and by RNA polymerase I in presence of Factor B and C was then analyzed by electrophoresis on 2·5% polyacrylamide gel. From Fig. 12 it is clear that in the absence of any factor, RNA polymerase I synthesizes most probably, in a non-specific manner, small amounts of RNA of very low molecular weight (4S and smaller). In the presence of the initiation Factor B, the synthesis is highly stimulated and in this case RNA of higher molecular weight (10 –20S) is synthesized. However, there is no appreciable change in the size-distribution of RNA synthesized by the simultaneous addition of Factor B and C. Though, it cannot be said from this experiment whether Factor B initiates the synthesis of specific types of RNA, at least it helps to synthesize longer RNA molecules.

DISCUSSION

RNA polymerase from higher organisms has been reported earlier but due to some inherent difficulties with the system this enzyme could not be purified (Biswas and Abrams, 1962). In contrast, RNA polymerase from bacterial systems has been purified extensively and a homogeneous protein with its subunits has also been characterized (Burgess et al., 1969). It seems that the RNA polymerase from *E. coli* contains the following subunits: $\alpha\alpha\beta\beta'$ or $\alpha\alpha\beta\beta' w$ with σ and ρ factor. However, in case of higher organisms, the RNA polymerases have been resolved into two or three species and the requirements of these species were shown to be different. From rat liver, RNA polymerase I and II have been reported and according to the resolution obtained through DEAE Sephadex chromatography, polymerase I was Mg^{2+} and II Mn^{2+} dependent, respectively (Roeder and Rutter, 1970). The catalytic properties of the easily solubilized polymerase II (Mn^{2+} dependent) are, in general, similar to the properties described by others for soluble enzymes derived from several eucaryotic organisms (Roeder and Rutter, 1969; Widnell and Tata, 1966; Stein and Hausen, 1970). In the present case, according to the elution profile from a DEAE cellulose column, coconut polymerase I exhibits maximum activity with Mn^{2+} and II with Mg^{2+}. In case of sea urchin, three polymerases have been reported (Roeder and Rutter, 1970) and form I and II have some similarities with the two RNA polymerases isolated from the present system. Since nothing is known about the homogeneity of their preparation the requirements may vary in details. What can be generalized is that there are at least two forms of RNA polymerase present in the nuclei of eucaryotic cells. The earlier reports, however, suggest that one is associated with the nucleolus and the other with the nucleoplasm (Roeder and Rutter, 1970). With the present system, two forms are isolated from the chromatin that includes the nucleochromosomal apparatus. Not only the RNA polymerase but also two factors which can modulate the activity are also detected in the chromatin. At least 40% of the total activity has been obtained in the soluble nucleoplasmic sap in the present case and after further resolution

of this activity two forms (I and II) have been isolated. This at the best suggests that RNA polymerases as well as factors bound to chromatin may be in equilibrium with the free forms in the nucleoplasm. Comparison of our preparation with that of others may be fortuitous as in the latter cases sonication or other drastic procedures are commonly used for solubilization of polymerases. In the present case RNA polymerase I has been purified to homogeneity and contains at least 4 subunits as evidenced from gel electrophoretic pattern (Fig. 2). Its specificity for native DNA from homologous system is established. DNA from calf thymus is less effective than coconut DNA when factor B is used. With *E. coli* or λ DNA there is no increase in RNA synthesis by addition of Factor B.

In view of the complexity of chromosomal transcription, regulation of RNA synthesis by RNA polymerase in the eucaryotic cell seems to be a partial process of the whole control system. The role of factors in RNA synthesis by *E. coli* RNA polymerase has already been elucidated (Travers and Burgess, 1969; Sugiura et al., 1970). The core enzyme binds with the initiation factor σ and then interacts with the promoter site of operon forming an initiation complex. Synthesis of RNA starts from that point, extends through the cistron and finally terminates with the help of ρ factor (Roberts, 1969). It has also been shown that either σ or core polymerase is changed with the stages of development of phage T_4 in *E. coli* (Travers, 1969; Seifert et al., 1969). A similar phenomenon has been reported in case of sporulation of *Bacillus subtilis* (Losick et al., 1970). Interesting is the case that a single σ factor is present in *E. coli* suggesting no specificity of σ for a particular gene or promoter site. Very recently, Travers et al. (1970) has discovered in *E. coli* another factor (ψ_r) which is required in addition to the σ factor for the transcription of ribosomal RNA.

However, nothing was known about initiation or termination factors in eucaryotic cells. The report of a stimulatory factor for RNA polymerase isolated from calf thymus has appeared (Stein and Hausen, 1970). But the mechanism of stimulation by this factor has not been clarified yet. Our intention was to find out whether RNA polymerases and the factors are associated with the chromatin. Since chromatin contains DNA, RNA, histone and non-histone proteins, it is pertinent to ask the question whether the protein parts have any role in RNA synthesis. Histone has now been classified as general repressor for RNA polymerase (Georgiev, 1969). This repression can, however, be derepressed nonspecifically by many substances (Frenster, 1965). Non-histone protein has been found to have some role in derepression (Paul and Gilmour, 1968). Does non-histone protein contain any RNA polymerase activity? Is it possible that non-histone protein in the chromatin can modulate RNA synthesis in the eucaryotic cell by changing its composition and conformation during the division cycle? The present findings show that RNA polymerases are present in the non-histone part of chromatin. Surprisingly it has also been found that both initiation and termination factors are present in the same fraction of chromatin. That Factor B in the present case acts as initiator has been supported by the following evidence: 1) It can form complex with DNA and RNA polymerase; without RNA polymerase it cannot bind with DNA alone; 2) There is no promotive effect of Factor B when λ DNA or *E. coli* DNA is used; native homologous DNA is more effective than

DNA from thymus; 3) Rifampicin, which can inhibit initiation in case of *E. coli* RNA polymerase, also influences RNA polymerase in the present case if Factor B is added after the drug; 4) Factor B promotes β,γ-^{32}P-ATP incorporation and reaches a plateau quickly whereas the extension of RNA chain continues linearly with an initial lag (Fig. 4); 5) The product of the reaction without Factor B indicates the presence of low molecular weight (4S) RNA whereas the same with Factor B shows 10—20S RNA (Fig. 11).

It is known that RNA polymerases from nuclei of higher organisms are insensitive to the antibiotic rifampicin, in contrast to *E. coli* RNA polymerase (Mauro et al., 1969; Wehrli et al., 1968). This inhibition by rifampicin in the latter case is attributed to the interaction of the drug with the minimal enzyme (Mauro et al., 1969). The inhibition of RNA polymerase from an eucaryotic cell (coconut RNA polymerase I) has been first observed and reported from this laboratory (Mondal et al., 1970). It has also been found that if Factor B is first allowed to form complex with polymerase I, followed by the addition of rifampicin, no inhibition occurs. The block may thus lie in the formation of Factor B-enzyme system. The resistance of the preinitiation complex to rifampicin has been confirmed in the *E. coli* system also (Bautz and Bautz, 1970; Sippel and Hartmann, 1970). The inhibition by rifampicin of coconut RNA polymerase I can be completely reversed by high concentration of Factor B (Fig. 8). The present findings suggest that rifampicin probably binds at the same site of RNA polymerase, where the initiation factor also can bind. The relative sensitivity (or insensitivity) of RNA polymerase from different organisms may depend on the relative affinities of the drug and the factor for the enzyme site. In eucaryotic organisms, the affinity of the factor for the enzyme may be much stronger than that of rifampicin.

The question as to the action mechanism of Factor C is, however, complicated by the fact that when Factor C is added after a lapse of 10 min., promotive effect is indicated. By analogy with Roberts' ϱ factor, inhibition can be explained if the RNA synthesized in presence of Factor C records molecular weight lower than that synthesized without Factor C. However, no change in the pattern of RNA synthesis in presence or absence of Factor C could be demonstrated as is evident from Fig. 11. The stimulatory effect of Factor C may be explained by the fact that RNA synthesis would not proceed after a certain period if this is not terminated and dissociated from the template. At this point, should terminator be added, there would be a spurt in the RNA synthesis. In fact, this is the case with Factor C and this seems to be very similar to the effects caused by high ionic strength in the incubation medium (Fig. 10). *A priori* assumption in the case of Factor C is that without termination, dissociation of RNA from the template does not occur. But the increase which has been demonstrated in presence of Factor C is not adequate to suggest that Factor C simply acts as terminator. Since Factor C is not a homogeneous protein there might be other components that interfere with the reaction. In view of this fact Factor C was subjected to gel electrophoresis and each fraction was analyzed for terminator and inhibitor functions. It seems that there is another fraction in Factor C which can inhibit the polymerization (Fig. 9). All these facts taken together suggest that Factor C acts as terminator for RNA synthesis in the present case.

It is interesting to note here that RNA polymerases are present in the chromatin of coconut nuclei and a number of protein factors that can modulate RNA synthesis *in vitro* are also bound to the chromatin. RNA polymerases and the factors may not be solely bound to the chromatin. There might be an equilibrium between the bound and the free forms since RNA polymerase and the factors are to be separated from the chromatin followed by reassociation in order to recycle the process *in vivo*. It may also be expected that the structure of chromatin itself during different stages of growth may regulate RNA synthesis in eucaryotic cells.

Until very recently, almost all that could be said about the mechanism of RNA transcription both in procaryotes and eucaryotes was that it is catalyzed by the enzyme RNA polymerase. But it is now clear that although the basic enzyme possesses the catalytic activity which synthesizes phosphodiester bonds, the initiation and termination of transcription are assisted by protein factors. In spite of various speculations at present on the mode of action, recognition and control exerted by these factors on RNA synthesis during different phases of cell cycle, there is every reason to think that a universal concept will emerge.

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SECTION II

PROTEIN SYNTHESIS IN PLANTS

Symp. Biol. Hung. 13, pp. 115—126 (1972)

ROLE OF PHYTOCHROME IN THE CONTROL OF ENZYME ACTIVITY IN HIGHER PLANTS: PHOTOMODULATION AND PHOTODETERMINATION OF ENZYME SYNTHESIS

by

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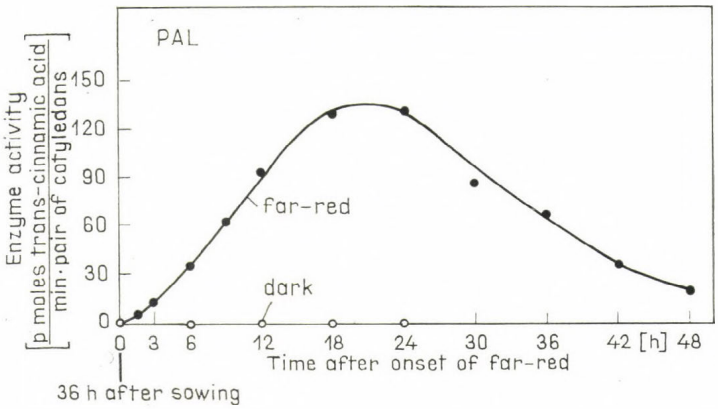
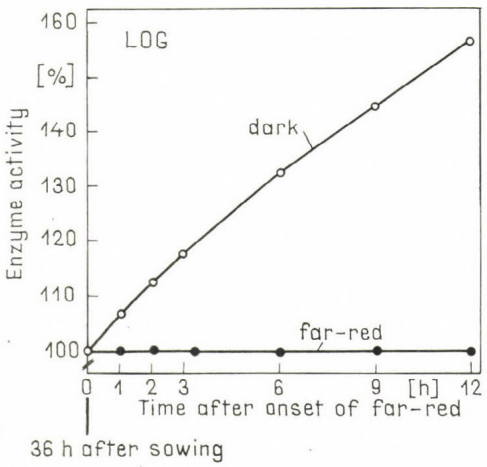
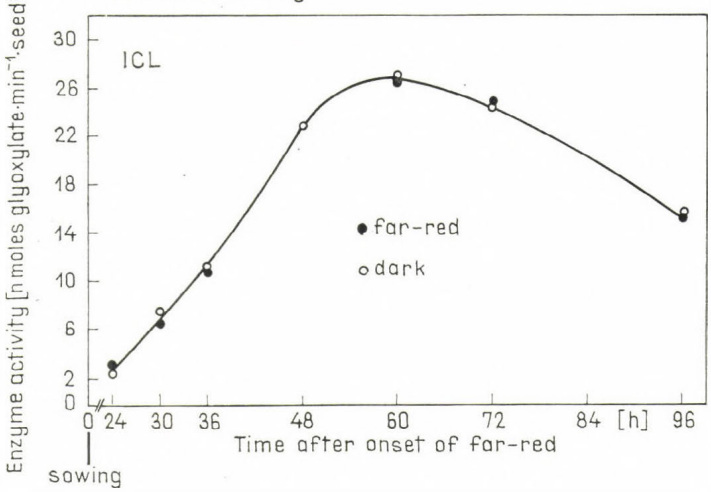
INTRODUCTION

P_{fr}, the active species of the phytochrome system, is an effector of apparent induction and repression of enzyme synthesis (Mohr, 1970). With respect to their control by P_{fr} we find three types of enzyme in the higher plant: (1) "inducible" enzymes, (2) "repressible" enzyme and (3) enzymes whose activity is not affected by P_{fr} (Fig. 1). The existence of these three functional classes of enzyme is predicted by the hypothesis of differential gene regulation (Mohr, 1966*a*, *b*) by P_{fr} which has been advanced and supported by Mohr and associates in the last few years (Fig. 2). This model includes a "primary" differentiation of the genome of a plant in at least 4 functional classes of gene: "active" genes, which are transcribed in the same way either in the presence or in the absence of P_{fr}, "potentially active" genes (P_{fr}), which can be activated by P_{fr}, "repressible" genes (P_{fr}), which can be inactivated by P_{fr}, and inactive genes, which are not transcribed either in the presence or in the absence of P_{fr} in the developmental stage under investigation. The pattern of functional gene types, which we call "primary" differentiation (or "pattern of competence"), is not fixed but is variable with respect to time and localization of the cell in the developing plant. Therefore P_{fr}-mediated responses usually show a strong dependence on the developmental stage and are limited to certain tissues or organs of the multicellular plant. According to this model P_{fr} exerts its function as a nonspecific "trigger" molecule on a preformed pattern of potential genetic activity to bring about the P_{fr}-mediated photoresponses which we call "secondary" differentiation.

The discovery of inducible enzymes, repressible enzymes, and enzymes not influenced by P_{fr} provides strong evidence for the hypothesis of differential gene regulation by P_{fr}. The double action mechanism of "primary" and "secondary" differentiation (Oelze-Karow and Mohr, 1970) appears to be the most logical explanation of the multiplicity (Mohr, 1966*a*, *b*), and specificity (Mohr, 1966*b*; Oelze-Karow and Mohr, 1970) of P_{fr} action, which are evident from many experiments. However, this hypothesis offers only a general framework for the understanding of the P_{fr}-mediated photomorphogenesis. There are still many holes in the model which need to be filled by experimental evidence at the molecular level.


PAL
Enzyme activity
[p moles trans-cinnamic acid
min·pair of cotyledons]
150
120
90
60
30
0
far-red
dark
0 3 6 12 18 24 30 36 42 [h] 48
Time after onset of far-red
36 h after sowing


LOG
160
[%]
140
130
120
110
100
Enzyme activity
dark
far-red
0 1 2 3 6 9 [h] 12
Time after onset of far-red
36 h after sowing


ICL
Enzyme activity [n moles glyoxylate·min⁻¹·seedling⁻¹]
30
26
22
18
14
10
6
2
0
far-red
dark
0 24 30 36 48 60 72 84 [h] 96
Time after onset of far-red
sowing

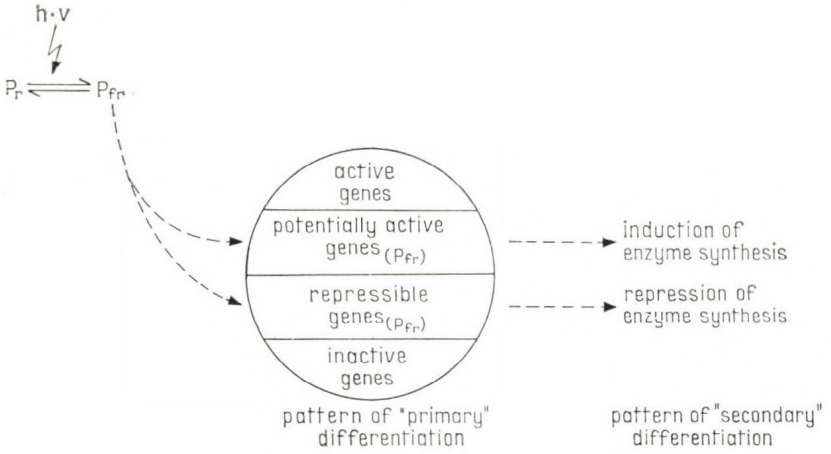


Fig. 2. A general model illustrating the hypothesis of differential gene activation and repression as the mode of action in differential induction and repression of enzyme synthesis by P_{fr}. (After Schopfer, 1967)

DEMONSTRATION OF PHYTOCHROME-MEDIATED ENZYME *DE NOVO* SYNTHESIS

One obvious prediction of the gene activity regulation hypothesis is the *de novo* synthesis of enzyme molecules under the influence of P_{fr}. Evidence for this comes mainly from experiments with inhibitors of DNA transcription (Actinomycin D) and RNA translation (Puromycin, Cycloheximide) (Drumm et al., 1971; Rissland and Mohr, 1967). These inhibitors were shown to reduce or prevent the effect of P_{fr} on extractable enzyme activity. However, these results are not generally accepted as unambiguous evidence for enzyme *de novo* synthesis. Therefore we recently used density labelling to approach more directly the problem of *de novo* synthesis of a P_{fr}-induced enzyme. This technique has been used successfully to demonstrate induced *de novo* enzyme synthesis in a number of plant systems (Filner et al., 1969). The P_{fr}-induced phenylalanine ammonia-lyase (PAL, cf. Fig. 1) of the mustard seedling appeared to be well suited to this experi-

←

Fig. 1. Examples representative of the three categories of enzyme observed in seedlings with respect to control by P_{fr}: a. induction of phenylalanine ammonia-lyase (PAL) (Mohr, 1970), b. repression of lipoxygenase (LOG) (Oelze-Karow et al., 1970), c. lack of control by P_{fr} of isocitrate-lyase (ICL) (Karow and Mohr 1967). Enzyme activities were measured in extracts from mustard seedlings (*Sinapis alba* L.) grown under standard conditions (Mohr, 1966*a*) in the dark for 0 (ICL) or 36 h (PAL, LOG) at 25 °C and then irradiated with continuous standard far-red light (Mohr, 1966*a*) (350 μWcm^{-2}). This irradiation establishes a low but virtually constant level of active P_{fr} in the tissue. According to Hartmann (1966) the P_{fr} functions under continuous irradiation in some short-lived excited state (P_{fr}*). Thus, in contrast to light pulse irradiation, continuous far-red irradiation virtually provides steady state conditions with respect to the active P_{fr} species and this allows the study of phytochrome-mediated responses undisturbed by changes in the concentration of the effector molecule

ment, since it is an extremely stable enzyme which does not show separation into isoenzymes on gelelectrophoresis (Fig. 3).

In the density labelling experiment the enzyme was extracted from cotyledons (which are virtually devoid of PAL in the dark, cf. Fig. 3) of seedlings grown in the presence of 80 per cent deuterium oxide and 20 per cent water

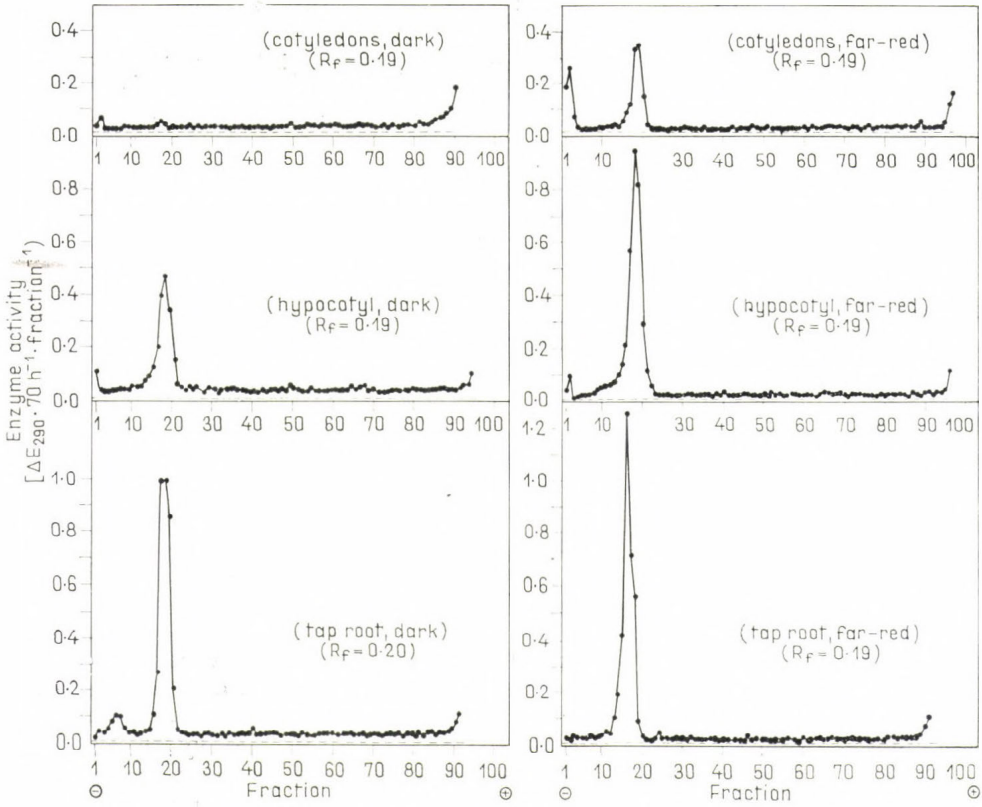


Fig. 3. Polyacrylamide gel electrophoresis pattern of phenylalanine ammonia-lyase from the organs of dark-grown (60 h) and far-red-grown (36 h dark + 24 h far-red) mustard seedlings. The enzyme always appears as a single band in this system (7% acrylamide) with a R_f value of about 0·19 (7% acrylamide). (After Schopfer, 1971)

under far-red light. When this enzyme preparation was subjected to an equilibrium centrifugation on an isopycnic CsCl density gradient, the PAL activity was concentrated at a buoyant density of 1·272 kg $\cdot l^{-1}$ while the enzyme from the control preparation (seedlings grown on distilled water) was located at about 1·257 kg $\cdot l^{-1}$ (Fig. 4). From the 1·2 per cent increase of buoyant density one can calculate that about 1—2 deuterium atoms were incorporated in each amino acid of the PAL molecule. Since only amino acids synthesized *de novo* in the basic metabolism of the cell can carry stable incorporated deuterium label into newly synthesized proteins, and since it is likely that breakdown of reserve proteins in the mustard coty-

ledons provides the bulk of amino acids for protein synthesis, this relatively low degree of labelling is to be expected. The experiments clearly show that the PAL extracted from the cotyledons of the mustard seedling grown under the influence of P_{fr} is not the product of activation of a "pro-enzyme" molecule preformed in the seed. The enzyme is rather a product

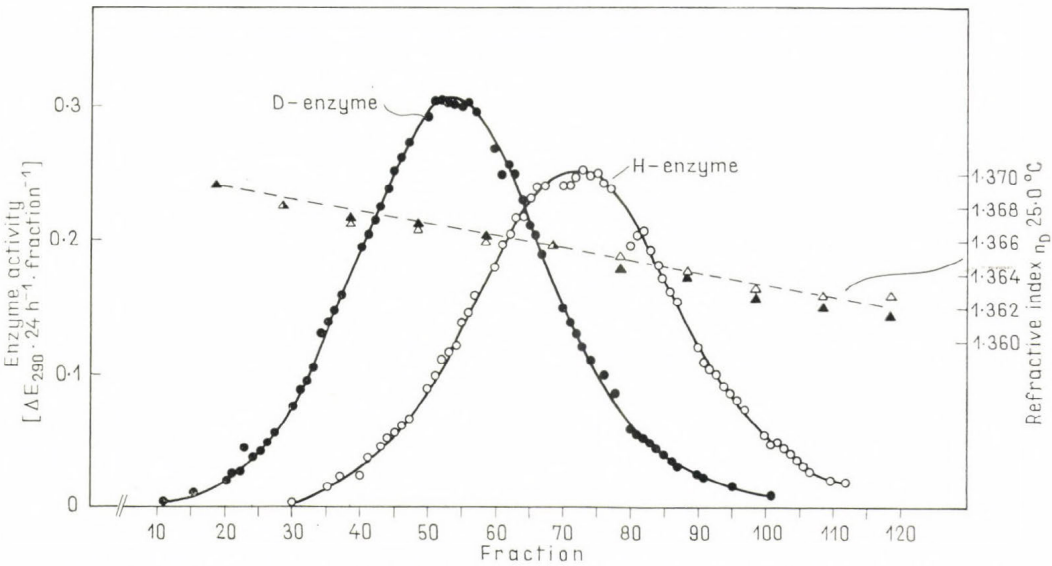


Fig. 4. Activity profiles following isopycnic density gradient centrifugation in CsCl of phenylalanine ammonia-lyase extracted from the cotyledons of mustard seedlings grown on 80 per cent deuterium oxide ("D-enzyme"), and on distilled water ("H-enzyme"), respectively. The seedlings were grown on H_2O (80 per cent D_2O) in the dark for 36 h (85 h) and then irradiated with continuous far-red light for 16 h (30 h) before extraction of the enzyme. (After Schopfer and Hock, 1971)

of *de novo* protein synthesis from amino acids. These results suggest that the term "induction of enzyme synthesis by P_{fr}" may be justifiable also for other enzymes controlled by P_{fr}. But, of course, additional rigorous evidence is required to support this statement.

PHOTOMODULATION AND PHOTODETERMINATION OF ENZYME SYNTHESIS

Further information on the molecular mechanism of P_{fr}-mediated enzyme regulation can be obtained from the study of the kinetics of enzyme activity after P_{fr} is formed in and after P_{fr} is removed from the tissue. It is a general experience with the mustard seedling that under appropriate conditions (i.e. when the lag phase is abolished by preirradiation) enzyme synthesis starts within a few minutes when the seedling is irradiated with continuous far-red light (Mohr, 1970; Rissland and Mohr, 1967). Also repression of enzyme synthesis by P_{fr} is a very rapid process (cf. Fig. 1b).

Figures 5 and 6 show the time-course of amylase and glycolate oxidase formation in the cotyledons of the mustard seedling after the continuous far-red light has been turned off. In both cases we observe a transient phase of about 6—8 h and then the amount of enzyme in the tissue remains at a constant level for at least a further 20 h, suggesting that

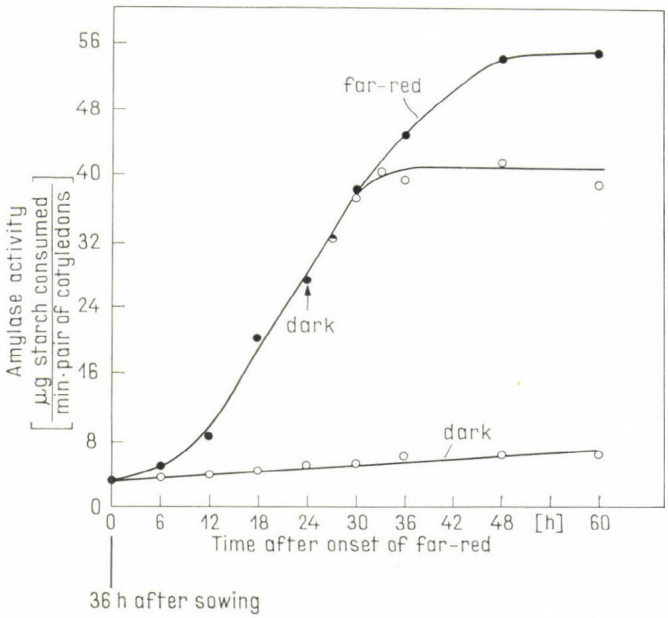


Fig. 5. Time-course of amylase activity in the cotyledons of the mustard seedling in the dark, under continuous far-red light, and after transfer from far-red light to darkness (arrow). (After Drumm et al., 1971)

the enzymes are not subject to turnover. The transient time between the end of irradiation and attaining the constant enzyme level gives an estimate of the velocity of decay of intermediates which are involved in the induction process. The $P_{fr\ (ground\ state)}$ remaining in the tissue after the light has been turned off drops to an ineffective level within a short time: $\tau_{\frac{1}{2}}$ ca. 45 min (Marmé, 1969). However, the threshold concentration of active $P_{fr\ (ground\ state)}$ which may vary in different responses is not yet known for the two enzymes (Figs 5 and 6). The transient time may also depend on the lifetime of the mRNA's involved in the synthesis of the particular enzyme. From the data shown in Figs 5 and 6 it may be concluded that the effective lifetime of the mRNA's of amylase and glycolate oxidase synthesis in the mustard seedling is shorter than 6—8 h under our experimental conditions. This assumes that transcription ceases after P_{fr} has become inactive and that there is no turnover of the enzyme. Furthermore, the time-courses shown in Figs 5 and 6 indicate that the induction of these two enzymes is a fully reversible process. The intermediates of the molecular path $P_{fr} \rightarrow$ enzyme synthesis

are relatively unstable and disappear completely after a few hours. Following the terminology of the animal embryologist P. Weisz (1967) we use the term "modulation" for this type of response. P_{fr}-mediated "photomodulation" is a widespread phenomenon in the mustard seedling e.g. synthesis of anthocyanin (Lange et al., 1971), ascorbic acid (Bienger and Schopfer,

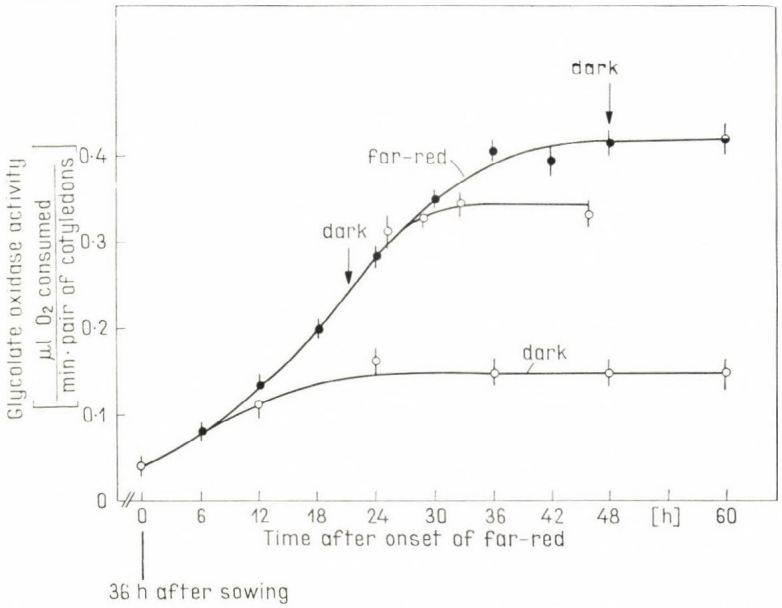


Fig. 6. Time-course of glycolate oxidase activity in the cotyledons of mustard seedlings in the dark, under continuous far-red light, and after transfer from far-red light to darkness (arrows). (After Van Poucke and Barthe, 1970)

1970) and carotenoids (Schnarrenberger and Mohr, 1970); repression of lipoxygenase synthesis (Oelze-Karow et al., 1970) and hypocotyl elongation (Schopfer and Oelze-Karow, 1971).

Recently we investigated a photoresponse of the mustard seedling which shows a completely different kinetic pattern: the P_{fr}-mediated increase of peroxidase activity. The appearance of this enzyme in the axis of the seedling (hypocotyl plus taproot = rest seedling) is not influenced by the presence of P_{fr} (Fig. 7) up to about 5—6 days after germination. (After this time the energy reserves of the seedling are depleted and reliable results can no longer be obtained.) This means that the gene (or genes) responsible for peroxidase synthesis belongs to the category of "active" genes (cf. Fig. 2) in the seedling axis. In the cotyledons, however, P_{fr} formed by continuous far-red light induces an increase in peroxidase activity (Fig. 8). Obviously the peroxidase gene (or genes) belongs to the category of "potentially active" genes (cf. Fig. 2) in the cotyledons. Red-far-red induction reversion experiments (Table 1) show that the operational criteria for the involvement of P_{fr} in this response are fulfilled.

The ability of the cotyledons to produce peroxidase under the influence of P_{fr} is strongly dependent on the stage of development: P_{fr} is effective only when it is formed before about 96 h after sowing. However, per-

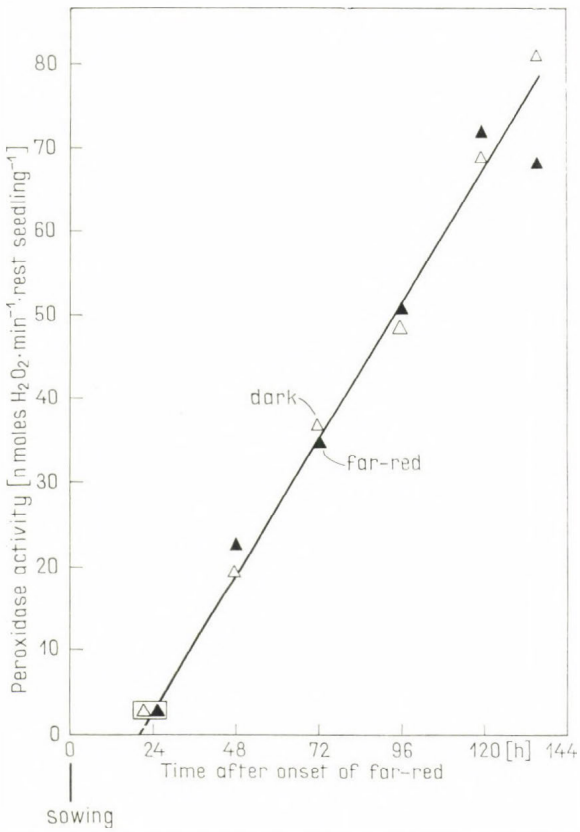


Fig. 7. Time-course of peroxidase activity in the seedling axis (hypocotyl plus taproot = rest seedling) of mustard seedlings in the dark and under continuous far-red light (onset of irradiation immediately after sowing)

oxidase activity can be increased only after about 96 h after sowing. The formation of P_{fr} in the cotyledons leads to enzyme synthesis in a period during which the competence to respond to P_{fr} is already lost. Obviously the induction process is clearly separated in time from the realization of the response. Figure 9 shows that formation of P_{fr} before 48 h after sowing is also ineffective in the induction of peroxidase activity. However, if the seedlings are irradiated in the proper time of "primary" differentiation (e.g. 24 until 72 h after sowing, cf. Fig. 9) and then transferred to darkness, peroxidase accumulates at the same rate as under continuous far-red light for at least 2 and a half days. Similar results are obtained when the irradia-

tion lasts for 48 h longer before onset of darkness (120 h after sowing). The deviation of these far-red → dark kinetics from the far-red kinetics after about 130 h is probably an artefact due to reetiolation of the seedlings in the dark which leads to a faster exhaustion of reserve materials from the cotyledons.

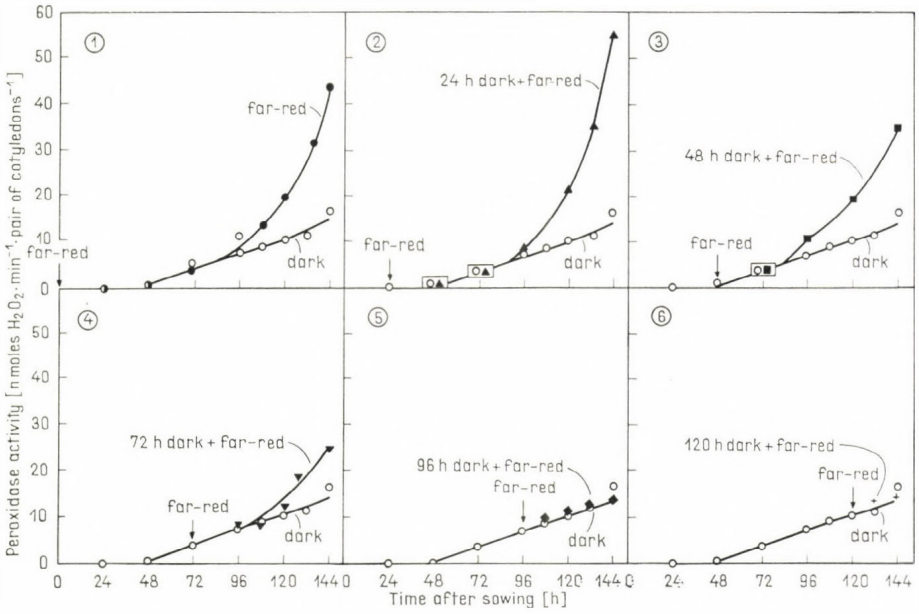


Fig. 8. Time-course of peroxidase activity in the cotyledons of mustard seedlings in the dark and under far-red light (onset of irradiation at 0, 24, 48, 72, 96 h after sowing)

It appears from these data that the P_{fr}-mediated induction of increased peroxidase synthesis is a virtually irreversible process at least during the time in which the mustard seedling can be used for experimentation under our conditions. We therefore use the term "determination" in contrast to the rapidly reversible "modulation" to characterize this type of response. So far the increased rate of peroxidase activity is the only response we know of that is due to "photodetermination" by P_{fr} in the mustard seedling. However, it has to be emphasized that reversible modulation of the *rate* may lead to an irreversible change in the *amount* of the product of a response, if the product is stable. Thus "photomodulation" of the rate of enzyme synthesis can result in "photodetermination" of the amount of enzyme present in the cell.

As to the molecular mechanism, it is obvious that we need a more complicated model for the "photodetermination" than we need for the "photomodulation" of the rate of enzyme synthesis. Since P_{fr} rapidly disappears from the system in the dark, we need at least one stable intermediate in

Table 1

The control by $P_{Ir(\text{ground state})}$ of peroxidase activity in the cotyledons of mustard seedlings as shown by red-far-red induction reversion experiments The data of two slightly different experiments show that the effect of short irradiations with red light can be reduced by immediately following short irradiations with far-red light

Irradiation program	Peroxidase activity $\left[\frac{\text{n moles } H_2O_2 \text{ consumed}}{\text{min·pair of cotyledons}}\right]$
24 h dark	0·0
144 h dark	14·3 ± 0·1
24 h dark + 5 min red + 4×(12 h dark + 5 min red) + 72 h dark	21·9 ± 1·3
24 h dark + 5 min far-red + 4×(12 h dark + 5 min far-red) + 72 h dark	14·8 ± 0·9
24 h dark + 5 min red + 5 min far-red + 4×(12 h dark + 5 min red + 5 min far-red) + 72 h dark	18·0 ± 1·0
24 h dark + 48 h continuous red + 72 h dark	24·7 ± 0·6
24 h dark + 48 h continuous far-red + 72 h dark	33·7 ± 1·3
24 h dark + 3 min red + 4×(12 h dark + 3 min red) + 48 h dark	10·94 ± 0·06
24 h dark + 10 min far-red + 4×(12 h dark + 10 min far-red) + 48 h dark	9·47 ± 0·03
24 h dark + 3 min red + 10 min far-red + 4×(12 h dark + 3 min red + 10 min far-red) + 48 h dark	9·84 ± 0·06

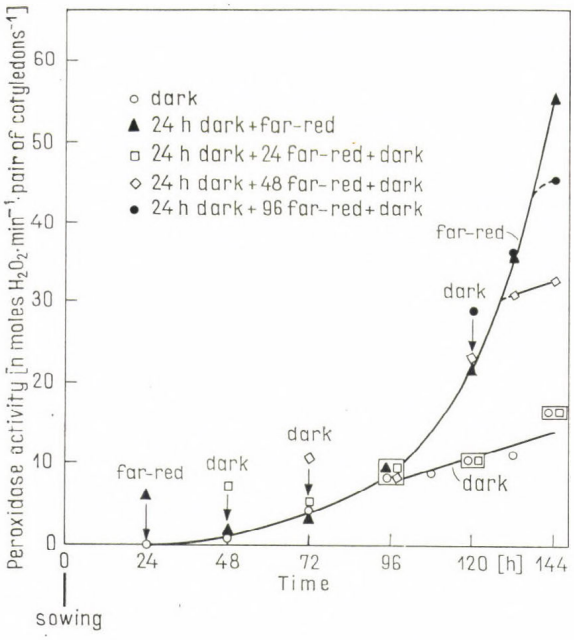


Fig. 9. Time-course of peroxidase activity in the cotyledons of mustard seedlings under different periods of far-red irradiation (onset of irradiation at 24 h after sowing; transfer to darkness at 48, 72, 120 h after sowing)

the metabolic chain between P_{fr} and peroxidase synthesis which acts as a "transmitter" of the primary effect of P_{fr} to the peroxidase synthesizing mechanism. Apparently the "transmitter" can be formed in the presence of P_{fr} only in an early stage of "primary" differentiation (about 48—96 h after sowing) and can act only at a later stage of "primary" differentiation (about 96—120 h after sowing) resulting in an increased synthesis of peroxidase. At this later stage the "transmitter" apparently cannot be formed any longer or is inactivated rapidly after formation. A relatively stable mRNA or a hormone-like substance are possible candidates for this "transmitter". — At any event we can conclude from these considerations that P_{fr} can influence the differentiation of the mustard seedling on the level of enzyme synthesis in two different ways, by "photomodulation" and by "photodetermination", and that these two types of response cannot be produced by the same molecular mechanism.

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END-PRODUCT REPRESSION OF NITRATE REDUCTASE IN *LEMNA MINOR* L.

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SUMMARY

The pattern of metabolic control regulating the formation of nitrate reductase in *Lemna minor* has been studied. The induction of the enzyme is shown to be inhibited by ammonia and amino acids, these compounds when added to induced plants result in a rapid decrease in enzyme level. In the case of ammonia and asparagine it is shown that they act at the cellular level rather than by inhibiting nitrate accumulation. The rate of induction is shown to be dependent on the nitrogen source, plants are grown on, prior to induction. Thus the rate of induction in asparagine grown plants is five times that in ammonia grown plants. The kinetics of repression suggest an immediate response to the presence of the repressors, possible mechanisms of repression are discussed.

INTRODUCTION

The specific mechanisms regulating enzyme synthesis in higher plants are as yet little understood. Recently Glasziou (1969) has reviewed this field and has suggested that there is evidence for at least five possible levels of control. These include various forms of transcriptional control (e.g. thymidine kinase — Hotta and Stern, 1965; acid phosphate — Reid and Bieleski, 1970; nitrate reductase — Ingle et al., 1966), translational control (e.g. invertase — Glasziou et al., 1966) and control over enzyme degradation (e.g. phenylalanine ammonia lyase — Engelsma, 1966).

Nitrate reductase is of particular interest since studies with a variety of higher plants indicate a rapid substrate (nitrate) induction which appears in most plants dependent upon m-RNA synthesis (see e.g. Beevers et al., 1965; Ingle et al., 1966). Such studies have been interpreted as being indicative of a mechanism similar to the bacterial regulator-operator system (Glasziou, 1969). In the absence of genetical evidence this hypothesis is, of course, only speculative. In fact one investigation suggests the increase observed in nitrate reductase activity does not involve the *de novo* synthesis of the enzyme (Ingle, 1968).

Another aspect of the control operating over nitrate assimilation and one which has been studied in less detail is the repressive or inhibitory effect of end products such as ammonia and amino acids. The first demonstration that certain amino acids inhibit the induction of nitrate reductase in higher plants was with culture tobacco cells (Filner, 1966).

Inhibition of nitrate reductase induction by ammonia has been observed in *Lemna minor* (Sims et al., 1968) and the related species *Spirodella oligorrhiza* (Ferguson, 1969). More recently Stewart (1972) has shown a variety

of amino acids inhibit the induction of both nitrate and nitrite reductase in *Lemna minor*.

The results described here concern the role of ammonia and amino acids in controlling the level of nitrate reductase in *Lemna minor*.

METHODS

The experimental material used in these studies was a strain of *Lemna minor*, raised from a single plant. This strain has been grown in axenic culture in the laboratory for a period of six years. The basal growth medium and conditions for growth were those described previously (Stewart, 1972). In the induction experiments the plants used were from six day cultures. It was found necessary to use cultures of a fixed age since rate of induction and maximum level of enzyme formed are both dependent on the age of the culture (Orebamjo unpublished results). The methods for the extraction and assay of nitrate reductase were as described previously (Stewart, 1972). The extraction, fractionation and determination of amino acids were carried out according to the methods of Stanley (1964). Nitrate was estimated as nitrite after enzymic reduction (Stewart and Orebamjo in preparation). The source of enzyme was a partially purified preparation of nitrate reductase from nitrate-adapted yeast *(Torulopsis utilis)*. Ammonia was estimated by the method of McCullough (1967).

Nitrate reductase activities are expressed as μ moles NO_2 produced per hour per gram fresh weight. The results are expressed on a fresh weights basis since previous studies have shown the protein content of plants grown on various nitrogen sources is similar.

Table 1

Effect of amino acids and ammonia on nitrate reductase induction

Additions	μ moles NO_2/h/g.f.w. (Nitrate reductase activity)	Growth as % of that on NO_3
None	4·8	100
Ammonia	0·5	97
Alanine	0·8	70
Arginine	0·7	0
Asparagine	0·3	100
Aspartic	1·9	60
Glutamate	2·2	65
Glycine	0·6	68
Isoleucine	2·1	0
Threonine	1·0	0

Plants grown on 1 mM asparagine were transferred to medium containing 0·5 mM nitrate and 5 mM amino acid (or ammonia) for 16 h prior to extraction.

RESULTS

Amino acids and ammonia as repressors of nitrate reductase

The results in Table 1 show the effect of ammonia and several amino acids on the induction of nitrate reductase. It is clear that all the compounds tested exert an inhibitory effect on the induction. The inhibition is particularly marked in the case of ammonia, alanine, asparagine and arginine. The effectiveness of these compounds as inhibitors of the induction does not appear to be directly related to either the extent to which they are utilized as sole nitrogen sources or the extent to which they inhibit growth. This is well illustrated by arginine and isoleucine. Neither of these is utilized as a sole nitrogen source, both completely inhibit growth on ammonia or nitrate but while arginine inhibits the induction by 80 to 90% isoleucine inhibits the induction by less than 50%.

Table 2

Effect of amino acids on nitrate reductase level in induced plants

Treatment	μ moles NO_2/h/g.f.w.
Control	5·4
+ 5 mM alanine	0·8
+ 5 mM asparagine	0·7
+ 5 mM glutamine	2·9
+ 5 mM glycine	0·7

Plants grown for 24 h on 0·5 mM nitrate were transferred to medium containing 0·5 mM NO_3 and 5 mM of appropriate amino acid for 24 h prior to extraction.

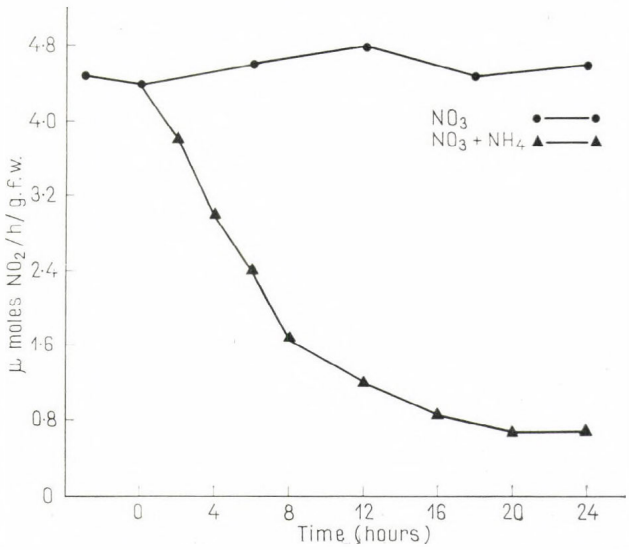

Fig. 1. Time course for ammonia repression of nitrate reductase. Plants were grown on 1·0 mM nitrate for 24 h prior to the addition of 7·5 mM ammonia (NH_4Cl). ● — ● NO_3 1 mM; ▲ — ▲ NO_3 1 mM + NH_4Cl 1 mM

A similar effect of amino acids has been noted by Filner (1966) who interprets this inhibition of induction as being evidence for repression by the end products of nitrate assimilation.

Ammonia and amino acids are also effective in reducing the level of nitrate reductase when added to already induced cultures (Table 2 and Fig. 1). The effectiveness of amino acids in reducing the level of induced enzyme is similar to their effectiveness in inhibiting the induction. The time course of repression by ammonia is shown in Fig. 1. There appears to be an immediate response on adding ammonia and after four hours the activity has decreased by almost 20%.

Table 3

Concentration dependance of asparagine and glycine repression

Concentration repressor	Nitrate reductase: μmoles NO_2/h/g.f.w.	
	Asparagine	Clycine
0 (1 mM NO_3)	5·06	5·06
+ 1 mM	1·64	1·83
+ 5 mM	1·24	1·29
+10 mM	0·65	0·64
0 (5 mM NO_3)	5·83	5·83
+ 5 mM	1·82	1·97
+10 mM	1·09	1·13

Plants were grown for six days on 1 mM asparagine prior to transfer (see above for treatments). Enzyme activity was determined after 20 h of induction.

In Table 3 the effect of varying concentrations of asparagine and glycine on the induction is shown. It is evident that the extent to which they inhibit the induction is dependent on the relative concentration of inducer and repressor.

Effect of ammonia and asparagine on nitrate accumulation

The range of compounds active as repressors of nitrate reductase raises a number of questions regarding their mode of action and in particular whether or not they act through a common mechanism. The simplest mechanism which could account for their effect involves inhibition of nitrate uptake. To investigate this possibility the effects of ammonia and asparagine on nitrate uptake have been examined (Table 4). It is evident that although ammonia and asparagine inhibit the induction of the enzyme they do not inhibit the accumulation of nitrate. The tissue concentration of nitrate is in fact higher in plants grown for short periods in the presence of ammonia and asparagine. This effect is observed when either limiting amounts or an excess of nitrate is supplied.

Previous experiments (Stewart, 1972) which examined the effect of nitrate concentration on the induction indicated that increasing the nitrate level above 0·25 mM resulted in little increase in enzyme level. The time-course

Table 4

Effects of ammonia and asparagine on intracellular nitrate level

A. Plants supplied with 2·0 μ moles NO_3 (1 mM) for 24 h

Treatment	μ moles NO_3/g.f.w.
Nitrate	0·70
Nitrate + 5 mM ammonia	1·05
Nitrate + 5 mM asparagine	1·00

B. Plants supplied with 100 μ moles NO_3 (1 mM) for 48 h

Treatment	μ moles NO_3/g.f.w.
Nitrate	5·47
Nitrate + 5 mM ammonia	7·27
Nitrate + 5 mM asparagine	6·15

Approximately 0·3 g plant tissue (initial weight) treated as described above.

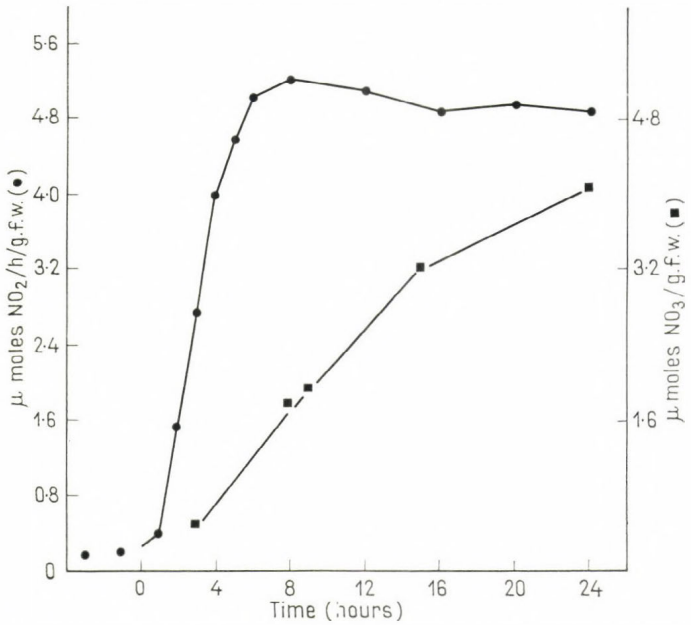

Fig. 2. Time course of nitrate reductase induction and nitrate accumulation
Plants were grown on 1 mM asparagine prior to transfer to 5 mM nitrate. • — • Nitrate reductase: ■ — ■ Nitrate concentration

of nitrate reductase induction and nitrate accumulation (Fig. 2) indicates that an increase in nitrate level above 1 to 2 μ moles per gram fresh weight is not reflected in a corresponding increase in enzyme level. Thus the nitrate reductase level reaches an apparent steady-state after 8 to 10 h while nitrate accumulation continues beyond 20 to 24 h. These results together with those obtained previously (Table 3) suggest that it is relative intracellular concentrations of inducer and repressor(s) which are important in determining the level of nitrate reductase.

Effect of pre-treatment of the kinetics of induction

The rate of induction shown in Fig. 2 is considerably faster than that reported previously (Stewart, 1968). While one of the factors contributing to this is the age of the cultures used, another and more important factor is the nitrogen source the plants were grown on prior to being transferred to nitrate. It can be seen from the results in Fig. 3 that the initial rate of induction with plants pre-grown on asparagine is nearly five-times as fast as that in plants pre-grown on ammonia. Since the growth rate and general nitrogen status of the plants on ammonia and asparagine are similar an explanation of these results in terms of ammonia "toxicity" is unlikely.

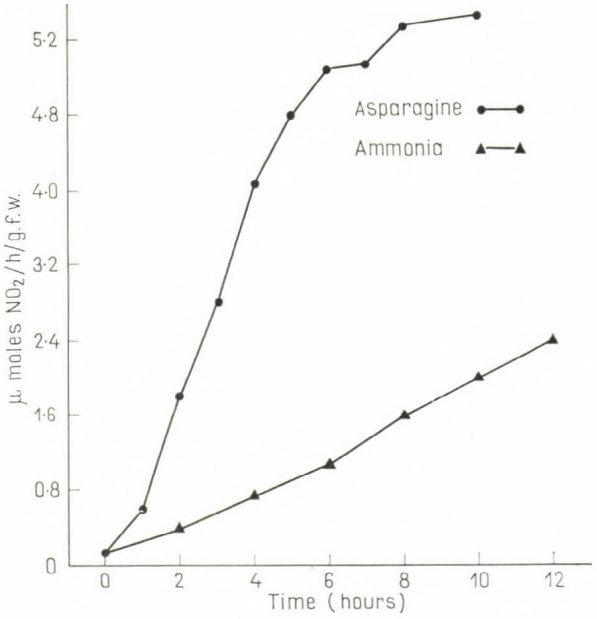

Fig. 3. Time course of nitrate reductase induction of plants pregrown on ammonia or asparagine
Plants used in this experiment were sub-cultured on either 1 mM asparagine (● — ●) or 1 mM NH_4Cl (▲ — ▲) for period of 30 days prior to being transferred to 5 mM NO_3. The actual cultures used were 6 days old. (Unpublished results T. O. Orebamjo.)

There would appear then to be a carry over effect in ammonia grown plants which results in a slower rate of induction.

These observations may be relevant in considering the nature of repression. It could be, for example, that in plants grown on ammonia the total amino pool is higher than on asparagine or that some specific pool component is higher, resulting in a slower rate of induction.

The total amino pool of plants grown on nitrate, ammonia and asparagine is shown in Table 5. Asparagine grown plants contain the highest soluble amino acid levels, greater than that on ammonia. If the difference in rate

Table 5

Total amino acid levels

Treatment	μg α-NH_2N/gram fresh weight
Ammonia	468
Asparagine	584
Nitrate	425

Plants were grown for six days on 2 mM of appropriate nitrogen source prior to extraction.

of induction in ammonia and asparagine grown plants is not related to the total amino pool levels then it seems probable that some specific component or components might be responsible. Analysis of the main components indicates that the relevant differences appear to be in the levels of ammonia and arginine. On ammonia the arginine level is 1·85 μ moles per gram fresh weight and that of ammonia is 8·3 μ moles per gram fresh weight while on asparagine they are 0·5 and 2·9 μ moles respectively.

The effect of pre-treatment with other amino acids prior to induction has been examined (Table 6). These results indicate that 24 h pre-

Table 6

Effect of pre-treatment on rate of nitrate reductase induction

Treatment	Units/h
Asparagine (> 30 days)	950
Ammonia (12 h)	650
Ammonia (24 h)	300
Ammonia (5 days)	255
Ammonia (> 30 days)	200
Aspartic (24 h)	875
Arginine (24 h)	320
Glutamate (24 h)	895

Plants pregrown on 2 mM ammonia or amino acids for varying periods of time prior to induction on 5 mM NO_3. Plants used in these experiments were grown on 1 mM asparagine. One unit of enzyme represents an activity of 1 nmole/h/g fresh weight. The initial rate of induction was determined from the increase in activity over the first six hours. (Unpublished results T. O. Orebamjo.)

treatment with arginine markedly reduces the rate of induction (by over 50%), relative to that of asparagine grown plants. A similar pre-treatment with glutamate and aspartate has little effect on the subsequent rate of induction. It is interesting to note that as the length of pre-treatment with ammonia is increased there is a progressive decrease in the rate of induction, suggesting that there is a build up of some compound which decreases the rate of induction. From the effects of arginine pretreatment and the preliminary data obtained from the amino pool composition arginine would seem a possible candidate for this compound.

DISCUSSION

The results described here concern for the most part the pattern of metabolic control regulating the formation of nitrate reductase rather than the precise molecular mechanisms through which such control is mediated. In relation to such mechanisms the results indicate that ammonia and asparagine at least, act at the cellular level rather than by inhibiting nitrate accumulation and in this way reducing the inducer pool. A similar conclusion has been reached by Ferguson (1969) and by Heimer and Filner (1970). Since ammonia and asparagine are as effective as nitrate when supplied as sole nitrogen sources non-specific effects arising from toxicity can be disregarded and these compounds can be regarded as end product repressors. In the case of amino acids such as isoleucine and threonine which cannot be utilized as sole nitrogen sources and are toxic to plants grown on nitrate or ammonia, the possibility that their inhibitory effects on nitrate reductase induction are non-specific, arising from toxicity cannot be eliminated. The question which arises in relation to those amino acids which inhibit the induction but are not toxic is whether the repression is really multivalent or is mediated through a common metabolite? The results from the pre-treatment experiments while by no means unequivocal suggest the possibility that repression might be specifically mediated by compounds such as ammonia and arginine. There are, however, difficulties in extrapolating the amino pool data to the *in vivo* situation since such measurements mask any intracellular compartmentation and hence the existence of a cytoplasmic repressor pool. One approach currently being used to investigate the problem of multivalent versus specific repression is to study the kinetics of induction in the presence of different repressors. Preliminary studies of the time course of induction in the presence of asparagine indicate the degree of inhibition is constant at all times during induction. If these observations can be confirmed with other amino acids then their conversion to a common repressor is unlikely. While multivalent repression would seem to permit flexibility in the control of nitrate assimilation the possibility that a nitrogen-rich storage compound such as arginine is the specific repressor is an attractive alternative hypothesis.

One feature of the results from the represssion experiments which seems of importance in considering the level at which repression occurs is the immediate response in enzyme level on adding ammonia to induced plants (Fig. 1). Assuming that the increase in enzyme activity observed during induction represents *de novo* synthesis it is unlikely that m-RNA synthesis

is limiting since at the time of ammonia addition the enzyme is at the fully induced level. This being so it is possible then that repression could act at a post-transcriptional step in protein synthesis. One mechanism which would account for repression might act at the translation step, inhibiting in some way the formation or release of the polypeptide. An alternative possibility is that the repressors might activate a protein dependant inactivation system similar to that described by Travis, Jordan and Huffaker (1969) for the dark inactivation of barley nitrate reductase. Further studies on the rate of nitrate reductase loss under different conditions should enable a distinction to be made between the effects of repressors on enzyme formation and those on enzyme stability.

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AMINO ACID ANALOGUES AND PROTEIN SYNTHESIS IN PLANTS

by

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INTRODUCTION

Protein synthesis can be influenced by many types of reagent. A number of antibiotics and several modified pyrimidine and purine bases antagonize the protein-synthesizing system at an early step, causing aberration of the DNA replication or transcription processes. By contrast, another group of substances influence protein synthesis either by limiting the supply of amino acids or by restricting their incorporation into polypeptide chains. Substances of this latter type are normally called amino acid analogues. To be effective, an analogue molecule should possess a structure closely akin to that of one of the twenty normal constituents of protein. Ideally, an analogue should be an 'isostere' (Richmond, 1962) of a protein amino acid, and possess almost identical pK values. Chemists have 'custom-built' many substances to fit these criteria and one of the most successful molecules has been *p*-fluorophenylalanine; the substitution of a single F atom for an aromatic proton leaves the molecule inappreciably larger than phenylalanine, and the ionization of the distant amino and carboxyl groups is hardly affected. Analogues may affect the size of metabolic pools by competing for sites on the permease enzyme systems governing the uptake of the normal amino acid into cells or by acting as 'false' feedback inhibitors or repressors of key enzymes involved in amino acid biosynthesis. Alternatively, protein synthesis can be impaired by analogues functioning as competitive substrates, or occasionally as inhibitors, of particular aminoacyl-tRNA synthetases: such competition can lead to either fewer protein molecules being completed, or to the production of anomalous protein molecules, containing analogue residues, having impaired biological function (Fowden et al., 1967).

Plants synthesize a great variety of amino acids, and a recent estimate places their number at almost 200 (Fowden, 1970). It is then not surprising that with so many plant amino acids available, some of the molecules should behave as analogues. For example, detailed investigations have been made of canavanine antagonism of arginine metabolism, of ethionine and selenomethionine interaction with systems utilizing methionine, and of the competitive behaviour observed for 2-amino-4-methylhex-4-enoic acid and azetidine-2-carboxylic acid with enzymes implicated in phenylalanine and proline metabolism, respectively (Fowden et al., 1967). We now wish to present new data concerning naturally-occurring imino acids that act as proline analogues and also some preliminary information regarding the amino acid substrate specificity of preparations of glutamyl-tRNA synthetase obtained from different plants.

Azetidine-2-carboxylic acid, the lower homologue of proline, was one of the earliest described examples of a natural amino acid exhibiting analogue behaviour. This imino acid occurs fairly widely, and in high concentration, in species assigned to the family Liliaceae (Fowden and Steward, 1957). It strongly inhibits the growth of seedlings, which do not themselves synthesize the imino acid (Fowden, 1963), and also restricts the growth of cultures of *Escherichia coli* (Fowden and Richmond, 1963). The extent of growth inhibition was correlated with the percentage replacement of proline residues in cellular protein by those of azetidine-2-carboxylic acid. Similar observations have been recorded with a chemically-synthesized analogue of proline, namely 3,4-dehydroproline (Fowden et al. 1963). However, azetidine-2-carboxylic acid is not a component of protein molecules in plants producing the imino acid. This difference was shown to depend upon the differential abilities of the prolyl-tRNA synthetase to activate proline and its analogue molecules. Enzyme from a plant such as *Phaseolus aureus*, whose growth is strongly inhibited by both azetidine-2-carboxylic acid and dehydroproline, activated these imino acids at rates (determined at enzyme-saturating concentrations of the substrates) of 38 and 57%, respectively, of that determined for proline. The prolyl-tRNA synthetase from *Convallaria majalis* (another azetidine-2-carboxylic acid-producing species) behaved similarly, but the enzyme from *Asparagus officinalis*, a liliaceous plant that does not produce azetidine-2-carboxylic acid, could be likened to that from *Phaseolus*, i.e. it activated the analogue (Peterson and Fowden, 1965); azetidine-2-carboxylic acid also markedly inhibited the growth of seedlings of *Asparagus*. These results strongly suggest that an evolutionary mutation, affecting the conformation of the active site of prolyl-tRNA synthetase molecules, has occurred in those species producing azetidine-2-carboxylic acid; as a result, the modified enzyme can selectively discriminate against azetidine-2-carboxylic acid and so protect these species against an otherwise toxic product (Fig. 1).

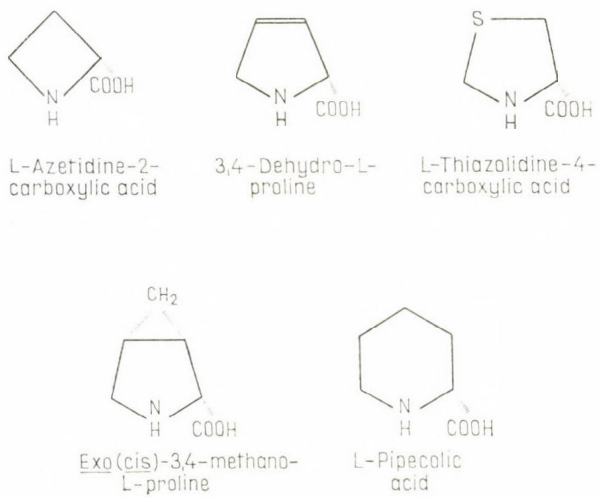

Fig. 1. Imino acid structure

In the past two years, interesting isolations of azetidine-2-carboxylic acid have been made from seedlings of the legume, *Delonix regia* (Sung and Fowden, 1969) and from the nitrogenous fraction obtained during the bulk processing of sugar beet (Knobloch and

Fowden, unpublished results). We also have established that azetidine-2-carboxylic acid forms a major constituent of seedlings of four other legume genera, including *Parkinsonia*. The imino acid generally cannot be detected in the dry seeds of these legumes using normal chromatographic procedures: this is also true for sugar beet extracts, where azetidine-2-carboxylic acid is only present at about onefiftieth of the concentration of proline and therefore is detected only after considerable fractionation of the amino acid complex has been accomplished. These identifications in plants totally unrelated to the Liliaceae have enabled us to extend our comparative study of prolyl-tRNA synthetase enzymes from different azetidine-2-carboxylic acid-producing species.

On *a priori* grounds, two distinct prolyl-tRNA synthetases might exist in the legume species — the enzyme located in the dry seed, which lacks azetidine-2-carboxylic acid, might resemble that of *Phaseolus* and activate azetidine-2-carboxylic acid significantly, whilst the prolyl-enzyme synthesized during the growth of seedlings presumably would discriminate against the imino acid, now rapidly accumulating in the plant tissues. However, purified preparations of prolyl-tRNA synthetase obtained from *Delonix* or *Parkinsonia* failed to activate azetidine-2-carboxylic acid, irrespective of whether the enzyme originated from dry seeds or from seedlings (Table 1). Obviously, evolutionary processes have eliminated the ancestral-type enzyme capable of activating azetidine-2-carboxylic acid (which presumably could have functioned satisfactorily in relation to protein synthesis in the developing seed), and have led to the simplest situation in which a single azetidine-2-carboxylic acid-resistant enzyme serves to activate proline throughout the plant.

Table 1 also presents kinetic parameters for preparations of prolyl-tRNA synthetase obtained from several other species, including seedlings of sugar beet *(Beta vulgaris*, var. *media)*. The enzyme from the latter species activated azetidine-2-carboxylic acid very significantly in comparison with the normal substrate proline (V_{max} for azetidine-2-carboxylic acid was 73 % of the similar value for proline); the slightly lower affinity of the enzyme for azetidine-2-carboxylic acid than proline is confirmed by the relative K_m values. These kinetic data show a very close similarity with those determined for prolyl-enzyme from other species lacking detectable azetidine-2-carboxylic acid, e.g. *P. aureus, Hemerocallis fulva,* and *Ranunculus bulbosa*. Therefore, although *Beta vulgaris* is able to synthesize azetidine-2-carboxylic acid, the plant apparently has not a need for a specialized prolyl-enzyme capable of discriminating against the analogue, because azetidine-2-carboxylic acid never reaches concentrations within the plant's cells that would lead to effective competition with proline at the active sites of the synthetase.

Data obtained for several other proline analogues are also given in Table 1. Prolyl-enzymes prepared from the three azetidine-2-carboxylic acid-producing plants activated 3,4-dehydroproline; in each case, the maximum reaction rates determined with this analogue were slightly less than 50 % of the corresponding V_{max} value determined for proline. Plants having prolyl-tRNA synthetases capable of using azetidine-2-carboxylic acid as a substrate activated dehydroproline somewhat more effectively, and V_{max} and K_m values closely approached those determined for proline, e.g.

Table 1

Kinetic parameters determined for proline and several of its analogues using prolyl-tRNA synthetase preparations from various higher plants

All data are calculated from reaction rates determined using ATP-$^{32}PP_i$ exchange procedures based on methods described by Peterson and Fowden (1965) and Anderson and Fowden (1970)

Plant species		Pro	A2C	DHPro	*N*-MeGly	*N*-EtGly	*N*-MeAla	MPro	TPro
**Parkinsonia*	K_m	$4{\cdot}35\times10^{-4}$	∞	$2{\cdot}2\times10^{-3}$	$4{\cdot}5\times10^{-2}$	—	—	$7{\cdot}1\times10^{-3}$	$\sim6\times10^{-2}$
aculeata (seed)	V_{max}	100	0	49	22	<3	5+	42	~70
**Delonix*	K_m	$1{\cdot}82\times10^{-4}$	∞	$7{\cdot}8\times10^{-4}$	3×10^{-1}	—	—	$4{\cdot}6\times10^{-3}$	—
regia (seed)	V_{max}	100	0	49	15	4	3+	22	~66
**Convallaria*	K_m	$4{\cdot}5\times10^{-4}$	∞	$1{\cdot}4\times10^{-3}$	—	—	—	$\sim2{\cdot}5\times10^{-3}$	—
majalis (seed)	V_{max}	100	0	44	19+	<3	<3	~36	—
Beta vulgaris	K_m	$4{\cdot}5\times10^{-4}$	$2{\cdot}2\times10^{-3}$	$5{\cdot}0\times10^{-4}$	—	—	—	—	—
(seedling)	V_{max}	100	73	89	~50	3+	10+	<3+	22+
Hemerocallis	K_m	$6{\cdot}25\times10^{-4}$	$5{\cdot}3\times10^{-3}$	$7{\cdot}4\times10^{-4}$	$1{\cdot}0\times10^{-1}$	—	—	—	—
fulva (leaf)	V_{max}	100	75	87	74	~4	11+	<3	—
Phaseolus	K_m	$1{\cdot}37\times10^{-4}$	$1{\cdot}43\times10^{-3}$	$2{\cdot}8\times10^{-4}$	$6{\cdot}7\times10^{-2}$	$\sim2\times10^{-2}$	$\sim7\times10^{-2}$	∞	$\sim2\times10^{-2}$
aureus (seed)	V_{max}	100	55	93	80	~5	17	0	~35
Ranunculus	K_m	$2{\cdot}9\times10^{-4}$	$2{\cdot}0\times10^{-3}$	$3{\cdot}6\times10^{-4}$	$1{\cdot}43\times10^{-1}$	∞	—	∞	—
bulbosa (leaf)	V_{max}	100	66	73	80	0	10	0	—

Key to substrate abbreviations: Pro, **L**-proline; A2C, **L**-azetidine-2-carboxylic acid; DHPro, **DL**-3,4-dehydroproline; *N*-MeGly, *N*-methylglycine; *N*-EtGly, *N*-ethylglycine; *N*-MeAla, *N*-methyl-**L**-alanine; MPro, *exo(cis)*-3,4- methano-**L**-proline; TPro, **L**-thiazolidine-4-carboxylic acid (**L**-thioproline).

K_m values are expressed as molar concentrations; the K_m for dehydroproline is expressed with respect to the **L**-form.

V_{max} values are expressed as percentages of the values determined for proline.

* Plants characterized by high concentrations of azetidine-2-carboxylic acid.

+ For these determinations, saturating substrate concentrations may not have been reached.

for enzyme from *Beta vulgaris*, the V_{max} value for dehydroproline was 89% of that for proline, whilst the respective K_m values were $5{\cdot}0 \times 10^{-4}$ and $4{\cdot}5 \times 10^{-4}$ M.

N-Methylalanine and several *N*-alkylglycines acted as substrates for a prolyl-tRNA synthetase prepared from *E. coli* by Papas and Mehler (1970). These substances show some analogy to proline by possessing an imino-N, whose attached carbon atoms may attain a configuration resembling a proline molecule having an unclosed heterocyclic ring. We have tested three of these *N*-substituted compounds as substrates for the prolyl-enzymes obtained from our range of higher plant species. Enzyme-substrate affinities, as reflected in K_m values (Table 1), were relatively low, but the values were of the same order as those determined by Papas and Mehler (1970) for *E. coli* enzyme (e.g. $2{\cdot}5 \times 10^{-1}$ M for *N*-methylglycine). There is an indication that these *N*-substituted derivatives (which, like dehydroproline have molecules that are smaller than proline) are activated more effectively, relative to proline, by prolyl-enzymes from species failing to discriminate against azetidine-2-carboxylic acid.

Table 1 also illustrates our findings for two analogues, thiazolidine-4-carboxylic acid (thioproline) and *exo(cis)*-3,4-methanoproline, that have slightly larger molecules than proline. Thioproline is an example of a chemically-synthesized analogue, that has not been demonstrated as a natural constituent of higher plants. *Cis*-3,4-methanoproline is a major component of the soluble nitrogen pool of seeds of *Aesculus parviflora* (Fowden et al., 1969). It has recently been synthesized, together with the *trans* isomer, by Fujimoto et al. (1971). Both substances were activated by selected prolyl-tRNA synthetase preparations and, in general, thioproline was the better substrate for individual enzymes. When V_{max} values, relative to proline, are compared for enzyme preparations from the two types of plant, it is clear that the behaviour of these larger analogue molecules contrasts sharply with that determined for analogues smaller than proline: both 3,4-methanoproline and thioproline were activated better by prolyl-enzymes from species that discriminated against azetidine-2-carboxylic acid, than by enzymes that activated the latter imino acid.

By extending these ideas, one may logically propose that the geometry of the active sites of prolyl-tRNA synthetases discriminating against azetidine-2-carboxylic acid, *e.g.* those from *Delonix* or *Convallaria*, is such that binding of molecules somewhat larger than proline is possible; however, by displaying this flexibility, the fit of small analogues, of which azetidine-2-carboxylic acid is the smallest of those being considered, is presumably too loose and inaccurate for firm binding to occur. Conversely, the active sites of the prolyl-enzymes from species activating azetidine-2-carboxylic acid are presumed to be smaller, thereby facilitating the formation of appropriate ligand bonds to azetidine-2-carboxylic acid, whilst restricting the binding of larger analogue molecules.

Our colleagues, Tristram and co-workers, have performed related studies on this range of proline analogues, being especially concerned with interaction upon the processes of proline uptake and biosynthesis in bacterial systems. Azetidine-2-carboxylic acid and dehydroproline both cause inhibition of growth of bacterial cultures and they strongly antagonize, by competition, the uptake of ^{14}C-proline into cells of *E. coli* (Tristram

and Neale, 1968); both analogues also act as feed-back inhibitors of the early steps in the enzymic synthesis of proline from glutamate (Tristram and Thurston, 1966). More recently, this group of workers have demonstrated that *cis*-3,4-methanoproline exhibits these same properties, markedly inhibiting growth and limiting ^{14}C-proline uptake of bacterial cells and effectively inhibiting the proline biosynthetic pathway in selected strains of *E. coli*. The *trans* isomer shows these properties to a lesser degree, whilst pipecolic acid has never been observed to act as a proline analogue (Rowland and Tristram, 1972).

STUDIES WITH GLUTAMIC ACID ANALOGUES

Numerous plant products may be regarded as being glutamic acid homologues or substituted derivatives. α-Aminoadipic acid, the simplest of these compounds, occurs fairly widely in plants but only in quite low concentrations. Various γ-substituted glutamic acids accumulate to much higher concentrations in particular species. For example, *threo*-γ-hydroxyglutamic acid occurs sporadically among species of the Liliaceae, including *Hemerocallis* (Fowden and Steward, 1957), and it has also been isolated from various ferns (Virtanen and Hietala, 1955): the *erythro*-isomer is not known as a higher plant constituent. Branched-chain C_6 amino acids related to glutamate are represented by:

(a) γ-Methyleneglutamic acid, and its amide γ-methyleneglutamine, which occur sporadically among members of the Leguminosae and Liliaceae and also in selected fern genera.

(b) *Erythro*-γ-methylglutamic acid, which has a very similar distribution to the compounds in (a), although usually in lower concentration: this compound, however, accumulates in *Phyllitis scolopendrium* (Blake and Fowden, 1964) and reaches a remarkable concentration in seed of *Caesalpinia bonduc* (Watson and Fowden, unpublished).

(c) γ-Hydroxy-γ-methylglutamic acid exists as the 2(S),4(S)-isomer in certain legumes (Sung and Fowden, unpublished), in *Pandanus* (Jadot et al., 1967), and in ferns (Blake and Fowden, 1964), whilst the diastereoisomeric 2(S),4(R)-form has been isolated from the fungus *Ledenbergia* (Jadot et al., 1967).

(d) *Cis*- and *trans*-forms of α-(carboxycyclopropyl)glycine, i.e. compounds that can be regarded as modifications of glutamic acid by the substitution of an additional —CH_2— group across the β and γ carbon atoms: these cyclopropane derivatives have been isolated from a number of genera of the Sapindaceae and from some species of *Aesculus* (Fowden et al., 1969).

Certain species producing γ-methyleneglutamic acid and γ-methyleneglutamine also contain γ-ethylideneglutamic acid (Fowden, 1966; Gmelin and Larsen, 1967); the related γ-ethylglutamic acid occurs in seed of *C. bonduc* (Watson and Fowden, unpublished). The structures of some of these amino acids are shown below as their Fischer projections (Fig. 2).

When we commenced our studies on the glutamyl-tRNA synthetase, no definitive information about this enzyme had been published for plant

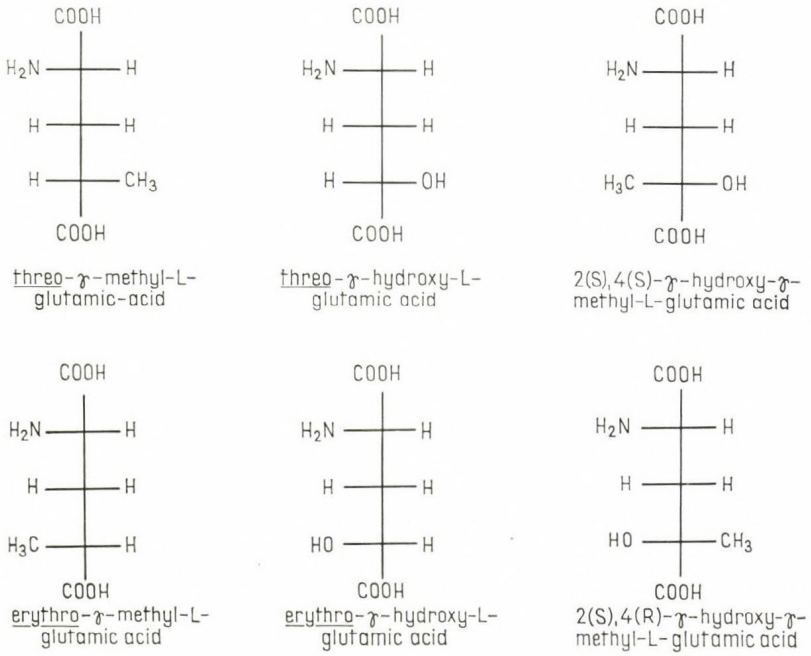

Fig. 2. Substituted glutamic acid structures

systems. Almost all studies surveying the levels of individual aminoacyl-tRNA synthetases in various plant tissues had recorded either very low or zero activities for the glutamyl-enzyme; such results are difficult to rationalize with the high content of glutamate in many plant proteins. Our initial experiments provided an explanation for many of the previous negative findings, for we observed that passage of buffered plant extracts through columns of Sephadex G-25 caused a preferential retardation of the glutamyl-tRNA synthetase peak, in relation to the main protein band which eluted earlier and contained almost all other aminoacyl-tRNA synthetases. Clearly, in many earlier studies the glutamyl-enzyme has been discarded in fractions from Sephadex G-25, not expected to contain enzymes. A further complication lies in the relative instability of the enzyme, a feature detrimental to the enhancement of the enzyme's specific activity by fractionation procedures.

Data relating to the amino acid specificity of the glutamyl-tRNA synthetase are given for three plant species in Table 2. The plants studied are (i) *P. aureus*, in which there is no record of occurrence of any of the substituted glutamic acids described above, (ii) *H. fulva*, a liliaceous species producing *threo*-γ-hydroxyglutamic acid, and (iii) *C. bonduc*, a legume whose seed contains *erythro*-γ-methyl-, γ-ethylidine- and γ-ethyl-glutamic acids. The results clearly show that enzymes from species producing particular glutamate analogues fail to activate the plant's own products, e.g. *threo*-γ-hydroxyglutamic acid shows no substrate activity for the glutamyl-enzyme from *Hemerocallis*, whereas the enzyme obtained from *P. aureus*

Table 2

Kinetic parameters determined for glutamic acid and several of its analogues using glutamyl-tRNA synthetase preparations from various higher plants
All data are calculated from reaction rates determined using ATP-$^{32}PP_i$ exchange procedures based on methods described by Peterson and Fowden (1965) and Anderson and Fowden (1970)

Substrate		*Plant species*		
		Phaseolus aureus seed	*Hemerocallis fulva* leaf	*Caesalpinia bonduc* seed
L-Glutamic acid	K_m	$7{\cdot}21\times10^{-3}$	$5{\cdot}24\times10^{-3}$	$9{\cdot}3\times10^{-3}$
	V_{max}	100	100	100
Erythro-γ-methyl-**L**-glutamic acid	K_m	$1{\cdot}55\times10^{-2}$	$2{\cdot}81\times10^{-2}$	∞
	V_{max}	68·1	40·2	0
Threo-γ-methyl-**DL**-glutamic acid	K_m	—	∞	—
	V_{max}	55·2 (75 mM)	0	20·1 (75 mM)
Threo-γ-hydroxy-**L**-glutamic acid	K_m	$2{\cdot}11\times10^{-2}$	∞	$5{\cdot}21\times10^{-2}$
	V_{max}	54·7	0	23·6
Erythro-γ-hydroxy-**DL**-glutamic acid	K_m	—	—	∞
	V_{max}	58·2 (75 mM)	34·2 (75 mM)	0
2(S), 4(S)-γ-hydroxy-γ-methyl-**L**-glutamic acid	K_m	$3{\cdot}43\times10^{-2}$	$1{\cdot}25\times10^{-1}$	∞
	V_{max}	42·2	Calculated as 10·2	0
2(S), 4(R)-γ-hydroxy-γ-methyl-**L**-glutamic acid	K_m	—	∞	∞
	V_{max}	38·2 (75 mM)	0	0

The following analogues of glutamic acid were also tested at concentrations of 75 mM, but no pyrophosphate exchange was detected: γ-methylene-**DL**-glutamic acid, γ-ethyl-**L**-glutamic acid, γ-ethylidene-**L**-glutamic acid, γ-ethyl **L**-glutamate, β-methyl-**DL**-glutamic acid, β-hydroxy-**DL**-glutamic acid, **DL**-α-aminoadipic acid, *cis*-**L**-α-(carboxycyclopropyl)glycine, *trans*-**L**-α-(carboxycyclopropyl)glycine.
K_m values are expressed as molar concentrations with respect to the **L**-form.
V_{max} values are expressed as percentages of the values determined for glutamic acid.

shows an affinity for a variety of γ-hydroxy and γ-methyl substituted glutamic acids. The discriminatory behaviour shown by particular enzymes seems to be stereospecific, for *erythro*-γ-hydroxyglutamic acid does serve as a substrate for the *Hemerocallis* enzyme. The same type of stereospecific discrimination is observed for the glutamyl-enzyme from *C. bonduc*, which fails to activate the natural *erythro*-γ-methylglutamic acid isomer, but shows an affinity towards the non-natural *threo*-isomer. An enzyme that fails to activate the *threo*-γ-hydroxy derivative, also will not utilize the *threo*-γ-methyl isomer (see *Hemerocallis*); similarly, failure of the *C. bonduc* enzyme to accept its own product, *erythro*-γ-methylglutamic acid, as a substrate is coupled with the non-utilization of *erythro*-γ-hydroxyglutamic acid. The sizes of hydroxy and methyl groups are closely alike and presumably the substitution of either group at an appropriate position on the glutamic acid molecule may cause complete steric hindrance of substrate binding at the enzyme's active site. α-Aminoadipic acid and several substituted glutamic acid derivatives, including those possessing larger sub-

stituent groups at the γ-position, or others having a β-substituent, were not activated by any of the glutamyl-tRNA synthetases.

These preliminary studies concerning the glutamyl activating enzyme from plants provide another example of an enzyme that has probably been subject to conformational modification in particular species to ensure the exclusion of particular substrates from its active site. Enzyme from *P. aureus* might be considered as an ancestral type associated with the lowest order of substrate discrimination. The two other species studied in Table 1 then might produce mutant forms of glutamyl-tRNA synthetase, whose molecules show subtle changes of conformation (possibly caused by substitution, deletion, or addition of amino acid residues in the polypeptide chain); these modified enzymes then discriminate more precisely between various amino acid substrates and fail to bind those molecules that are normal products of the plant under investigation.

SUMMARY

For many years, the non-protein amino acids have been regarded merely as a further group of unusual secondary products of plants. Certainly, many aspects of their behaviour and role in plants are still little understood, but their adoption in analogue studies has given them a new importance in recent years, and we may confidently expect that their increasing use in this way will provide further insights into the mechanisms controlling amino acid and protein biosynthesis.

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THE ROLE OF ATP SULPHURYLASE IN THE BIOSYNTHESIS OF CYSTEINE IN HIGHER PLANTS

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INTRODUCTORY REVIEW OF SULPHATE METABOLISM IN HIGHER PLANTS

Inorganic sulphate is the primary source of sulphur utilized by plants for the biosynthesis of all the sulphur-containing compounds found in plants, the most important being the amino acids cysteine and methionine. The synthesis of cysteine and methionine from sulphate by plants and micro-organisms is of great biological importance since animals are dependent on plants and microorganisms for their supply of cysteine and methionine. Animals, however, do have a limited capacity to synthesize cysteine from sulphate, but the sulphate in turn originates from the oxidation of sulphur containing substrates of plant and microbial origin (Roy and Trudinger, 1970).

The pathway of assimilatory sulphate metabolism is now fairly well established in yeast and *E. coli* though the details of the reactions involving the enzyme complexes PAPS* reductase and sulphite reductase are still not completely understood. The pathway of sulphate incorporation into cysteine has recently been reviewed in detail (Roy and Trudinger, 1970); the pathway is summarized in Fig. 1 using the nomenclature of Bandurski (1965).

The incorporation of sulphate-sulphur into cysteine has been demonstrated in a variety of photosynthetic and non-photosynthetic plant tissues (Wilson, 1962; Ellis, 1963; Thompson, 1967) but the pathway of sulphate metabolism in higher plants is not yet clear. The role of sulphite and sulphide as intermediates in the biosynthesis of cysteine from sulphate in plants has been deduced from nutrition experiments (Wilson, 1962; Roy and Trudinger, 1970) and is consistent with the rudiments of the pathway of sulphate metabolism described in yeast. The enzyme serine sulphydrase has been described in spinach leaf tissue (Brüggemann et al., 1962; Brüggemann and Waldschmidt, 1962) and Ellis (1963) has reported that serine enhanced the synthesis of cysteine in beetroot slices, thus demonstrating the role of this enzyme in the biosynthesis of cysteine *in vivo*. Sulphite reductase activity has been extracted and purified from plants (Tamura, 1965; Asada, 1967; Asada et al., 1969) and some progress has been made on characterizing the sulphite reductase complex and the nature of the electron donor (Asada et al., 1969). Mayer (1967) has reported that sulphite reductase activity is associated with chloroplasts.

* Abbreviations: APS, adenosine 5′-sulphatophosphate; APSe, adenosine 5′-selenophosphate; PAPS, adenosine 3′-phosphate 5′-sulphatophosphate.

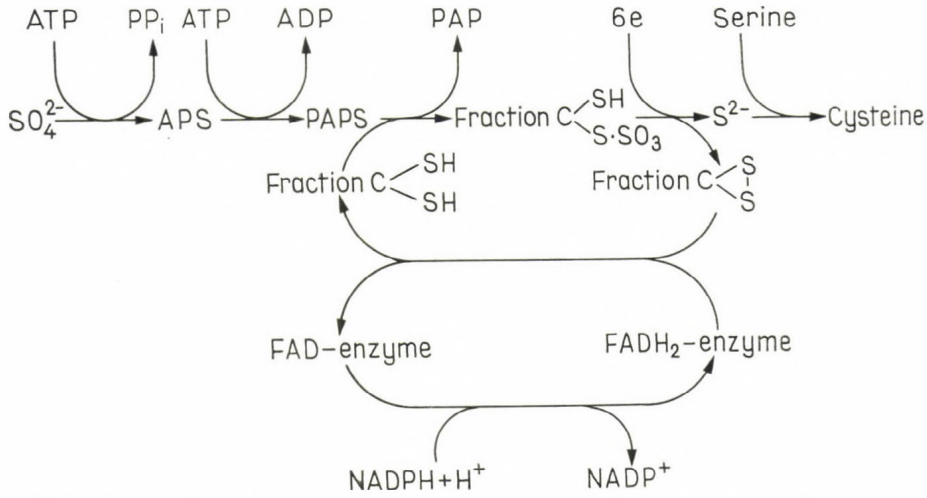

Fig. 1. Pathway of assimilatory sulphate metabolism in yeast (adapted from Bandurski, 1965). Fraction C is a heat-stable low molecular weight protein

Considerable uncertainty surrounds the earlier part of the pathway in plants. In yeast, the enzyme ATP: sulphate adenylyltransferase (trivial name ATP sulphurylase) catalyzes the formation of APS from ATP and sulphate:

$$ATP + SO_4^{2-} \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} APS + PP_i$$

Another enzyme, ATP adenylylsulphate 3'-phosphotransferase (trivial name APS kinase) catalyzes the activation of APS by ATP to form PAPS:

$$APS + ATP \overset{Mg^{2+}}{\rightleftharpoons} PAPS + ADP$$

The synthesis of [^{35}S]APS has been demonstrated in isolated illuminated chloroplasts (Asahi, 1964) and in extracts of various plant tissues (Ellis, 1969) using [^{35}S]sulphate as substrate. Adams and Johnson (1968) and Adams and Rinne (1969) have reported ATP sulphurylase activity in crude extracts of several plant species using the molybdate substrate method of Wilson and Bandurski (1958) but this method only provides indirect evidence for ATP sulphurylase activity and supplies very little information about the properties of the enzyme. PAPS reductase activity and synthesis of PAPS have been reported in *Chlorella* (Wedding and Black, 1960; Levinthal, 1967; Hodson et al., 1968) but there is considerable uncertainty whether PAPS is an intermediate in sulphate metabolism in higher plants. Mercer and Thomas (1969) reported that unilluminated chloroplast fragments synthesized both APS and PAPS when incubated with pyrophosphatase isolated from yeast. Asahi (1964) and Balharry and Nicholas (1970) on the other hand, did not detect the synthesis of PAPS in illuminated chloroplasts though Asahi (1964) concluded that PAPS is an intermediate in sulphate metabolism in spinach leaf because chloroplasts were unable

to reduce sulphate to sulphite unless ATP sulphurylase and APS kinase from yeast were added to illuminated chloroplasts.

The problem of whether PAPS is an intermediate in the metabolism of sulphate has also been encountered in *Salmonella pullorum;* in this organism, APS, but not PAPS, was synthesized from sulphate whilst the organism incorporated sulphate-sulphur into cysteine (Kline and Schoenhard, 1968). *S. pullorum*, however, contains a powerful 3'-nucleotidase which would hydrolyze PAPS to APS and orthophosphate. By including a 3'-nucleotide (e.g. 3'-AMP) to act as a substrate of 3'-nucleotidase whilst monitoring for APS kinase activity, then the synthesis of PAPS was detected. 3'-nucleotidase has been recorded in several plant species (Sung and Laskowski, 1962; Walters and Loring, 1966; Hanson and Fairley, 1969) and it is possible that the activity of this enzyme might have confounded attempts to detect PAPS in plant tissue, though Ellis (1969) found that plant extracts fortified with extracts of a bacterial mutant (*E. coli*, 4—2), which lacks ATP sulphurylase but contains APS kinase, synthesized PAPS. Since PAPS accumulated, Ellis reasoned that enzymes degrading PAPS were absent from the plant extract used. Another interpretation could be that PAPS only accumulated because the rate of synthesis of PAPS by the plant plus bacterial system exceeded the rate of degradation of PAPS. It may be significant that the synthesis of APS by plant extracts bears no relation to the synthesis of PAPS by plant extracts fortified with the mutant bacterial extract (Ellis, 1969). The action of a sulphatase (Roy and Trudinger, 1970) in plant extracts which hydrolyzes the P—O—S bond of sulphatophosphates bearing a 3'-phosphate group (e.g. PAPS) could also explain the failure to detect PAPS in plant extracts. We are currently conducting experiments similar to those of Kline and Schoenhard (1968) in an attempt to resolve whether 3'-nucleotidase activity is confounding attempts to demonstrate the synthesis of PAPS in extracts of higher plants.

Our main interest in the sulphate pathway in plants to date has been to obtain direct evidence for the occurrence of ATP sulphurylase in plant tissues, to purify the enzyme and to study its properties with special reference to substrate specificity. Our interest in substrate specificity arose in the first instance from ecological and physiological studies which established that some soils of northwestern Queensland (Australia) and Wyoming (U.S.A.) contain high concentrations of selenium and support plant species which accumulate selenium (e.g. *Neptunia amplexicaulis* and *Astragalus racemosus*) which in turn are toxic to stock that graze on these species (McCray and Hurwood, 1964; Rosenfeld and Beath, 1964; Shrift, 1969). Studies with non-accumulator species have shown that the selenium of selenate is incorporated into cysteine and methionine in lieu of sulphur (Peterson and Butler, 1962) resulting in the synthesis of inactive proteins when the selenoamino acid analogue is incorporated into protein. Accumulator species on the other hand characteristically synthesize derivatives of selenocysteine (Shrift, 1969) e.g. Se-methylselenocysteine. Whilst it has been argued that the synthesis of compounds such as Se-methylselenocysteine are detoxification mechanisms (Peterson and Butler, 1962), there are many reports of reactions in selenium accumulator species involving selenoamino acids for which the reaction with the corresponding sulphur

amino acid has not been detected, i.e. enzymes of selenium accumulator species differentiate between selenium compounds and the corresponding sulphur analogue (Shrift, 1969). We wondered whether the ATP sulphurylases of selenium accumulator species might also differentiate between sulphate and selenate since Wilson and Bandurski (1958) have reported that both selenate and sulphate are substrates of yeast ATP sulphurylase. An example of differentiation between a natural substrate and a toxic analogue is afforded by the prolyl-tRNA synthetase from *Polygonatum multiflorum* (Liliaceae) which differentiates between proline and azetidine-2-carboxylic acid whereas the prolyl-tRNA synthetases from non-liliaceous species are unable to differentiate between these two compounds; azetidine-2-carboxylic acid is toxic to those species in which the prolyl-tRNA synthetase is unable to differentiate azetidine-2-carboxylic acid from proline (Peterson and Fowden, 1965).

STUDIES OF SPINACH-LEAF ATP SULPHURYLASE BY THE PYROPHOSPHATE EXCHANGE ASSAY

Asahi (1964), Ellis (1969) and Mercer and Thomas (1969) have all reported the synthesis of APS from sulphate either in chloroplasts or crude plant extracts; these authors used [^{35}S]sulphate as substrate and separated [^{35}S]APS from sulphate by chromatography. The yield of APS in all cases was very low and suggests that assay methods based on the measurement of APS production are unsuitable for monitoring ATP sulphurylase activity. Wilson and Bandurski (1958) reported that the equilibrium of the reaction catalyzed by yeast ATP sulphurylase lies strongly towards ATP and sulphate ($\Delta G = +11$ kcal/mol); this would explain the low yields of APS reported in studies of the forward reaction even when the forward reaction was measured in the presence of either an endogenous or exogenous pyrophosphatase. Clearly then, assays based on the reverse reaction, or studies at equilibrium ($\Delta G = 0$), must be more sensitive than assays based on the forward reaction given the premise that only true substrates of the reaction catalyzed by ATP sulphurylase are to be used.

Marcus (1959) reported that when $MgSO_4$ was used in lieu of $MgCl_2$ as a source of Mg^{2+} for studying the aminoacyl-tRNA synthetases of spinach-leaf tissue by the [^{32}P]PP_i-ATP exchange assay, much higher endogenous exchange was observed. We reinvestigated this report and confirmed that crude dialyzed extracts of spinach-leaf tissue catalyze sulphate-dependent incorporation of [^{32}P]pyrophosphate into ATP (Shaw and Anderson, 1971). This observation is consistent with the operation of the back reaction of ATP sulphurylase:

$$[^{32}P]ATP + SO_4^{2-} \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} APS + [^{32}P]PP_i$$

The sulphate dependent PP_i-ATP exchange assay for measuring ATP sulphurylase has many advantages over the various methods employing the forward reaction: pyrophosphatase is not required, the correct substrates are employed, very short incubation times are required and the method is extremely sensitive. The stimulation of ATP-PP_i exchange by sulphate

and the sensitivity of the assay with crude dialyzed spinach extracts is illustrated in Fig. 2. Balharry and Nicholas (1970, 1971) have described another technique for measuring ATP sulphurylase which utilizes the back reaction; the method involves the formation of ATP (measured by the luciferin-luciferase assay) from APS. The method of Balharry and Nicholas (1970, 1971) is probably even more sensitive than the ATP-PP_i exchange assay but requires more sophisticated equipment and cannot be used to study the kinetics of sulphate nor to study sulphate analogues. Both methods are well suited for monitoring activity during purification.

The ATP-PP_i exchange assay is not without its problems. Fluoride stimulated sulphate-dependent pyrophosphate exchange of crude extracts approximately 2 to 3-fold. Since fluoride is a potent inhibitor of Mg^{2+} dependent alkaline pyrophosphatase and spinach-leaf tissue contains a very active pyrophosphatase (El-Badry and Bassham, 1970), then this suggests that the endogenous pyrophosphatase interferes with the pyrophosphate exchange assay of ATP sulphurylase. This conclusion was confirmed by a study of the effect of pyrophosphate concentration, with and without fluoride, on sulphate-dependent incorporation of pyrophosphate into ATP (Fig. 3). Sulphate-dependent pyrophosphate incorporation was approximately maximal at 2 to 4 mM pyrophosphate. At low concentrations of pyrophosphate in the absence of fluoride, pyrophosphate incorporation was much less than in the presence of fluoride. Sulphate-dependent pyrophosphate incorporation in the absence of fluoride, relative to the exchange with fluoride, gradually increased as the concentration of pyrophosphate was increased until at 4 mM the two exchange rates were approximately equal.

ATP sulphurylase of spinach was purified approximately 1000-fold and separated from pyrophosphatase by a combination of ammonium sulphate

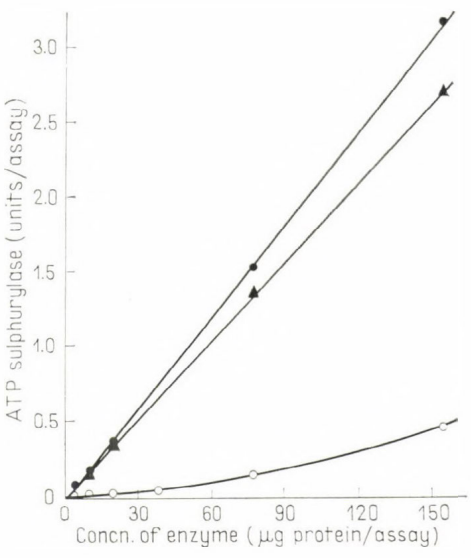

Fig. 2. Effect of concentration of crude dialyzed spinach extracts upon sulphate-dependent ATP-PP_i exchange(▲). Standard assays were conducted at 35 °C for 15 min and contained 2 µmol Na_2K_2ATP, 10 µmol $MgCl_2$, 2 µmol [^{32}P]$Na_4P_2O_7$ (approximately 0·5 µCi), 40 µmol K_2SO_4, 10 µmol NaF, 100 µmol tris-HCl buffer (pH 7·8) and enzyme in a total volume of 1 ml. Activities with (●) and without (○) K_2SO_4 are also included. A concentration of 0·1 mg protein per assay represents the protein extracted from approximately 40 mg fresh weight of tissue. [^{32}P]ATP was isolated and measured as described by Shaw and Anderson (1971). ATP sulphurylase activity was calculated by the method of Davie, Koningsberger and Lipmann (1956) and is expressed as sulphate-dependent pyrophosphate exchange in nmol/min (ATP sulphurylase units). Undialyzed extracts contained negligible sulphate-dependent pyrophosphate exchange

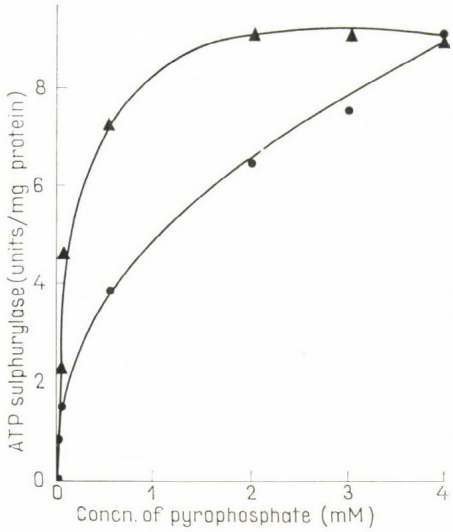

Fig. 3. Effect of concentration of [^{32}P] sodium pyrophosphate (of constant specific radioactivity) upon ATP sulphurylase activity of crude dialyzed spinach extracts with (▲) and without (●) sodium fluoride (10 μmol). All other conditions of the assays were as described in Fig. 2

fractionation, gel filtration on Sephadex G-200 and chromatography on DEAE-cellulose (Table 1, Fig. 4). Pyrophosphatase activity, in addition to ATP sulphurylase activity with and without fluoride, was monitored at each stage of purification. The higher activity of ATP sulphurylase in the presence of fluoride was closely correlated with pyrophosphatase activity. Purified enzyme contained no pyrophosphatase activity and was virtually insensitive to fluoride.

G. W. Skyring and P. A. Trudinger at the Baas Becking Geobiological Laboratory, Canberra (personal communication), have found that purified ATP sulphurylase, prepared by the method described in Table 1, contains one major protein band which is associated with ATP sulphurylase activity and four minor bands, some of which might be isozymes. Gel filtration on Sephadex G-200 indicates that the enzyme has a molecular weight between 200,000 and 300,000 but we have no detailed information about the exact molecular weight and sub-unit components to date though Tweedie and

Table 1

Typical purification of ATP sulphurylase from spinach and separation from pyrophosphatase

ATP sulphurylase activities are shown using sulphate (40 mM) and selenate (40 mM) as substrates with and without 10 mM-sodium fluoride. No pyrophosphatase activity was detected in any fraction in the presence of 10 mM-fluoride. Virtually no sulphate-dependent pyrophosphate exchange was detected in undialyzed crude extracts. ATP sulphurylase and pyrophosphatase activities were assayed as described in Fig. 1 and Fig. 4 respectively

Treatment	Protein (mg)	ATP sulphurylase (units/mg protein)				Pyrophosphatase (units/mg protein)
		with sulphate		with selenate		
		with fluoride	without fluoride	with fluoride	without fluoride	
Dialyzed crude extract	2750	4·67	3·23	1·44	0·70	0·42
$(NH_4)_2SO_4$ fraction	143	25·1	3·99	6·31	0·31	0·80
Sephadex G-200 (ATP sulphurylase peak)	26·2	105	105	29·5	29·7	0·04
DEAE-cellulose	0·75	4300	4640	1290	1300	0

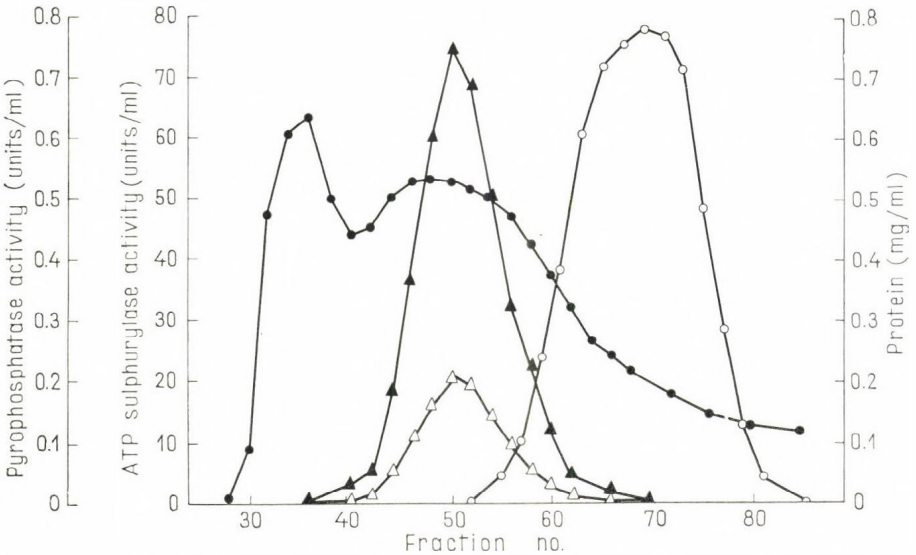

Fig. 4. Separation of ATP sulphurylase and pyrophosphatase activities of spinach by gel filtration on Sephadex G-200. Sulphate-dependent PP_i-ATP exchange (▲), selenate-dependent PP_i-ATP exchange (△), pyrophosphatase activity (○) and protein (●). Assay conditions for the pyrophosphate exchange activities were as described in Fig. 2 except that sodium fluoride was omitted and selenate-dependent exchange was determined using potassium selenate (40 μmol) in lieu of potassium sulphate. Pyrophosphatase assays were conducted at 35 °C for 15 min and contained 5 μmol $Na_4P_2O_7$, 20 μmol $MgCl_2$, 100 μmol tris-HCl (pH 8·0) and enzyme in a final volume of 1 ml. Activity is expressed as μmol orthophosphate released/min (pyrophosphatase units); phosphate was measured by the method of Allen (1940)

Segel (1971) have reported that the ATP sulphurylase from *Penicillium chrysogenum* has a molecular weight of 440,000 and is comprised of 8 subunits. Purified spinach enzyme requires either Mg^{2+} or Co^{2+} (10 mM) for optimum activity; Mn^{2+}, Ni^{2+} and Zn^{2+} were less effective. The pH optimum of the enzyme was 7·5 to 9·0 and the enzyme was virtually insensitive to sulphydryl group reagents.

Neither crude extracts nor purified enzyme catalyzed ADP-P_i exchange demonstrating that spinach-leaf tissue does not contain ADP sulphurylase activity as reported in yeast (Robbins and Lipmann, 1958*a*) nor an enzyme catalyzing ADP-P_i/sulphate exchange as also reported in yeast (Grunberg-Manago et al., 1966). Purified enzyme also does not catalyze pyrophosphate exchange with AMP, ADP, or GTP, nor orthophosphate exchange with ATP, ADP, AMP, deoxyATP or GTP. Purified enzyme, however, catalyzed deoxyATP-PP_i exchange; the synthesis of [^{32}P]deoxyATP was confirmed by chromatography. The affinity of ATP sulphurylase for deoxyATP (K_m 0·84 mM) was less than for ATP (K_m 0·35 mM) and V (deoxyATP) was 30% V (ATP) indicating that ATP is the preferred substrate. Competition experiments with deoxyATP and ATP demonstrated that deoxyATP competed with ATP for the same active site on the enzyme (Fig. 5). Asahi

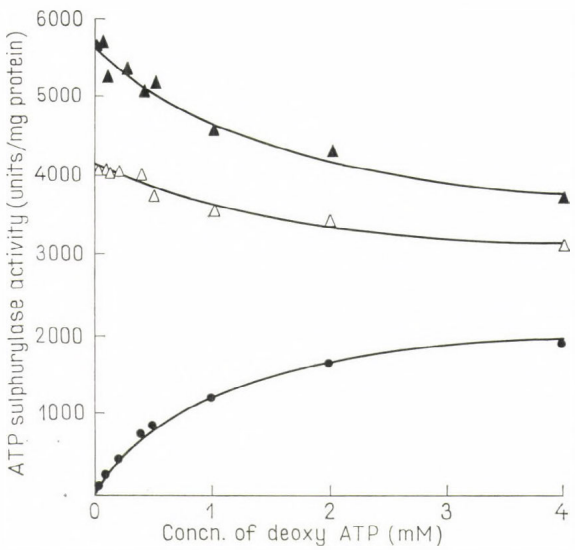

Fig. 5. Effect of concentration of deoxyATP on sulphate-dependent pyrophosphate exchange in the presence of 0·5 mM (△), and 1·0 mM ATP (▲) and in the absence of ATP (●) using purified enzyme in the absence of sodium fluoride. All other conditions of the assay were as described in Fig. 2

(1964) and Balharry and Nicholas (1970) have demonstrated that ATP sulphurylase is a chloroplast enzyme; since chloroplasts are the main sites of ATP synthesis in leaf tissue then, taken collectively, the evidence suggests that ATP is the preferred substrate *in vivo*.

The affinity of ATP sulphurylase for sulphate is not high (K_m 3·1 mM) and suggests that quite high concentrations of sulphate must be located in the chloroplast for APS synthesis to proceed *in vivo*. Sulphate is the only form of sulphur activated by ATP sulphurylase; sulphite, thiosulphate, dithionate, persulphate, dithionate and metabisulphite were not activated. Selenite was also inactive. The only true alternative substrate of sulphate in the pyrophosphate exchange assay was selenate as instanced by the constant ratio of sulphate- to selenate-dependent pyrophosphate exchange during purification (Table 1, Fig. 4). The affinity of ATP sulphurylase for selenate (K_m 1·0 mM) was greater than for sulphate (K_m 3·1 mM) though V (selenate) was only 30% V (sulphate). The kinetics of sulphate/selenate competition experiments (Fig. 6) are consistent with the kinetics of two substrates competing for one enzyme (Pocklington and Jeffery, 1969). The synthesis of [^{32}P]ATP using selenate as substrate was confirmed by chromatography; no unlabelled AMP was detected. Wilson and Bandurski (1958) reported that molybdate was an analogue of sulphate in the ATP sulphurylase assay. Purified spinach leaf enzyme did not catalyze molybdate-dependent pyrophosphate exchange but substantial amounts of AMP were synthesized. Molybdate inhibited sulphate-dependent pyrophosphate exchange thus confirming Wilson and Bandurski's hypothesis that molybdate competes with sulphate and that a stable adenylate of molybdate is not formed.

Spinach leaf ATP sulphurylase has many properties in common with the ATP sulphurylases of yeast (Robbins and Lipmann, 1958*b*; Wilson and Bandurski, 1958) and *Penicillium chrysogenum* (Tweedie and Segel, 1971). All three enzymes are stable, require either Mg^{2+} or Co^{2+} as cofactors, have similar broad pH optima and the yeast and spinach enzymes are insensitive to sulphydryl group reagents. The three enzymes differ from

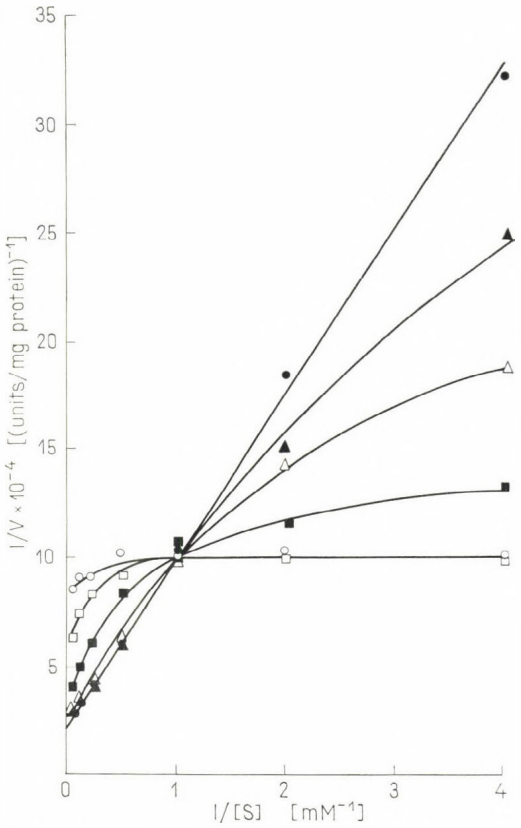

Fig. 6. Double reciprocal plot of the effect of concentration of potassium sulphate [S] on the activity of purified ATP sulphurylase (v) in the presence of 0·125 mM (▲), 0·5 mM (△), 2 mM (■), 20 mM (□), and 40 mM potassium selenate (○) and in the absence of selenate (●). All other conditions of the assay were as described in Fig 2 except that fluoride was omitted

the ATP sulphurylases from animal tissues which are labile and sensitive to sulphydryl group reagents (Panikkar and Bachhawat, 1968; Levi and Wolf, 1969).

SYNTHESIS OF APS BY COUPLING PURIFIED ATP SULPHURYLASE WITH Mg^{2+} DEPENDENT ALKALINE PYROPHOSPHATASE

Attempts to synthesize APS from ATP and [^{35}S]sulphate using highly concentrated purified ATP sulphurylase were unsuccessful, presumably because of the very unfavourable free energy change. Accordingly, we prepared partially purified Mg^{2+} dependent alkaline pyrophosphatase from spinach (free from ATP sulphurylase activity). Addition of pyrophosphatase to assays containing ATP, [^{35}S]sulphate, Mg^{2+} and ATP sulphurylase resulted in the synthesis of a [^{35}S]-labelled product which was absorbed by activated charcoal; the product was eluted with 0·1 M NH_3 in 50% (v/v) ethanol and subjected to chromatography. The product ran with R_F identical to APS (Fig. 7); this result was confirmed by rechromatography in several solvents. Thus we were able to utilize the absorption of APS by

charcoal to separate APS from sulphate and to quantify the synthesis of APS by radiochemical procedures. We propose to use this technique with [^{75}Se]selenate as substrate to establish whether APSe is synthesized.

The requirements for the synthesis of APS in the coupled enzyme system are shown in Table 2; ATP, Mg^{2+}, ATP sulphurylase and pyrophosphatase are all required. Fluoride, an inhibitor of pyrophosphatase, inhibits the

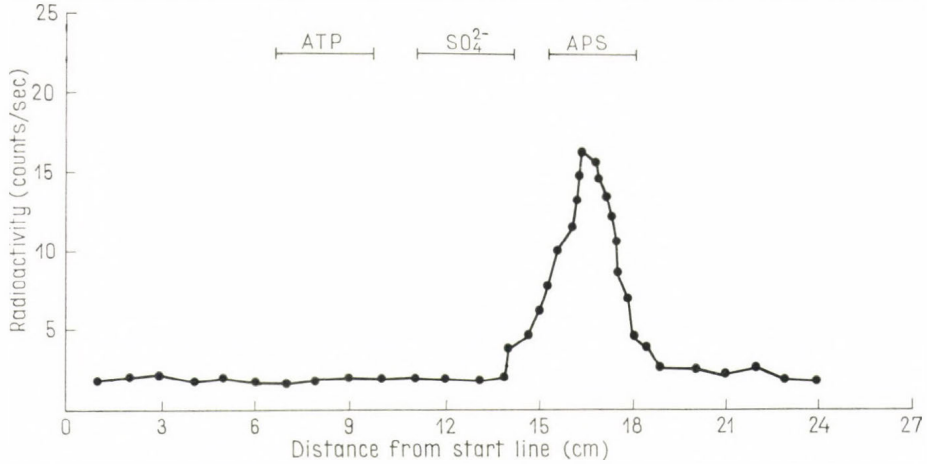

Fig. 7. Radiochromatogram trace of the [^{35}S]-product synthesized in a standard coupled enzyme system. Assays were conducted at 35 °C for 60 min and contained 40 μmol [^{35}S]K_2SO_4 (50 μCi), 20 μmol $MgCl_2$, 10 μmol Na_2K_2ATP, 100 μmol tris-HCl (pH 8·0), 50 units purified spinach-leaf ATP sulphurylase and 1 unit Mg^{2+} dependent alkaline pyrophosphatase (partially purified from spinach) in a final volume of 1 ml. The reaction was terminated by heating (100 °C for 1 min) and the radioactive product was adsorbed to charcoal. The product was eluted from charcoal with 0·1 M-NH_3 in ethanol (50%, v/v), applied to chromatograms and developed in propan-1-ol — ammonia — water (6 : 3 : 1) for 24 h; marker spots of ATP, sulphate and APS are shown

Table 2

Effect of omitting components of the standard assay and the effect of including sodium fluoride (10 mM) on the synthesis of APS in the coupled enzyme system

Results are expressed as a percentage of the standard assay for each experiment. The absolute activities of the standard assays for experiments 1, 2, 3 and 4 were 23·8, 44·1, 33·2 and 33·3 nmol/h respectively. Assays were as described in Fig. 7

Assay	Activity (%)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Standard minus ATP sulphurylase	0	0	0	0
Standard minus pyrophosphatase	0	0	3	0
Standard minus ATP	—	0	0	—
Standard minus Mg^{2+}	—	—	—	3
Standard plus fluoride	15	18	50	45

synthesis of APS, confirming the requirement for pyrophosphatase and not some contaminant in the pyrophosphatase preparation. Mg^{2+} dependent alkaline pyrophosphatase, like ATP sulphurylase, is a chloroplast enzyme (El-Badry and Bassham, 1970) and we conclude that one of the many functions of pyrophosphatase is to hydrolyze pyrophosphate as it is formed in the chloroplast to augment the yield of APS by lessening the free energy change for the synthesis of APS by approximately 7 kcal/mol.

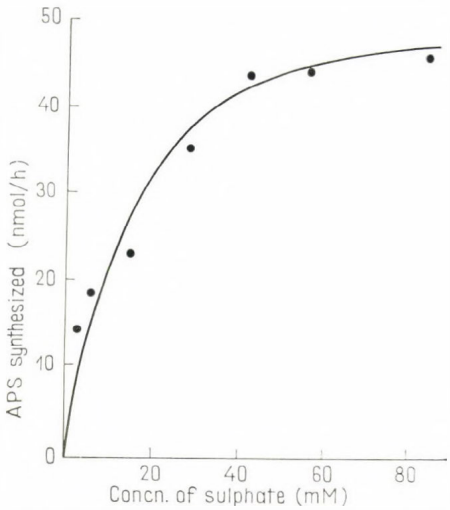

Fig. 8. Effect of concentration of potassium sulphate on the synthesis of APS in the coupled enzyme system. All other conditions of the assay were as described in Fig. 7

The concentrations of ATP and sulphate required for maximum rate of production of APS are high (Figs 8 and 9) and are consistent with the studies of ATP sulphurylase by the PP_i-ATP exchange assay; taken collectively, this datum implies that high concentrations of sulphate and ATP are required, probably in the chloroplast, for APS synthesis *in vivo*. Uptake of sulphate by green leaf tissue has been reported (Penth and Weigl, 1969) but we have been unable to find any published data on the concentration of sulphate in chloroplasts. Bradfield et al. (1970), however, have reported that mycelia of *Aspergillus nidulans* grown in 0·5 mM sulphate accumulated sulphate

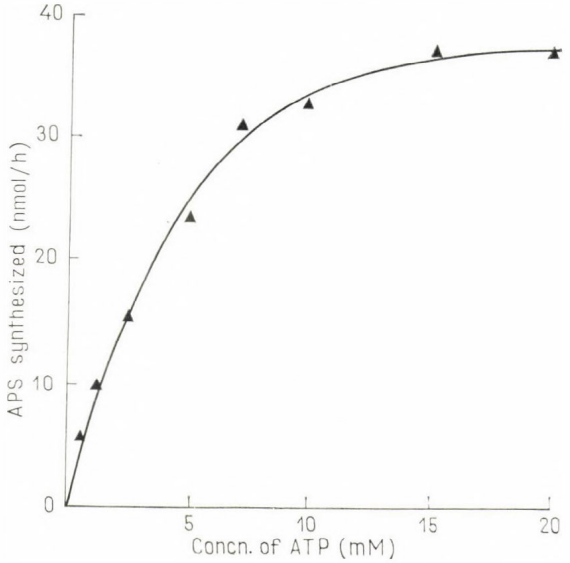

Fig. 9. Effect of concentration of ATP on the synthesis of APS in the coupled enzyme system. All other conditions of the assay were as described in Fig 7

equivalent to 43 mM internal concentration. Asada et al. (1968) have reported that sulphate (1—100 mM) inhibits photophosphorylation of isolated spinach-leaf chloroplasts but does not inhibit photosynthetic electron transport; the inhibition of photophosphorylation by sulphate was reversible. Whilst the uncoupling effect of sulphate on isolated chloroplasts could be an artifact of extraction, it is possible that the inhibition of ATP synthesis by sulphate could be due to the utilization of electron flow to generate a chemiosmotic gradient which is expended on accumulating sulphate ions. ATP levels inside the chloroplast could also be depleted by sulphate activation. Failing these alternatives, then the whole concept of the chloroplast as the site of sulphate activation must be seriously questioned. Ellis (1963) has reported that beetroot slices incorporate sulphate-sulphur into cysteine, thereby implying ATP sulphurylase activity in non-photosynthetic tissue. Subsequently, Ellis (1969) has reported the synthesis of APS in tomato root extracts. It would be very interesting to know the subcellular location and properties of the ATP sulphurylase(s) of non-photosynthetic plant tissues.

ATP SULPHURYLASE AND SELENATE METABOLISM

Selenate is clearly a substrate of ATP sulphurylase from spinach (a non-accumulator of selenium); we are currently trying to establish whether APSe is synthesized when selenate is used as substrate in lieu of sulphate in the coupled enzyme assay. Selenate competes very favourably with sulphate in the pyrophosphate exchange assay (Fig. 6). Since the sulphate uptake mechanism of plants will also take up selenate (Leggett and Epstein, 1956), then the synthesis of APS under field conditions will be decreased by externally available selenate. It remains to be seen whether APSe is synthesized in non-accumulator species and then metabolized in lieu of APS to give rise to the selenium analogues of cysteine and methionine.

We have commenced a study of the ATP sulphurylases of *Astragalus* species grown in soil of uncontrolled sulphate and selenate status; these studies have been limited to crude extracts to date. Crude extracts of leaf-tissue from *A. sinicus* and *A. hamosus* (non-accumulators of selenium) catalyze sulphate- and selenate-dependent pyrophosphate exchange and the kinetics of these reactions appear to be very similar to the kinetics of the purified ATP sulphurylase from spinach leaf; K_m (sulphate) approx. 3—4 mM, K_m (selenate) approx. 1 mM, V (selenate) approx. 28—33 % V (sulphate). Extracts of leaf-tissue from *A. bisulcatus* and *A. racemosus* (selenium accumulator species) also catalyzed sulphate- and selenate-dependent pyrophosphate exchange and the affinities for sulphate and selenate were similar to the affinities of the non-accumulator species. However, the V (selenate)/V (sulphate) ratios of *A. bisulcatus* and *A. racemosus* were slightly less than the ratios of non-accumulator species suggesting that the yield of APS relative to APSe at specified concentrations of sulphate and selenate would be greater in leaf-tissue of accumulator species. Experiments are in progress to purify the ATP sulphurylases from accumulator and non-accumulator species of *Astragalus* so that the kinetics and substrate specificities of the enzyme(s) from each species can be examined in detail.

SUMMARY

APS, bound-sulphite and sulphide are intermediates in the incorporation of sulphate-sulphur into cysteine in plants; it is not yet known whether PAPS is an intermediate in the metabolism of sulphate in higher plants. ATP sulphurylase, the enzyme catalyzing the synthesis of APS, was detected in spinach-leaf tissue by a pyrophosphate exchange assay. The enzyme was purified 1000-fold and some properties of the purified enzyme are reported. Selenate is an alternative substrate (in lieu of sulphate) of spinach-leaf ATP sulphurylase; this suggests that selenate, if available to the plant, could be incorporated in lieu of sulphate to synthesize the selenium analogues of cysteine and methionine.

Synthesis of APS from sulphate and ATP was demonstrated *in vivo* by coupling purified ATP sulphurylase with partially purified Mg^{2+} dependent alkaline pyrophosphatase; some kinetic parameters of the coupled system are reported. Synthesis of APS in leaf tissue probably occurs in chloroplasts but there is evidence that APS is also synthesized in root tissue.

ATP sulphurylase activity was detected in crude extracts of leaf-tissue from *Astragalus bisulcatus* and *A. racemosus*, species which characteristically accumulate selenium from soil and synthesize derivatives of selenocysteine. The kinetics of the ATP sulphurylase from accumulator species were similar to the enzymes from non-accumulator species of *Astragalus* as well as spinach except that V (selenate)/V (sulphate) ratios in the accumulator species were slightly lower than the ratios in leaf-tissue of non-accumulator species. This suggests that the yield of APS relative to APSe at specified concentrations of sulphate and selenate would be greater in leaf-tissue of selenium accumulator species.

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PLASTID SPECIFIC TRANSFER RIBONUCLEIC ACIDS IN *PHASEOLUS VULGARIS*

by

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In plants, protein biosynthesis can take place in the cytoplasm, in the chloroplasts and in the mitochondria. We have begun a comparative study of the first steps of protein synthesis, the reactions leading to the attachment of the aminoacids to their cognate tRNA's, in these 3 systems. We have shown that N-formyl-methionyl-tRNA is present in bean chloroplasts, but not in the cytoplasm (Burkard et al., 1969). We are reporting here the results of experiments showing that bean chloroplasts contain other specific tRNA species which are not found in the cytoplasm, are only aminoacylated by chloroplast enzyme preparations, and are preferentially synthesized upon exposure of dark-grown plants to light.

MATERIAL AND METHODS

French bean plants *(Phaseolus vulgaris)* were grown from seeds for 10 to 12 days, then placed in the dark for 2 days (to deplete the leaves of starch) and the young leaves were harvested. The leaves were washed, the mid-ribs and lateral veins were removed, and the leaves were freeze-dried at —25° for 48 hrs. The chloroplasts were then extracted by the nonaqueous technique of Charlton et al. (1967). Controls of our preparations by electron microscopy showed that we had intact chloroplasts, without appreciable cytoplasmic contamination and with negligible contamination by microorganisms (Burkard et al., 1970).

Etioplasts were obtained from etiolated leaves (after growth for 2—3 weeks in the dark) by the same technique, except that somewhat higher densities (1·35 and 1·37 instead of 1·32 and 1·34 respectively) had to be used in order to collect the etioplasts.

The preparation of the tRNA's and of the aminoacyl-tRNA synthetases from the cytoplasm, the chloroplasts or the etioplasts and the attachement of aminoacids to tRNA's were performed by methods previously described (Burkard et al., 1970). The ^{14}C- or ^{3}H-aminoacyl-tRNA's were fractionated by reverse-phase chromatography using either RPC-2 (Weiss and Kelmers, 1967) or RPC-5 (Pearson et al., 1971) system.

RESULTS

When the capacity of chloroplast tRNA to accept leucine is determined, the results differ according to the source of the enzyme used in the assay: with a homologous enzyme preparation (obtained from chloroplasts) 2·25

nmoles of leucine can be attached per mg of tRNA whereas with a cytoplasmic enzyme preparation only 1·30 nmoles of leucine are attached (Burkard et al., 1970). This lower fixation is not the consequence of limiting enzyme concentration or of limiting incubation time: even if the enzyme concentration (Fig. 1a) or the incubation time (Fig. 1b) is increased, the attachment of leucine to chloroplast tRNA in the presence of cytoplasmic enzyme never exceeds 60% of that obtained in the presence of chloroplast enzyme. These results could be explained if some $tRNA^{leu}$ species present in the chloroplasts could be charged only by chloroplast enzymatic preparations and not by cytoplasmic ones. In order to test this hypothesis we undertook the fractionation of cytoplasmic and chloroplast $tRNA^{leu}$.

A co-chromatography of leucyl-tRNA's from hypocotyls (cytoplasm) and from leaves (cytoplasm + chloroplasts) shows the existence of a small extra-peak present in leaf tRNA's but not in hypocotyl tRNA's (Fig. 2), which suggests that this extra-peak represents chloroplast $tRNA^{leu}$.

As chloroplast tRNA's represent only a small proportion of total leaf tRNA's, chloroplast specific $tRNA's^{leu}$ could not be conveniently studied in such a mixture; we therefore compared the elution profiles of cytoplasmic

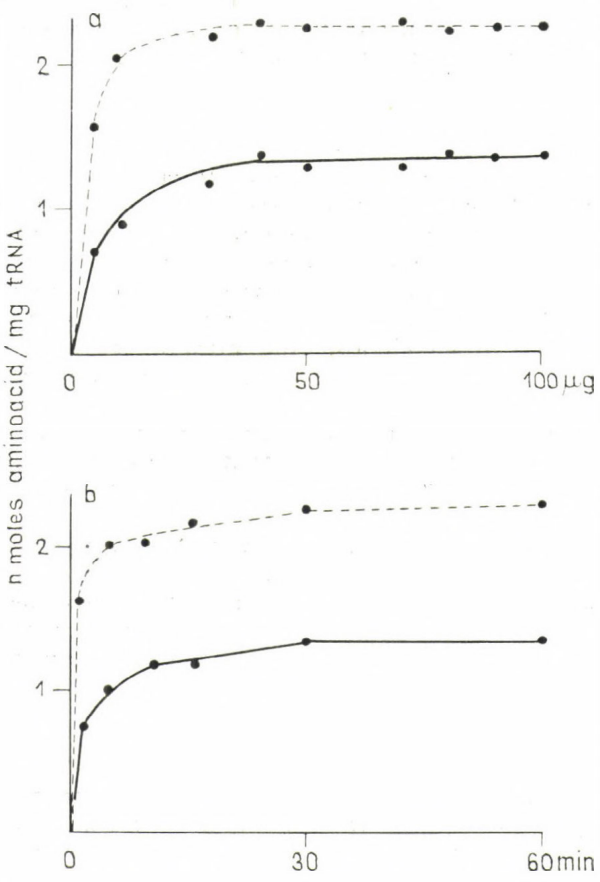

Fig. 1. Attachment of leucine to chloroplast tRNA's catalyzed by cytoplasmic enzyme (●———●), or chloroplast enzyme (● - - ●), as a function of enzyme concentration (a) and of time (b)

leucyl-tRNA's (charged with a cytoplasmic enzyme preparation) and of chloroplast leucyl-tRNA's (charged with a chloroplast enzyme preparation). As can be seen on Fig. 3, there are 2 peaks of cytoplasmic leucyl-tRNA, and 6 peaks of chloroplast leucyl-tRNA.

In the cytoplasmic tRNA's, there are only 2 leucyl-tRNA peaks, no matter which enzyme is used for the aminoacylation (Fig. 4). But from the 6 leucyl-tRNA peaks found in the chloroplast tRNA's, only the first 2 peaks can be charged by the cytoplasmic enzyme (those which are superimposable to the 2 cytoplasmic leucyl-tRNA peaks), whereas the other 4 peaks can only be charged by a chloroplast enzyme preparation (Fig. 5).

A somewhat similar situation is observed with valine: whereas a chloroplast enzyme preparation allows 1·95 nmoles of valine to be attached per mg of chloroplast tRNA, only 0·70 nmole can be attached per mg of chloroplast tRNA when a cytoplasmic enzyme is being used (Burkard et al., 1970). Chromatography of cytoplasmic valyl-tRNA charged with a cytoplasmic enzyme preparation reveals 3 peaks, while 5 peaks can be seen in the elution profile of chloroplast valyl-tRNA charged with a chloroplast enzyme preparation (Fig. 6). From these 5 peaks, 2 are superimposable to cytoplasmic valyl-tRNA peaks.

As it has been suggested that there may be a control of cellular differentiation not only at the level of transcription, but also at the level of translation, and as it has been postulated that tRNA's may play a role

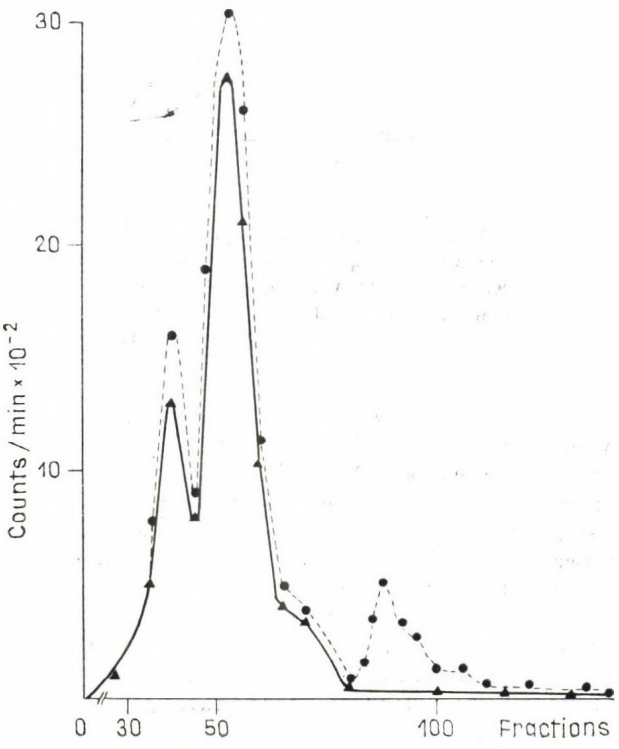

Fig. 2. Reverse phase chromotography (RPC 2) of leaf and hypocotyl leucyl-tRNA's. ● - - - ● leaf ^{14}C leucyl-tRNA's charged with leaf enzyme ●——● hypocotyl ^{3}H leucyl-tRNA's charged with hypocotyl enzyme NaCl gradient from 0·25 to 0·7 M in sodium acetate buffer 0·01 M (pH 4·7) $MgCl_2$ 0·01 M

in regulation at the translational level, it was of interest to study the possible qualitative or quantitative changes in tRNA's during the differentiation of etioplasts into chloroplasts. The elution profiles after co-chromatography of chloroplast and etioplast leucyl-tRNA's are shown on Fig. 7. The 6 peaks of leucyl-tRNA are present in both types of plastids,

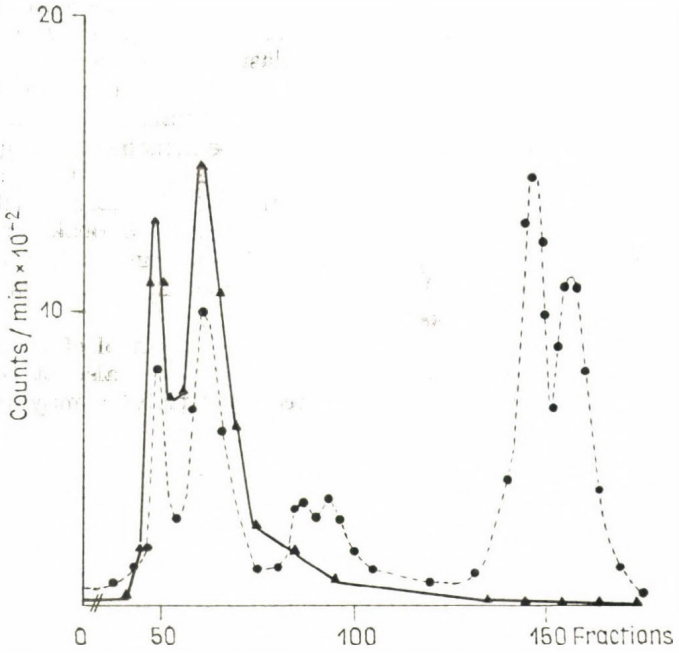

Fig. 3. Reverse phase chromatography (RPC 2) of cytoplasmic and chloroplast leucyl-tRNA's. ▲——▲ cytoplasmic ^{3}H leucyl-tRNA's charged with cytoplasmic enzyme; ● - - - ● chloroplast ^{14}C leucyl-tRNA's charged with chloroplast enzyme- NaCl gradient from 0·35 to 0·7 M in sodium acetate buffer 0·01 M (pH 4·7) $MgCl_2$ 0·01 M

but their relative levels are different. If peak I (which is present in the cytoplasm and in both types of plastids) is taken as a reference and if the ratio of the levels tRNA chloroplast/tRNA etioplast for this peak is considered to be 1, it can be seen that the level of leucyl-tRNA in chloroplasts is smaller (as compared to that in the etioplasts) for peak II, but is higher in peaks III and IV (taken together), as well as in peaks V and VI.

The elution profiles after co-chromatography of chloroplast and etioplast valyl-tRNA's are shown on Fig. 8. The five peaks are present in both types of plastids, but here also their relative levels are different. Here peak II was taken as a reference, as it is present not only in the plastids but also in the cytoplasm. If the ratio of the levels tRNA chloroplast/tRNA etioplast for this peak is considered to be 1, one can see that the level of

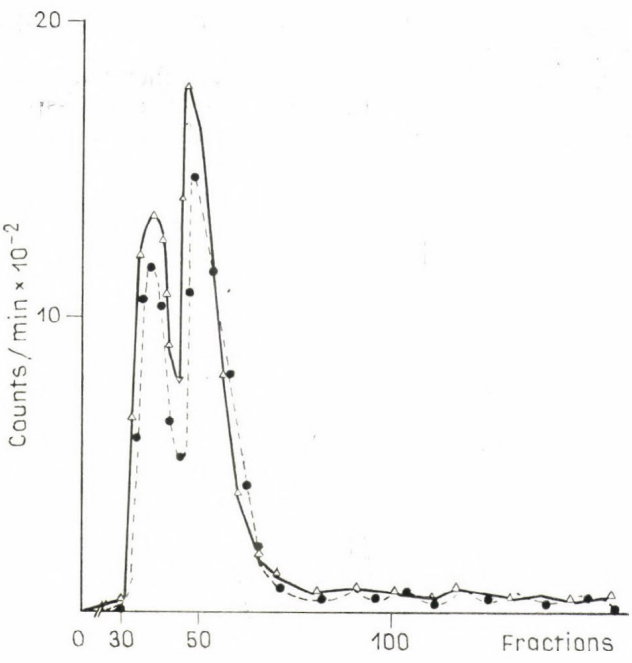

Fig. 4. Reverse phase chromatography (RPC 2) of cytoplasmic leucyl-tRNA's. ● - - - ● cytoplasmic ^{3}H leucyl-tRNA's charged with cytoplasmic enzyme; △——△ cytoplasmic ^{14}C leucyl-tRNA's charged with chloroplast enzyme. NaCl gradient as in Fig. 3

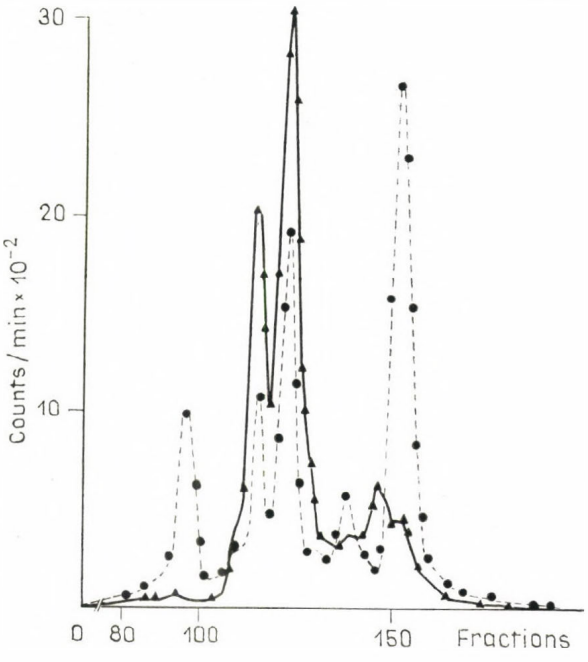

Fig. 5. Reverse phase chromatography (RPC 2) of chloroplast leucyl-tRNA's. ● - - - ● chloroplast ^{3}H leucyl-tRNA's charged with chloroplast enzyme; ▲——▲ chloroplast ^{14}C leucyl-tRNA's charged with cytoplasmic enzyme. NaCl gradient as in Fig. 3

valyl-tRNA in the chloroplasts is higher (as compared to that in the etioplasts) for peaks I and V.

These results suggest that in a higher plant such as *Phaseolus vulgaris* light can stimulate the synthesis of plastid-specific tRNA species. Our results can be compared to those of Barnett et al. (1969) and Reger et al.

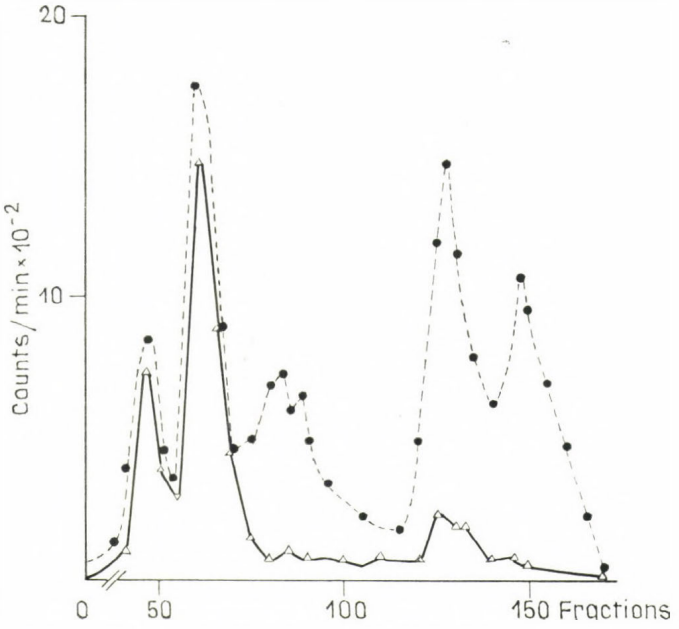

Fig. 6. Reverse phase chromatography (RPC 5) of cytoplasmic and chloroplast valyl-tRNA's. △——△ cytoplasmic ^{3}H valyl-tRNA's charged with cytoplasmic enzyme; ● - - - ● chloroplast ^{14}C valyl-tRNA's charged with chloroplast enzyme. NaCl gradient from 0·4 M to 0·8 M in sodium acetate buffer 0·01 M (pH 4·7) $MgCl_2$ 0·01 M

(1970) who have reported the existence of light-induced $tRNA^{ile}$ and $tRNA^{phe}$ species in algal chloroplasts. Furthermore Williams and Williams (1970) have observed that only certain $tRNA^{leu}$ isoacceptors are synthesized preferentially upon greening of etiolated leaves. More recently Merrick and Dure (1971) have reported an increase in certain isoaccepting $tRNA^{met}$, $tRNA^{val}$ and $tRNA^{ile}$ of cotton seedlings, and have suggested that the species which increase in relative concentration may be localized in the chloroplasts. Our experiments, performed with tRNA's obtained from isolated chloroplasts and etioplasts, demonstrate a preferential synthesis of plastid-specific tRNA species upon greening.

In summary, we have shown that in bean chloroplasts there are some $tRNA^{leu}$ and $tRNA^{val}$ species which co-chromatography with the cytoplasmic species, and some species which are plastid-specific and can only be charged by the chloroplast enzymes. The cytoplasmic and chloroplast

leucyl-tRNA synthetases have different specificities and must therefore be different; this is also true for cytoplasmic and chloroplast valyl-tRNA synthetases.

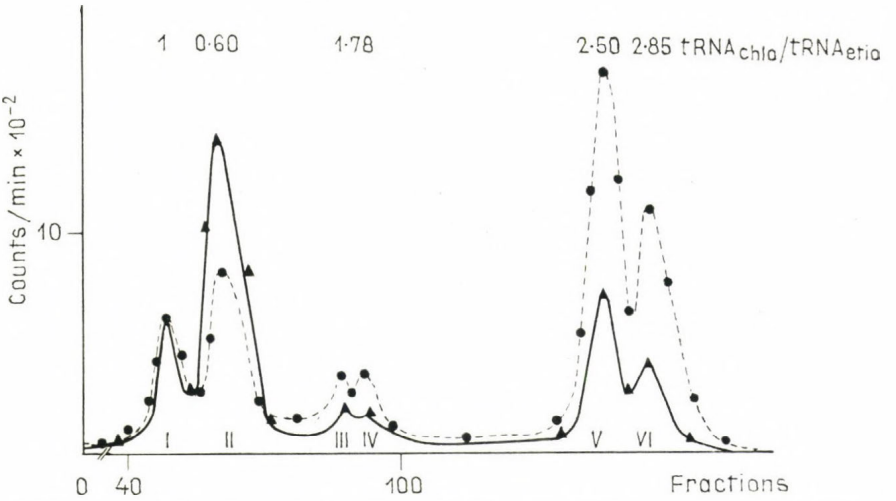

Fig. 7. Reverse phase chromatography (RPC 2) of chloroplast and etioplast leucyl-tRNA's. ● - - - ● chloroplast ^{3}H leucyl-tRNA's charged with chloroplast enzyme; ▲——▲ etioplast ^{14}C leucyl-tRNA's charged with etioplast enzyme. NaCl gradient as in Fig. 3

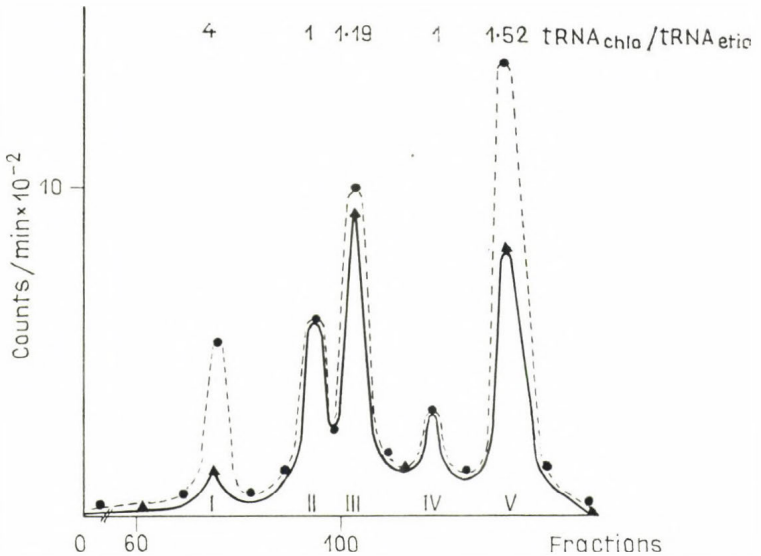

Fig. 8. Reverse phase chromatography (RPC 5) of chloroplast and etioplast valyl-tRNA's. ● - - - ● chloroplast ^{3}H valyl-tRNA's charged with chloroplast enzyme; ▲ ▲ etioplast ^{14}C valyl-tRNA's charged with etioplast enzyme, NaCl gradient as in Fig. 6

A comparison of the relative levels of plastid-specific $tRNA^{val}$ and $tRNA^{leu}$ in the chloroplasts and in the etioplasts has revealed differences which suggest that the plastid-specific tRNA's are preferentially synthetized upon greening.

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SOME PROBLEMS OF PHENYLALANINE POLYMERIZATION IN POLY-U DIRECTED WHEAT GERM SYSTEM

by

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At least three enzymatic reactions are involved in polypeptide chain elongation in the protein biosynthesis in eucaryotic cells. These are: a) the aminoacyltransferase I (TI, bindig enzyme) catalyzed binding of aminoacyl-tRNA molecule to the ribosomal acceptor site (A site); b) the peptide bond formation between the amino group of the newly bound aminoacyl-tRNA and the carboxyl group of the peptidyl-tRNA occupying the ribosomal donor site (P site), which is catalyzed by peptidyltransferase; and c) the aminoacyltransferase II (TII, translocase) catalyzed translocation of the newly lengthened peptidyl-tRNA from the A site to the P site.

Whereas considerable information has been obtained on the mechanism of the chain elongation in mammalian systems, the systematic analysis of this reaction in plant systems is still only beginning.

As it was showed in 1969 the polyuridilic acid-dependent phenylalanine incorporation into ribosome-bound peptide in *in vitro* plant systems requires two soluble factors (App, 1969; Jerez et al., 1969). In 1970 Legocki and Marcus developed a procedure for resolving the wheat germ supernatant fraction into two complementary transfer factors TI and TII both of which were required for poly U-catalyzed formation of polyphenylalanine.* Factor TI was characterized as a binding enzyme and factor TII as a translocase.

This communication describes the improved method of purification of both elongation factors TI and TII from wheat germ and presents some properties of the enzymatic binding reaction in poly U-directed system.

In these studies phenylalanine-tRNA molecules (phe-tRNA) were used as amino acid precursors for the binding and polymerization assays. The ribosomes were prepared from the wheat germ microsomal pellet resulting from the 160,000 g centrifugation during purification of the transfer factors and were purified as previously described (Legocki and Marcus, 1970).

The purification of both elongation factors is summarized in Table 1. The purification of factor TI involves four fractionation steps and provides 50-fold purified enzyme with relatively good yield. Our data suggest that in the wheat system a single soluble protein is involved in the enzymatic binding reaction. Its molecular weight according to preliminary estimations is about 200,000.

* In the referred paper TI was defined as T1 and TII as T2.

Table 1

The purification of factors TI and TII

Factor	Protein mg	Specific activity units/mg	Yield %
FACTOR TI			
Germ supernatant	3400	2·8	100
$(NH_4)_2SO_4$ 25–65%	1416	4·8	84
Sephadex G100	82	62·3	54
DEAE-cellulose	64	72·0	46
Hydroxylapatite	22	140·0	32
FACTOR TII			
Germ supernatant	3050	2·8	100
$(NH_4)_2SO_4$ 35–60%	1280	5·1	76
DEAE-cellulose	275	19·1	62
$(NH_4)_2SO_4$ 25–45%	72	59·7	41
Hydroxylapatite	9	367·3	37

Factor TII was purified by a similar procedure. The molecular weight of the 130-fold purified preparation is about 80,000. At the moment we are trying to purify both factors to the homogeneous state.

The activity of the binding enzyme was routinely expressed as the increase in the amount of ^{14}C phe-tRNA bound to the ribosomes in the presence of binding enzyme in standard reaction mixture (for the details see Legocki and Marcus, 1970).

The activity of the translocase can be defined by the measure of overall polypeptide elongation, i.e. by the factor TI-dependent incorporation of ^{14}C phenylalanine from phe-tRNA molecules into growing polypeptide chains in the presence of poly U. The another reaction which was generally employed to assay the translocation, is that with puromycin. The reaction is based on the assumption that puromycin reacts only with peptidyl-tRNA bound at the ribosomal P site. The facilitation of the reaction of ribosome-bound aminoacyl-tRNA with puromycin by aminoacyltransferase II (and GTP) has been interpreted on the basis of intraribosomal translocation of the aminoacyl-tRNA from the A site to the P site where it reacts with puromycin, forming the puromycin peptide (Brot et al., 1968).

In the wheat system, as it was shown earlier, the binding reaction at low Mg^{2+} concentration (about 5 mM) is absolutely dependent upon the enzyme while at higher concentrations, of the magnitude of 15—22 mM Mg^{2+}, the binding is enzyme independent (Legocki and Marcus, 1970). An advantage was taken from this observation to demonstrate some function of factor TII in the puromycin reaction. The following experiment was undertaken: ^{14}C phe-tRNA was bound non-enzymatically to ribosomes at 20·5 mM Mg^{2+}, then incubated with factor TII in the presence of GTP at low Mg^{2+} concentration (6·5 mM) and subsequently reincubated with puromycin. Table 2 shows that in the presence of factor TII and GTP there is a marked stimulation of the reaction of ribosomal bound phe-tRNA with puromycin. In the absence of TII no puromycin-peptide was formed.

Also, purified factor TI could not replace TII (translocase). The formation of a puromycin product was dependent upon the presence of ribosomes and poly U in the binding reaction (1st incubation). Under the conditions used about 40—55 per cent of the phe-tRNA present in the first incubation mixture was bound to the ribosomes at the moment of puromycin addition.

Table 2

Requirements for the formation of puromycin peptide

First incubation: BINDING — vol. 0·2 ml; 6 min, 20 °C. 50 mM Tris-acetate pH 8·0; 4 mM PEP; 16 μg PKinase; 71 mM KCl; 20·5 mM $MgAc_2$; 2·2 mM dithiothreitol; 10 μg poly U; 0·24 mg ribosomes; 18 $\mu\mu$ moles ^{14}C phe-tRNA (7800 cpm).
Second incubation: TRANSLOCATION — vol. 0·6 ml; 5 min, 30 °C. 20·5 mM $MgAc_2$ 6·5 mM.
Added: 30 μM GTP; 71 mM KCl; 6 μg TII.
Third incubation: PUROMYCIN REACTION — 0·62 ml; 30 min, 30 °C. Added: 5 mM pyromycin

Omission from 1st incubation	Omissions from 2nd incubation	Puromycin peptide* $\mu\mu$moles
None	None	2·95
None	None 0 °C	0·16
— Ribosomes	None	0
— Poly U	None	0·05
None	— TII	0
None	— GTP	0·10
None	— TII + TI	0·03
None	— TII + 20·5 mM Mg^{2+}	0·25

*The values were corrected for extractable radioactivity (0.60 $\mu\mu$moles) observed in the absence of puromycin.

A similar experiment with phe-tRNA non-enzymatically bound has been performed in *E. coli* system, except that the high Mg^{2+} concentration was maintained through all the three incubations (Brot et al., 1968). In the wheat germ system, if the Mg^{2+} concentration was not lowered during the incubation with the translocase, less than 10 per cent of puromycin product was formed. Such difference between the wheat and *E. coli* systems may suggest different Mg^{2+} optima for the translocation reactions. Now we are examining this question in more detail.

As already noted above, the enzymatic binding reaction requires GTP. The involvement of GTP in the enzymatic binding was first established in reticulocyte system by Arlinghaus et al. (1964). In bacterial systems GTP is also required for the binding of aminoacyl-tRNA to the ribosome-mRNA complex and its cleavage may be a prerequisite for peptide bond formation (Ono et al., 1969).

In order to ascertain whether the binding per se requires hydrolysis of GTP, 5'-guanyl-methylene-diphosphonate (GMPPCP) was used in these studies as a GTP analogue which cannot undergo enzymatic cleavage to GDP and P_1. However, the results of enzymatic binding in the presence

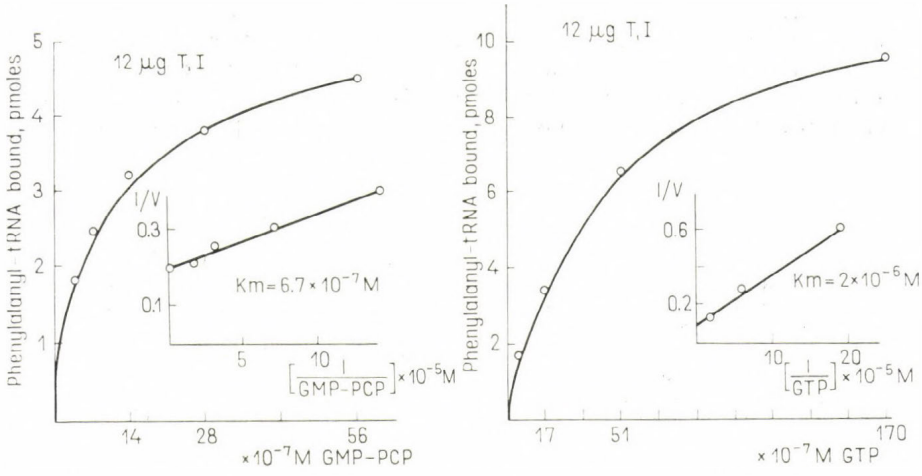

Fig. 1. The effect of GTP and GMP-PCP on the enzymatic binding

of GMPPCP are not univocal. GMPPCP gives in reticulocyte system, for instance, less than 10 per cent of the activity of GTP (Lin Syr-Yaung et al., 1969), whereas in bacterial systems it can substitute for GTP in the enzymatic binding (Ravel, 1967). In this case, however, peptide bond formation does not take place, while in the presence of GTP it does (Ono et al., 1969).

We have performed some preliminary studies with GMPPCP in the wheat germ system. We already ascertained that enzymatic binding is catalyzed by GMPPCP in the presence of the purified factor TI. Figure 1 presents the effect of various concentrations of GTP and GMPPCP on the

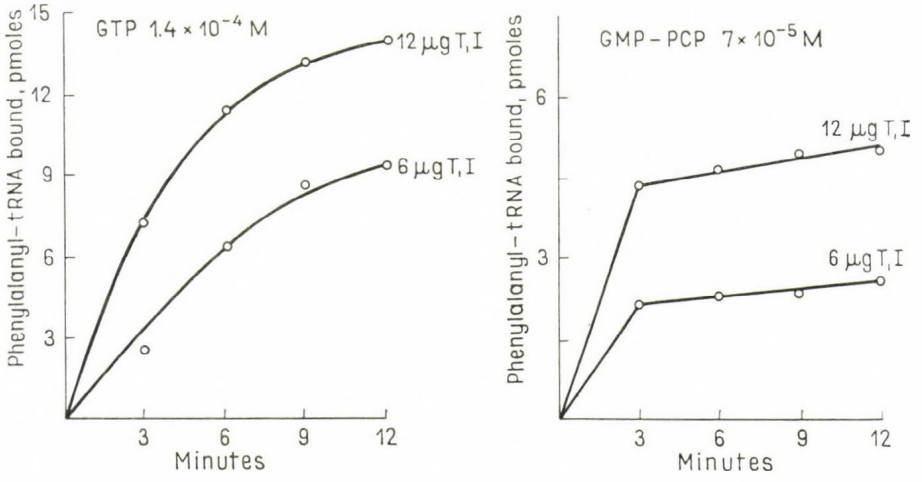

Fig. 2. The effect of GTP and GMP-PCP on the rate of enzymatic binding

rate of enzymatic binding. K_m values were calculated: $6{\cdot}7 \times 10^{-7}$ M for GMPPCP and 2×10^{-6} M for GTP. The binding ability in the presence of GMPPCP at the optimal Mg^{2+} concentration, 4·5 mM, (for polymerization, 6·7 mM Mg^{2+}) is about 40 per cent of that with GTP. However, the kinetics of binding in the presence of GTP and its analogue is quite different (Fig. 2). These kinetics might suggest a rapid equilibrium in the GMPPCP reaction. On the other hand, no increase in binding was observed in the experiments with twofold amounts of GMPPCP of ribosomes. It is possible that GMPPCP is attached stoichiometrically to binding enzyme and blocks further binding. We would like to examine this point more directly.

In order to determine whether the binding in the presence of GMPPCP does not lead to the formation of short peptides the reaction product was identified after hydrolysis with 1N NH_4OH by paper chromatography. In the presence of either GTP or GMPPCP 95 per cent of ^{14}C phenylalanine bound to the ribosomes was recovered in the region corresponding to monophenylalanine. Thus, it can be concluded that factor TI in presence of GMPPCP catalyzes the real binding of phe-tRNA to wheat germ ribosomes.

Summarizing the above considerations we suggest two possible ways of interpretation of the involvement of GMPPCP in enzymatic binding: a) the normal binding reaction involves two steps of which only the first one might be catalyzed by GMPPCP, or b) the possibility of the existence of two binding sites on plant ribosomes, which cannot be excluded, similarly to the reticulocyte system as postulated by Culp et al. in 1969. To answer the above mentioned problems more detailed studies are required and there are now in progress in our laboratory.

The author is grateful to Professor J. Pawelkiewicz for valuable discussion during the preparation of this manuscript. Some of the experiments presented here were performed in Dr. A. Marcus' laboratory at the Institute for Cancer Research in Philadelphia.

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INFLUENCE OF TRANSFER RNAs OTHER THAN tRNA^{phe} ON POLY-U DIRECTED SYNTHESIS OF POLYPHENYLALANINE IN A WHEAT EMBRYO SYSTEM*

by

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We have studied the influence of the concentration ratio of tRNA to ribosomal RNA on the rate of polyphenylalanine synthesis, using a system from wheat embryos.

Anderson (1969) has already investigated this problem with an *in vitro* system from E. coli, and found that the rate of polyphenylalanine synthesis was dependent on phe-tRNA^{phe} concentration.

We extracted ribosomes by the method of Allende and Bravo (1966) from viable wheat embryos prepared according to Johnston and Stern (1957), and stored them in liquid nitrogen. The supernatant was purified by precipitation with $(NH_4)_2SO_4$, between 40 and 80% saturation, passed through a Sephadex G 25 column and stored at —20 °C. The unfractionated transfer RNA used was extracted from commercial wheat germs by the phenol method (Monier et al., 1960) and accepted about 40 pmoles phenylalanine per O.D.$_{260\ nm}$ unit.

The polymerization of phenylalanine in our system was completely dependent on the addition of tRNA, as one can see from Table 1.

Table 1

Characteristics of phenylalanine polymerizating system from wheat embryos

System	Cpm
Complete	4170
Without tRNA	24
Without ribosomes	32
Without poly U	58
Without supernatant	37

The incorporating system contained in a final volume of 1 ml; 50 micromoles Tris-HCl, pH 7·5; 25 micromoles KCl; 7·5 micromoles $MgCl_2$; 1 micromole ATP; 0·5 micromoles GTP; 5 micromoles phosphoenolpyruvate; 10 micrograms pyruvate kinase; 10 milimicromoles ^{14}C-phenylalanine (specific activity 20 microcuries/micromole); 1 micromole 2-mercaptoethanol; poly U (0·12 micromoles P); 5 O.D.$_{260}$ units ribosomes; 200 micrograms proteins of the 100,000 g×supernatant; 100 micrograms of wheat embryo total transfer RNA.

The mixture was incubated for 30 min at 32 °C, then added with 2 ml of 10% TCA; the hot-TCA insoluble material was collected and washed on millipore filters (0·45 micron pore diameter).

* With the technical assistance of C. Greger.

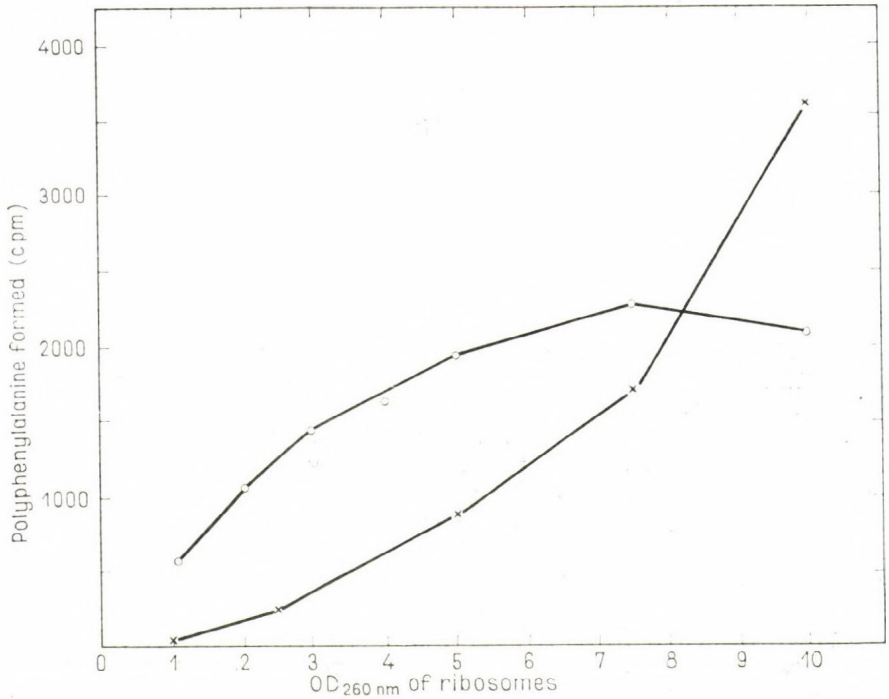

Fig. 1. Dependence of the rate of polyphenylalanine synthesis on transfer RNA/ribosomal RNA ratio, using unfractioned tRNA. The incorporating system was the same as described in Table 1, except that increasing quantities of ribosomes and two different amounts of unfractioned tRNA were used. a) ○——○ 100 micrograms unfractioned tRNA; b) ×——× 800 micrograms unfractioned tRNA;

In Fig. 1 the amount of polyphenylalanine (polyphe) formed using unfractionated tRNA is plotted against different ribosomal levels. It is evident that the optimal conditions depend on the tRNA/ribosomal RNA ratio. With quantities of ribosomes lower than 7·5 $O.D._{260\ nm}$ /test the polyphe formed was larger with 100 micrograms tRNA than with 800 micrograms tRNA/test. We made the same experiment using $tRNA^{phe}$ purified by benzoylated DEAE cellulose (B.D.cellulose) chromatography (Gillam et al., 1967) and reverse phase (R.P.) chromatography (system No. 4 of Weiss et al. (1968), accepting 1,500 pmoles phenylalanine/$O.D._{260\ nm}$ unit. As shown in Fig. 2, at ribosome concentrations lower than 4 O.D. units/test no difference was seen between the quantity of the polyphe formed with two different concentrations of $tRNA^{phe}$; with higher ribosome concentrations, the polyphe formed was dependent on the concentration of $tRNA^{phe}$.

The different results obtained with pure $tRNA^{phe}$ and with unfractionated tRNA at low ribosome concentrations induced us to think that an inhibitor of polyphe synthesis was present in the unfractionated tRNA preparations. We, therefore, checked the influence of different fractions obtained by B.D. cellulose chromatography of the total tRNA on polyphe synthesis

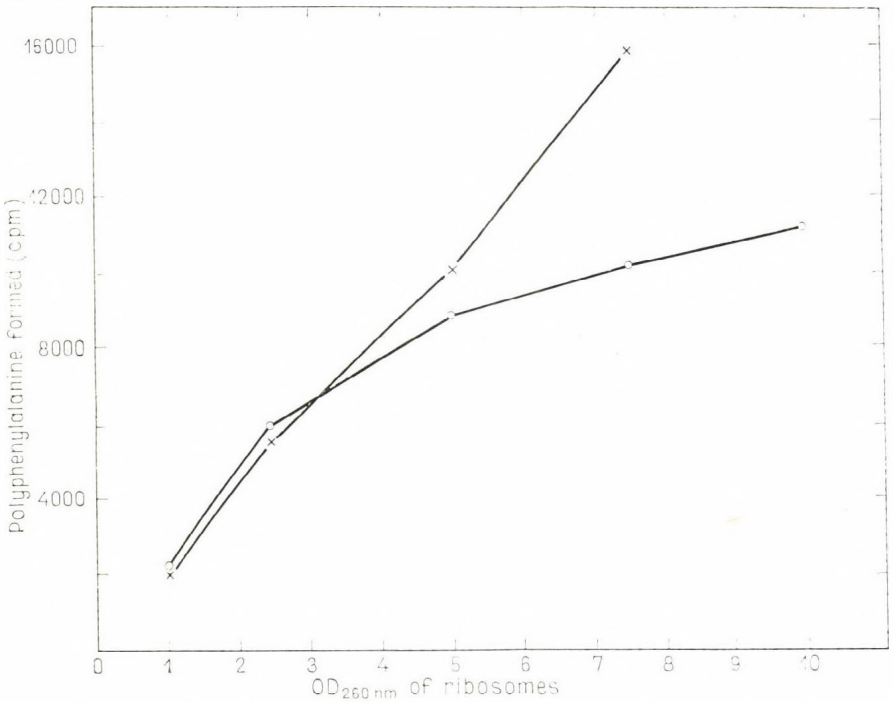

Fig. 2. Dependence of the rate of polyphenylalanine synthesis on transfer RNA/ribosomal RNA ratio, using purified $tRNA^{phe}$. The incorporation system was the same as described in Table 1, except that increasing quantities of ribosomes were added, and the unfractioned tRNA was replaced by $tRNA^{phe}$, purified as indicated in the text and accepting 1,500 pmoles phe/O.D.$_{260}$ units when tested under the conditions indicated in Table 2. o—— o 2 micrograms $tRNA^{phe}$; ×—— × 12 micrograms $tRNA^{phe}$

using purified $tRNA^{phe}$ in the incorporation mixture (Fig. 3). Only fraction IV showed a considerable inhibition.

The fact that only one fraction from the B.D. cellulose chromatography is inhibitory in our system indicates that this phenomenon is not merely an aspecific effect, as it would be, for instance, in the case of modification of cation concentration by large amounts of RNA in the incorporating mixture. The inhibition by increasing concentrations of fraction IV is shown in Fig. 4.

Fraction IV contains a very small amount of $tRNA^{phe}$ and some $tRNA^{phe}$ lacking the terminal CCA: the inhibitory effect could very well be due to this last component. We chromatographed fraction IV on a R.P. column (Weiss et al., 1968), checking phe acceptance by using both a partially purified phe tRNA-synthetase devoid of CCA pyrophosphorylase (Manzocchi, Lanzani, Menegus, unpublished results) and a crude enzyme containing CCA pyrophosphorylase activity. As shown in Fig. 5, the fractions with inhibitory activity were different from those containing $tRNA^{phe}$ lacking the terminal CCA.

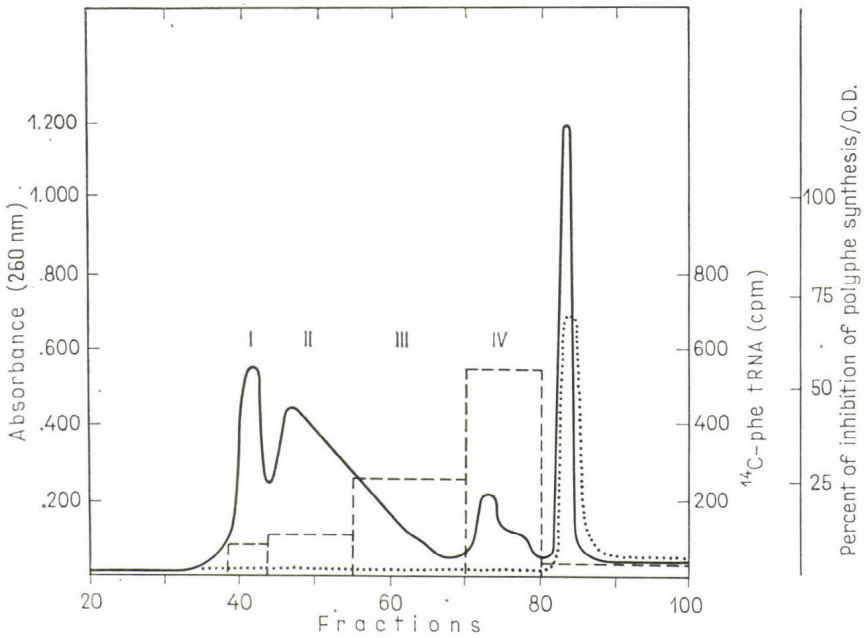

Fig. 3. Benzoylated-DEAE cellulose chromatography of wheat germ transfer RNA acylated with ^{14}C phe. 50 mg of tRNA were incubated with ^{14}C-phenylalanine in a 5 ml incorporating mixture with the same components as indicated in Table 2, with 100 units of purified phe tRNA synthetase (90 units/mg) protein) (Manzocchi, Lanzani, Menegus, unpublished results). After three phenol treatments and precipitation with ethanol, the tRNA was dissolved in 2 ml buffer 0·05 M Na acetate pH 5, 0·45 M NaCl, 0·01 M $MgSO_4$, and put on a B.D. cellulose column (cm 3×100) conditioned with the same buffer. The first three U. V. peaks were eluted with a 2 l gradient, 0·45—0·7 M, of NaCl; the fourth one was eluted with 500 ml I M NaCl, and the fifth peak (containing ^{14}C-phe tRNA) with 1 M NaCl plus 15% ethanol, always in the same buffer. Four groups of fractions were collected and concentrated in an Amicon ultrafiltration cell (membrane UM 10), dialyzed against buffer without NaCl and aliquots containing 50 micrograms RNA were tested in the incubation mixture for polyphenylalanine synthesis as described in Table 1, except that unfractionated tRNA was replaced by purified $tRNA^{phe}$ (50 micrograms/test). ——— Absorbance at 260nm; ..... Radioactivity (^{14}C phe-tRNA) cpm; - - - - - - Percent of inhibition of polyphenylalanine synthesis

The inhibitory fraction from R.P. chromatography did not show any fluorescence at the wavelength characteristic of the base Y of the $tRNA^{phe}$, and therefore it does not contain fragments of $tRNA^{phe}$ with the fluorescent base; moreover one single, symmetrical peak is obtained when it is chromatographed on a Sephadex G-100 column, and this indicates that neither small RNA fragments nor 5S RNA are present.

We tested the charging of either unfractionated tRNA or the R.P. inhibitory fraction with 17 different amino acids using a crude synthetase preparation from wheat embryos (Table 2). The total aminoacid acceptance accounted for 591·2 pmoles/O.D. unit for unfractionated tRNA, and for 355·4 pmoles/O.D. unit for the R.P. fraction. The transfer RNAs enriched

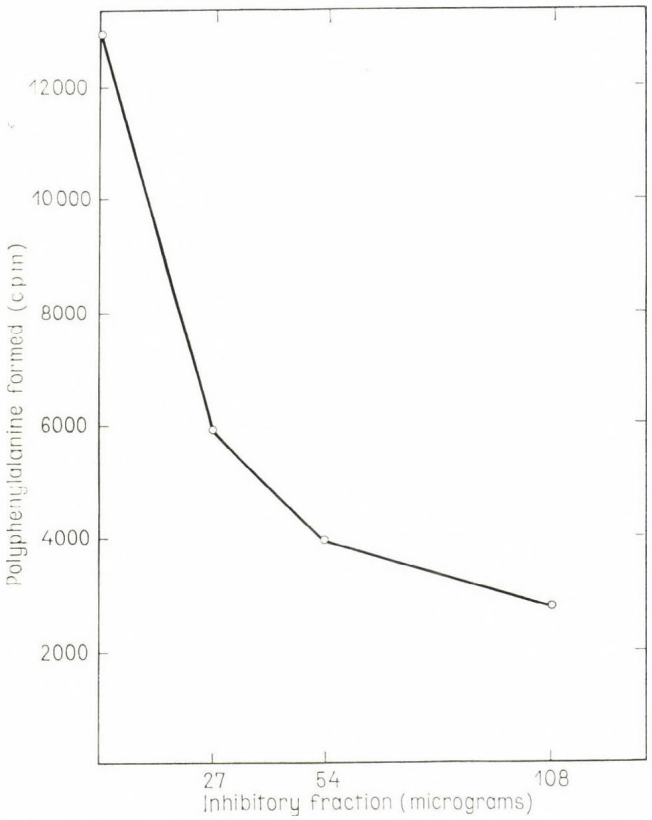

Fig. 4. Inhibition of polyphenylalanine synthesis by increasing amounts of the inhibitory fraction (fraction IV from B. D. cellulose chromatography). The same polyphe synthesis system as in Table 1 was used, except that unfractionated tRNA was replaced by 50 micrograms of purified $tRNA^{phe}$

in the R.P. fraction in comparison to the unfractionated tRNA were those of arginine, asparagine and methionine.

We decided to study the effect of charging each one of the three enriched tRNAs present in the R.P. inhibitory fraction with its own amino acid; after charging three aliquots of the inhibitory fraction, one with arginine, a second one with asparagine and a third one with methionine, we checked them in our system for polyphe synthesis. Since we wanted to eliminate the discharging operated by the enzymes of the supernatant in the phe-polimerizing system, we used a limiting amount of supernatant. However, since there was the possibility of a deficient charging of thet RNA^{phe} under these conditions, we added partially purified phe-synthetase (1·57 units) to the incorporating mixture.

As one can see from Table 3, in the absence of phe-tRNA synthetase, the charging of the R.P. fraction with arginine and asparagine did not

modify the inhibitory effect on polyphe synthesis, while the charging with methionine increased the inhibition from 55·4% to 68%.

Much to our surprise, the phe-tRNA synthetase addition inhibited by itself 70% of the phe-polymerization.

In the presence of phe-tRNA synthetase, the inhibition by uncharged fraction IV was 48%; charging with either arginine or asparagine did not

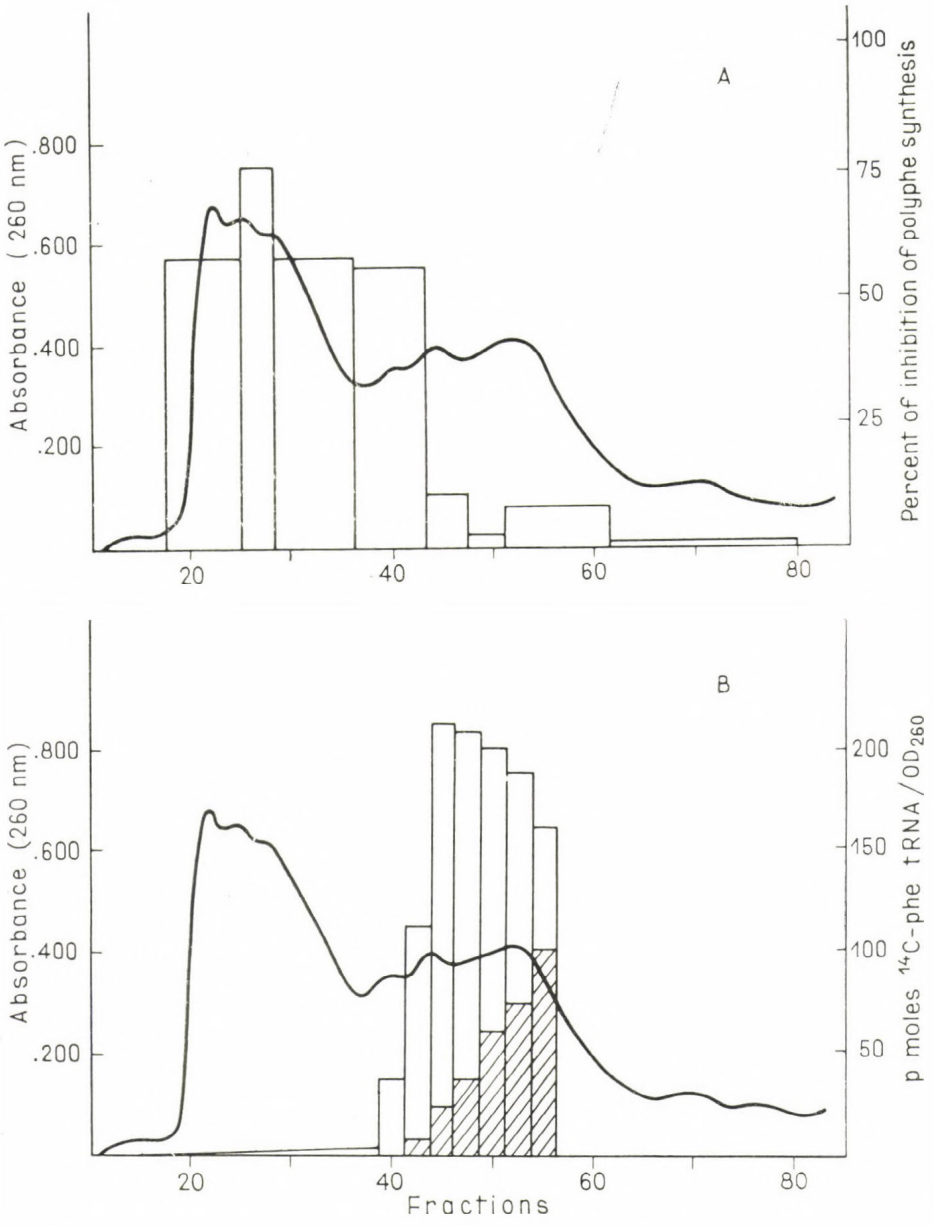

Table 2

Charging with different ^{14}C-aminoacids of unfractioned tRNA and of inhibitory R.P. fraction by a crude aminoacyl-tRNA synthetase

Aminoacid tested	Unfractioned tRNA pmoles/OD	Inhibitory R. P. fraction pmoles/OD
Glycine	53·2	0·704
Alanine	16·4	1·22
Valine	36·6	7·40
Methionine	24·8	51·60
Serine	22·8	2·08
Threonine	51·8	3·46
Asparagine	20·4	68·0
Lysine	127·0	20·4
Histidine	24·8	0·53
Arginine	30·4	174·0
Tyrosine	1·68	0·65
Tryptophan	8·25	3·8
Cystine	26·2	6·50
Proline	19·7	0·54
Isoleucine	87·2	8·2
Leucine	1·98	1·22
Phenylalanine	38·4	1·22

The incubation mixture for amino acid acceptance contained in a final volume of 0·12 ml: 6 micromoles Tris-HCl, pH 7·8; 1·8 micromoles $MgCl_2$; 4·8 micromoles KCl; 0·48 micromoles 2-mercaptoethanol; 24 micrograms bovine serumalbumine; 0·12 micromoles ATP; 28 micrograms crude aminoacyl-tRNA synthetase from wheat germ; 1·2 micromoles of ^{14}C aminoacid (specific activity 20 microcurie/micromole) and 1·2 $O.D._{260}$ units of RNA. After incubation at 32 °C for 20 minutes, a 0·1 ml aliquot was pipetted onto a Whatman 3 MM filter paper disk (22 mm diameter) and cold tricloroacetic acid insoluble radioactivity was courted.

←

Fig. 5. Reverse-phase chromatography of the inhibitory fraction (Fraction IV from B. D. cellulose chromatography). 390 $O. D._{260}$ units of fraction IV from B. D. cellulose in 30 ml of buffer 0·01 M Na acetate pH 5, 0·01 M $MgCl_2$, 0·45 M NaCl, were charged on a R. P. column, system 4 of Weiss et al. (1968) (cm 1×24; $t° = 24$ °C), and eluted with a 2 l gradient, 0·45—0·7 M, of NaCl in the aforesaid buffer. Eight group of fractions were collected, concentrated in an Amicon ultrafiltration cell (membrane UM10) and tested for inhibition on polyphenylalanine synthesis, as previously described, using 50 micrograms of purified $tRNA^{phe}$ and 50 micrograms of the fractions per test (Fig. 1a) A) Thick line: Absorbance at 260 nm; vertical bars: percent of inhibition. The acceptance for phenylalanine of each fraction was checked as described in Table 2 both with a purified phe tRNA synthetase (specific activity 90 units/mg protein) (7) and with a crude enzyme (Fig. 1b) B) Thick line: Absorbance at 260 nm; vertical bars: ^{14}C-phe charging with crude enzyme (empty area) and with purified enzyme (shaded area)

change again the inhibition; charging with methionine decreased the inhibition from 48% to 28·6%, exactly the opposite effect of the charging with methionine in the absence phe-tRNA synthetase.

Even though our results are too scanty to give a clear explanation of these data, we can at least attempt an interpretation. The polyphe synthesis system from wheat embryos at 7·5 mM Mg^{2+} could be similar to the one from reticulocites (Hardesty et al., 1969), in which the initiation is operated by uncharged $tRNA^{phe}$. Accordingly, in wheat embryo system the un-

Table 3

Inhibition of polyphenylalanine synthesis by the inhibitory R.P. fraction charged with different aminoacids, with and without purified phenylalanil-tRNA synthetase added

Inhibitor	− Phe-tRNA synthetase		− Phe-tRNA synthetase	
	Cpm	% of inhibition	Cpm	% of inhibition
	4717	0	1413	0
Uncharged	2102	55·4	745	48·1
Charged with methionine	1508	68·0	1037	28·5
Charged with asparagine	1935	59·8	677	52·0
Charged with arginine	1984	57·9	647	54·1

Three aliquots of 50 $O.D._{260}$ units of the R. P. inhibitory fraction were incubated in three incorporating mixtures of 2·5 ml final volume, with the same components in the concentrations described under Table 2, containing respectively ^3H arginine, ^3H methionine and ^3H asparagine (10^{-4} M, specific activity 600 microcuries/micromole). After 30 minutes incubation, the mixtures were treated three times with phenol, the RNAs were precipitated with ethanol, dissolved in Mg acetate 10^{-4} M, K acetate 10^{-4} M, pH 5, and washed in an Amicon ultrafiltration cell (membrane UM 10) to eliminate ATP co-precipitated with ethanol. 50 micrograms were tested for inhibition in a polyphe incorporating system as described under Table 1, with 50 micrograms purified $tRNA^{phe}$ and 25 micrograms protein of the supernatant. To the tests with phe-tRNA synthetase, 1·57 units (one unit is the amount of enzyme which catalyzes the incorporation of 1 nanomole phe into tRNA/min at 32 °C) of purified phe tRNA synthetase (specific activity 90 units/mg protein) were added. (Manzocchi, Lanzani, Menegus, unpublished results)

charged $tRNA^{met}$ could compete for that site, while charged $tRNA^{met}$ would be less competitive: this would also explain the inhibition by the phe-tRNA synthetase, which, charging completely and recharging continuously the $tRNA^{phe}$, would nearly eliminate the uncharged $tRNA^{phe}$ necessary for initiation.

It would also be interesting to know which of the two $tRNA^{met}$ species known to be present in wheat embryos (Tarrago et al., 1970) is present in the inhibitory fraction.

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Symp. Biol. Hung. 13, pp. 185—189 (1972)

2-(4-METHYL-2,6-DINITROANILINO)-N-METHYL PROPIONAMIDE: AN INHIBITOR OF PROTEIN SYNTHESIS IN HIGHER PLANTS

by

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INTRODUCTION

The compound is one of a class of dinitroanilines designed for use as pre-emergence herbicides.

It is a derivative of the amino acid alanine and posseses, therefore, an assymetric carbon atom (Fig. 1). Of the two possible optical isomers, one,

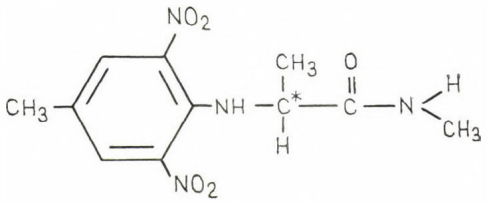

Fig. 1. 2-(4-methyl-2,6-dinitro anilino)-N-methyl propionamide

the D-form, is much more active than the L-form when looked upon as a seed germination inhibitor. It should perhaps be noted that it is the L-form of amino acids which predominate in plant proteins. D-amino acids are not incorporated into protein to any appreciable extent.

Inhibition of growth

The D-form is a powerful pre-emergence herbicide. For example, the concentration required to inhibit the germination of half the seeds in a lentil germination test is 5×10^{-7} M. Growth inhibition is not confined to higher plants, as growth of yeast and the fungus *Piricularia oryzae* is inhibited by low concentrations of the compound.

In contrast, bacterial growth is not affected by the presence of the compound. The nature of this resistance is being examined.

INHIBITION OF AMINO ACID INCORPORATION

It seemed that, because of the rather rapid effect on a variety of eucaryotic organisms, the compound was inhibiting a fundamental process, possibly protein synthesis.

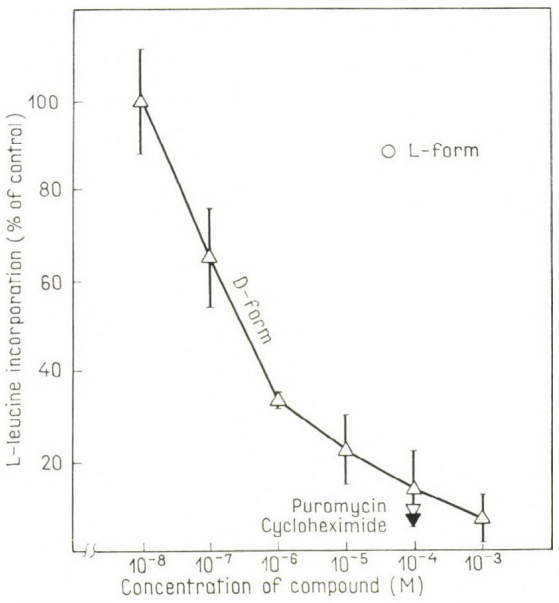

Fig. 2. The effect of the compound on the incorporation of ^{14}C-L-leucine into the TCA-insoluble fraction of wheat embryo. Embryos (100 mg), excised from dry wheat seed, were incubated in 0·01 M sodium acetate (pH 7·5), 1% sucrose, 0·02% chloramphenicol, and compound where necessary, for 4 h at 30° with shaking. Incubation in fresh buffer-sucrose compound solution, containing 0·31 μCi ^{14}C-L-leucine (344 μCi/μmole), followed for 1·5 h. Finally, the embryos were incubated for 1·5 h in buffered sucrose containing unlabelled, leucine, (0·1 M), after which the radioactivity associated with the TCA-precipitable fraction was estimated. Standard errors (three replicates) are indicated

Table 1

The effect of the D-form of the compound on the in vitro incorporation of ^{14}C-L-leucine into protein by a microsomal (polysomal) fraction from maize plumules

Concentration of compound (D-form)	Leucine incorporated (p moles per 45 min/mg of microsomal protein)	Fraction of aqueous control (%)
Experiment 1		
Control	46·4	100
1×10^{-6} M	49·5	107
1×10^{-5} M	47·9	103
1×10^{-4} M	40·6	88
Experiment 2		
Control	45·1	100
1×10^{-4} M	49·7	110
5×10^{-4} M	48·7	108
1×10^{-3} M	42·6	95

Polysome and supernatant fractions, and incubation conditions, were essentially those used by Mans and Novelli (1964). Supernatant protein per reaction: 0·15 mg. Polysomal protein: O 0·42 mg. 1—^{14}C-L-leucine (1 μ Ci, 50 μ Ci/μmole) was used.

In vivo experiments

We examined the effect of the compound on plant tissue which was actively incorporating amino acids into a TCA-insoluble product. Marked inhibition was found in several species and tissues and can be illustrated in the wheat embryo system. This system was inhibited by puromycin and cycloheximide, but to nowhere near the same degree by the L-form of the compound (Fig. 2.). We are at present examining the pattern of polysome formation in wheat embryos imbibed in the presence and absence of the compound, in an attempt to determine whether or not the compound prevents the formation of polysomes from stored ribosomes and messenger RNA.

In vitro experiments

Preliminary experiments with the maize plumule cell-free system of Mans and Novelli (1964) indicated that the compound had no effect upon the incorporation of leucine into the protein associated with a preformed polysomal complex (Table 1). This was later substantiated using the wheat embryo cell-free system (Fig. 3.). This indicated that amino acid activation, the formation of amino-acyl-tRNA, and peptide chain elongation, were not being affected.

It was found, however, that when the initiation complex (ribosome-m-RNA complex) had to form prior to amino acid incorporation, as in the case of the TMV-RNA-mediated system, the compound inhibited subsequent leucine incorporation. This indicated that the compound was interfering with the formation of an active messenger RNA-ribosome initiation complex (Fig. 3.). The L-form of the compound was inactive in this system.

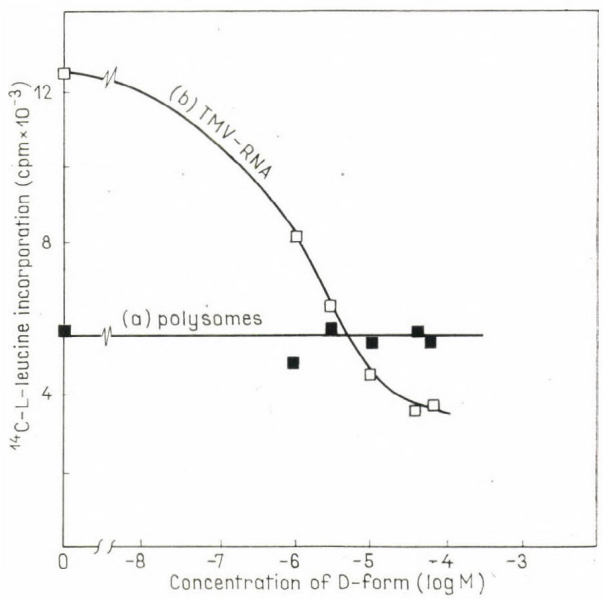

Fig. 3. Incorporation into a TCA-insoluble product, of ^{14}C-L-leucine as mediated by (a) wheat embryo polysomes, (b) tobacco mosaic virus RNA with wheat ribosomes. Isolation of polysomes (Weeks and Marcus, 1969), ribosomes, post-ribosomal supernatant fractions (Marcus et al., 1969) and tobacco mosaic virus (TMV) RNA (Marcus, 1970) were essentially as previously reported. Incubation conditions were as for Marcus (1970)

That the inhibition was relatively specific was indicated by the fact that the analogous system using the artificial "messenger" molecule, poly U, was not affected by the compound (Table 2). Attachment of the poly U to the ribosome is non-specific, and does not require the mediation of initiation factors in order to function (Staehelin, 1970). It may be noted that the poly U-stimulated incorporation of phenylanine in cell-free *E. coli* systems was also insensitive to the compound.

Table 2

Poly U stimulated incorporation of ^{14}C-L-phenylalanine by wheat embryo ribosomes. Effect of the D-form of the compound

Treatment	Phenylalanine incorporation (p moles/mg microsomal protein/15 mins) (mean of 2 estimations)
None	15·2
D-form (M)	
10^{-6}	20·1
10^{-5}	20·2
10^{-4}	18·7
10^{-3}	18·4
Poly U absent	0·1
Ribosomes absent	3·4

Ribosomes and post-ribosomal supernatant fractions were obtained as for Marcus (1970), as were incubation conditions. Reaction volumes (0·20 ml) included: 1·20 mg supernatant protein, 0·28 mg ribosomal protein, 20 μg poly U and 0·08 μCi ^{14}C-L-phenylalanine (0·64 n moles).

CONCLUSION

The compound (2-(4-methyl-2,6-dinitroanilino)-N-methyl propionamide) is a potent inhibitor of germination when in the D-form. One of its major sites of action is clearly on the protein synthesizing system of eukaryotic organisms. More specifically, the compound appears to interfere with the formation of an active messenger RNA-ribosome complex.

All investigations with this compound were based upon a ground-work performed on this and related compounds by the staff of the Shell Research Laboratories at Sittingbourne. In particular the skilled assistance of Miss V. C. Knell is acknowledged.

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SECTION III

NUCLEIC ACID AND PROTEIN SYNTHESIS IN CELL PARTICLES

Symp. Biol. Hung. 13, pp. 193—199 (1972)

THE CONTROL OF RIBOSOMAL RNA SYNTHESIS: THE TRANSCRIPTION PRODUCTS OF THE RIBOSOMAL GENES IN LEAVES AND ROOTS

by

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INTRODUCTION

Most organisms contain multiple copies of the genes which code for ribosomal RNA (rRNA). The number of these genes in higher plants varies from about 160 to 28,000 per diploid cell in different species (Birnstiel et al., 1970). Such a multiplicity is required to allow the very high rate of ribosome synthesis in a rapidly growing cell, perhaps 100 ribosomes per second or 5 to 10 million per cell cycle. A study of these genes provides an opportunity to elucidate some of the basic mechanisms of gene action and of the control of RNA synthesis. In this communication we show that the product of these genes (the precursor ribosomal RNA, pre-rRNA) varies in properties in different tissues of the same plant.

The structure of the ribosomal genes and the synthesis and maturation of the pre-rRNA have been extensively reviewed (Birnstiel et al., 1970; Maden, 1971; Loening, 1970). We give here an outline of those features which are of immediate relevance. The ribosomal genes are clustered in the nucleolar organiser and the DNA is concentrated in the central fibrillar region of a functioning nucleolus. The repeating unit or ribosomal RNA cistron is a DNA sequence which contains the structural genes for the 18S and 28S rRNA and some so-called spacer DNA. The spacer DNA is best defined as that region which is never transcribed. It is frequently taken, however, to include regions adjacent to the 18S and 28S ribosomal RNA regions which are transcribed. The ribosomal RNA is transcribed as a polycistronic precursor molecule (pre-rRNA) which contains the sequences of the RNA of the large and small ribosomal sub-units together with some additional sequences; these latter are discarded during maturation to rRNA. As far as is known the multiple gene copies are identical to each other in the ribosomal regions within the limits detectable by present methods. (This presents something of a puzzle since no mechanism is known by which individual genes can be prevented from gradually evolving away from each other.) There is, however, some evidence that the regions of non-transcribed spacer DNA could vary in length as shown by the beautiful pictures presented by Miller and Beatty (1969). We have also suggested

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that the ribosomal RNA precursor is heterogeneous, suggesting that some of the RNA which is discarded during maturation may vary in length (Loening, 1970; Rogers et al., 1970).

Most of the work on the ribosomal genes has been done in Brown's and Birnstiel's laboratories on *Xenopus*. Very little indeed is known about the structure of these genes in plants or in mammals. On the other hand, most of the detailed work on the pre-rRNA and its maturation has been done on mammalian cells, especially the HeLa cell, and comparisons have been made with other species. We ourselves (Loening et al., 1969) and Perry et al. (1970) have shown that the molecular weight of the ribosomal precursor varies from about 2·3 million in some plants to 4·4 million in mammals. It was especially striking to find that the amount of the excess RNA which is discarded during maturation is very much greater in the warm blooded animals, mammals and birds, than in any other organism. However, among the cold blooded animals and the plants the amount of excess RNA varies considerably between different species, and no simple correlation between molecular weight and evolutionary status could be made. Indeed differences between closely related species may be as large as between distant ones; for example we found about twice as much excess RNA in the bean pre-rRNA as in that of the pea. We suggested that the length of the pre-rRNA could vary with a shift in the initiation or the termination sites of the nucleolar RNA polymerase on the DNA (Loening et al., 1969). The changes in apparent size with evolution would then not represent any basic change in the structure of the gene but only in the amount of the spacer DNA which is transcribed. Since such a shift occurs so readily and since the ribosomal RNA precursor appeared to be heterogeneous, it is possible that not all the products of the multiple ribosomal genes are identical. It seems conceivable to us that a differentiating organism could make use of such differences in its developmental processes and therefore it was not unreasonable to search for differences in the ribosomal RNA precursor between different tissues of the same species. We show in this paper that such differences can indeed be found between the leaves, roots and hypocotyls of the mung bean *Phaseolus aureus*.

We have previously proposed a scheme for the synthesis and processing of the ribosomal RNA precursor of the cytoplasmic (not the chloroplast) rRNA in the mung bean leaf (Grierson et al., 1970 and in prep.). This scheme is summarised in Diagram 1. The evidence for scheme is based mainly on the molecular weights and the rates of labelling of the components of RNA determined by gel electrophoresis, on their ^{32}P base compositions, and on the ability of ribosomal RNA to compete with the precursors during hybridisation to DNA. The stages of processing are similar (with very different molecular weights) to those proposed for the HeLa cell by Weinberg and Penman (1970) and they differ from the scheme proposed for rat

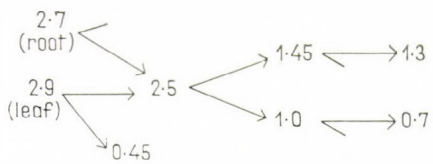


Diagram 1. Proposed steps of processing of the pre-rRNA in the mung bean. RNA components which can be detected by gel electrophoresis are indicated by their molecular weights in millions; the arrows indicate the proposed relationship between them (Grierson et al., 1970 and in prep.)

tissues by Egawa and Busch (1971). This difference may be explained by differences between these mammalian species (Grierson et al., 1970). We must stress, however, that this scheme is not proved and that there is much conflicting data about the arrangement of the different components in the precursor both from different laboratories and in different species. We do not know whether there is an underlying arrangement and scheme of processing which is common to all eukaryotes; nothing is known about the function of the excess RNA which is discarded during processing (in most organisms and in most situations this RNA has such a short half life in the cell that it cannot be detected); and it is not known definitely which is the 5' end of the molecule or on which end the terminal piece of excess RNA occurs. In the mung bean leaves we were fortunate in being able to find an RNA component with a molecular weight of about 0·45 million which was rapidly labelled and could be hybridised specifically to the isolated ribosomal genes (Grierson et al., 1970). These properties suggest that the molecule is the piece of excess RNA which is first cleaved from the ribosomal RNA precursor as shown in the diagram. Similar experiments with the mung bean root failed to show such a component and prompted a direct comparison between the leaf and the root.

RESULTS

Figure 1 shows a separation by gel electrophoresis of the RNA from leaves labelled with ^{32}P and RNA from roots labelled with ^{3}H. The optical density scan shows the two ribosomal RNA components with molecular weights of 0·7 million and 1·3 million. Next to these are small amounts of chloroplast RNA with weights of 0·56 and 1·1 million. In these young leaves and in roots we usually see a minor component with a molecular weight of 1·0 million which may be mitochondrial RNA. The chloroplast RNA does not become labelled during a pulse incubation at this stage. The radioactivity scan of the leaf RNA labelled with ^{32}P shows the components which were described in the diagram above. A comparison of this to the scan of the ^{3}H radioactivity of the root RNA shows the following: (1) The mobility of the root pre-rRNA is slightly greater than that of the leaf, with an apparent molecular weight of about 2·7 million. (2) The piece of excess RNA (molecular weight of 0·45 million) which is found in leaf is missing from the root RNA. (3) All the other components, i.e. the immediate precursors to the mature ribosomal RNA and the ribosomal RNA itself, are identical in leaf and root.

Although these results suggest that the molecular weight of the pre-RNA in the root is lower than that in the leaf one could question the validity of the relationship between electrophoretic mobility on gels and molecular weight. Some differences in the conformation of the RNA molecules could account for the difference, especially as these components migrate on gel electrophoresis as broad bands — as broad indeed as the differences being measured. It is also possible that the differences between the leaf and the root are a function of the time of labelling. Indeed a recent paper by Tiollais et al. (1971) has shown that there are small differences in the apparent molecular weight of the pre-rRNA in HeLa cells with time of labelling.

Such differences could be due to changes in the length of the molecule or to changes in its conformation. We have therefore repeated the experiment described in Fig. 1 under many different conditions using different

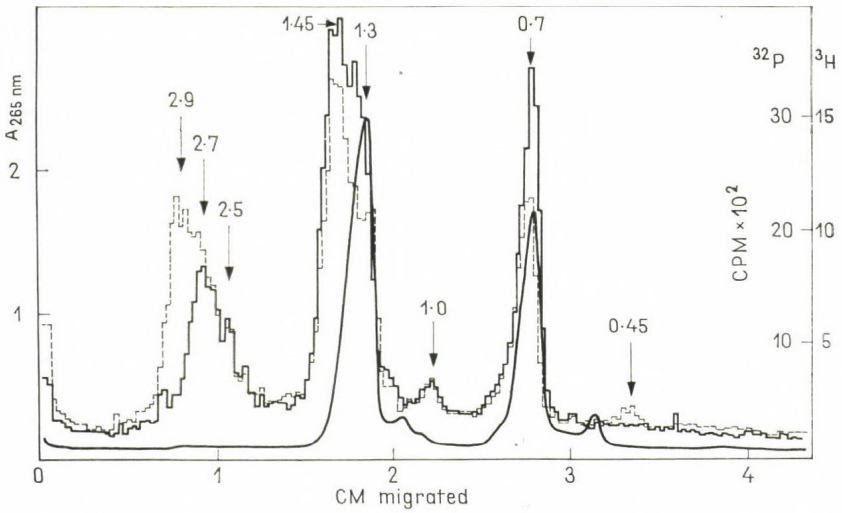
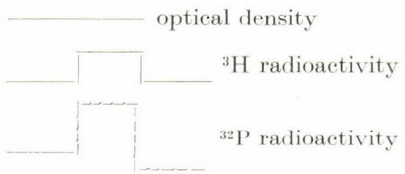


Fig. 1. Fractionation of a mixture of pulse-labelled leaf and root RNA. The leaves of 3 day old mung bean seedlings were labelled with ^{32}P by direct application of the isotope to the leaf surface. Roots of seedlings 30 h old were labelled with ^{3}H-uridine by adding isotope to the sand in which the seedlings were growing. Labelling was for 1½ h in each case. The roots and leaves were then mixed and the total cell RNA extracted and analyzed by electrophoresis on a 2·4% polyacrylamide gel. The molecular weights were determined from the mobilities using the rRNA as standards (Loening, 1969), and are indicated in millions.



labelling times, with the isotopes reversed and by electrophoresis in other buffers containing either a low salt concentration or magnesium ion. These experiments are described elsewhere (Grierson and Loening, 1972). In every case we obtained the same result and we are therefore confident that we are observing a true difference of molecular weight of the precursor between leaf and root. It remains, of course, possible that the molecular weight of the precursor which we observe is not in fact the primary transcription product at all. It is quite conceivable that cleavage of the molecule could begin at one end while synthesis is still continuing at the other. In such a case the excess RNA to be cleaved would have to be at the 5' end of the molecule and any further cleavage would be delayed until some time after

synthesis was completed, since the molecule with molecular weight of 2·7 million is observed. We think that this interpretation is unlikely, especially as it would suggest that the primary transcription product

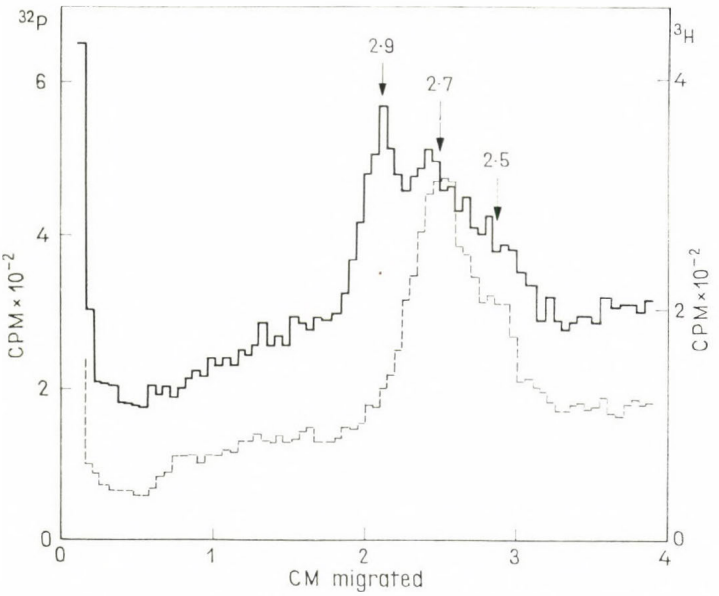


Fig. 2. RNA from hypocotyls compared to that from roots. Mung bean seedlings were grown for 2 days in the dark followed by 2 days in the light. Hypocotyl segments 2·5 cm long were excised and incubated in ^{3}H-uridine for 1¼ h. Roots were labelled with ^{32}P for 1¼ h by dipping intact seedlings in the isotope. RNA was extracted from the 2 tissues separately and RNA from 6 hypocotyls and 2 roots mixed and analyzed by electrophoresis on a 2·4% polyacrylamide gel; only the top 4 cm of the gel are shown

may never be observed in many tissues, even in the presence of inhibitors which prevent cleavage. However, a final proof that the true pre-rRNA in leaf and root is different must await the identification of the 5' end of the molecule in each case.

We have also examined the labelled RNA of the hypocotyls from the same bean seedlings. The separation shown in Fig. 2 suggests that the ribosomal RNA precursor is in this case more complex than that of the leaf or root. It appears to include the same components as in the leaf with molecular weights of about 2·9 and 2·5 million, but there also seems to be

a range of intermediate components. We do not know whether all these components occur in a single cell type within the hypocotyl.

Obviously we were interested to see to what extent these differences could also be found in other plant species, and have briefly examined the pulse-labelled RNA of wheat, tobacco, sunflower and french bean. In all cases, except perhaps tobacco, but including the secondary and tertiary leaves of the French bean, the leaf pre-rRNA had a slightly higher molecular weight than that in the root. We have not yet examined the lower molecular weight regions to investigate the pieces of cleaved excess RNA.

DISCUSSION

These results taken together suggest that there is a true difference of molecular weight between the pre-rRNA molecule synthesized in the leaf and that in the root. The molecular weights suggest that the difference is around 600 nucleotides out of a total length of somewhat less than 9,000. The 2·5 million components and all the other components appear to be identical in both tissues except that the root does not contain the 0·45 million piece of excess RNA. Therefore we suggest that the difference is due entirely to a smaller amount of excess RNA at one end of the molecule in the root, (this piece of excess RNA in the root would have a molecular weight of about 0·2 million and this would be difficult to detect unless it had a long half life). Thus root and leaf differ in the same way as closely related species, and we suggest that this is brought about by a shift in the initiation or termination sites of transcription. This immediately leads to a question about the heterogeneity of the ribosomal genes: (1) either the re-iterated genes are not identical and a different group of these genes is active in the leaf from that in the root or (2) they are identical but there is more than one initiation or termination site on each DNA stretch and different control elements are active in the two tissues. Any further interpretation of the results must depend on which of these possibilities is correct but the second one is attractive and some simple models can be constructed around it. One could, for example, suggest that the initiation or termination of transcription in the root and the leaf are controlled by different *sigma* or *rho* factors which alter the specificity of attachment of the polymerase. In this case, stimulation of ribosomal RNA synthesis by, for example, a hormone would lead to the synthesis of ribosomal RNA and to the production of the piece of cleaved excess RNA. The concentration of the latter would depend on the rate of RNA synthesis and on its half life; its specificity or molecular weight, however, would vary with the tissue. This molecule therefore has exactly the properties one would expect for a component which co-ordinates the synthesis of ribosomes with other functions and these functions would differ in different tissues. In other words a single hormone could be made to stimulate growth as well as development in different directions depending on a previous production of polymerase factors. It is tempting but premature to suggest models like this. Clearly we need more evidence about the nature of the ribosomal genes before we can interpret the results any further.

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RNA SYNTHESIS IN ISOLATED CHLOROPLASTS

by

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Chloroplasts are semi-autonomous cell organelles which synthesize DNA, RNA and proteins. In many respects they resemble prokaryotic systems more closely than they do the eukaryotic cells of which they are a part (sensitivity to certain inhibitors, structure of ribosomes, DNA not associated with histons, etc.). Therefore, it is of interest to ask, whether this analogy could be extended to the RNA-synthesizing system. The findings known hitherto on the nature of the RNA polymerase in chloroplasts are contradictory. Until now it was not possible to isolate and enrich RNA polymerase from plastids. However, comparative investigations on the action of transcription inhibitors could provide information about existing differences between nuclear and chloroplast polymerase and about similarities to the prokaryotic polymerase.

In this connection such inhibitors are interesting which inhibit specifically either the eukaryotic nuclear polymerase (α-amanitin) or the prokaryotic enzyme (rifampicin). Some investigations have been already done about the action of rifampicin on the RNA synthesis in chloroplasts, but they gave both positive (Bogorad and Woodcock, 1971; Galling, 1971; Surzycki, 1969; Surzycki et al., 1970) and negative (Bottomley et al., 1971; Spencer et al., 1971) results.

The problem of the information content in the chloroplast DNA is also widely an open one. It was shown by hybridization experiments with in vivo synthesized RNA, that chloroplasts are able to synthesize rRNA and tRNA, coded by the own DNA (Tewari and Wildman, 1970). But the real capacity of the chloroplasts to synthesize different RNA sepcies can only be solved by experiments with isolated chloroplasts. Although the capacity for DNA-dependent RNA synthesis has been reported in purified chloroplasts, the information on the nature of the in vitro synthesized RNA is meagre (Spencer and Whitfeld, 1967; Spencer et al., 1971; Tewari and Wildman, 1969; 1970).

This paper reports results from experiments, aimed to show, which species of RNA are made by the isolated chloroplasts from tobacco leaves and secondly to receive more information on the action of rifampicin on the RNA polymerase in plastids.

METHODS

Isolation of chloroplasts

Young leaves (3—5 cm) from *Nicotiana rustica* plants were homogenized in a mortar in the following extraction medium: 0·07 M Tris-HCl, pH 7·8; 0·5 M sucrose; 0·01 M $MgCl_2$; 0·01 M KCl; 0·004 M mercaptoethanol. The homogenate was filtered through 6 layers of muslin and then centrifuged for 5 min at 200 × g to pellet starch and most of the intact nuclei. The supernatant was centrifuged for 10 min at 2000 × g. The chloroplast pellet was resuspended in the extraction medium and centrifuged 10 min at 2000 × g. This pellet was suspended in two volumes of Tris-Mg-SH-medium (0·05 M Tris-HCl, pH 7·8; 0·01 M $MgCl_2$; 4 mM mercaptoethanol) and was centrifuged again at 2000 × g (crude chloroplast fraction).

For further purification the chloroplast suspension (5 ml, before washing with Tris-Mg-SH-medium) was layered on top of discontinuous sucrose gradients consisting of 10 ml 60%, 10 ml 45% and 10 ml 35% sucrose in extracting medium. After 20 min centrifugation at 4000 × g the fraction of intact chloroplasts was collected, diluted with extraction medium and centrifuged. The pellet was washed with Tris-Mg-SH medium.

To get most purified chloroplasts the chloroplast fraction from the sucrose gradient (without washing with Tris-Mg-SH medium) was suspended in extraction medium. 2 ml were mixed with a solution consisting of 5·5 ml glycerol and 2·5 ml sucrose (1·3 g sucrose dissolved in 0·07 M Tris-HCl, pH 7·8) and centrifuged for 30 min at 35 000 rpm in the SW 40 rotor (Spinco L2-65 B). The highly purified chloroplasts from the top of the tube were washed with Tris-Mg-SH medium.

Assay for RNA synthesis

The standard assay for RNA-polymerase contained crude or purified chloroplasts (0·1 mg chlorophyll) in 0·15 ml Tris-Mg-SH medium together with 0·125 μmoles each of GTP, CTP and UTP; 0·125 μC ^{14}C-ATP (spec. activity 30 mC/mM); 2·5 μmoles phosphoenolpyruvate; 10 μg pyruvate kinase. In some experiments ^{14}C-UTP was used instead of ^{14}C-ATP. After 20 min of incubation at 25 °C the reaction was stopped by adding 1 ml 0·2 N $HClO_4$ + 2% sodium pyrophosphate. After standing 30 min. at 0 °C the sediment was collected on filter paper discs and washed several times with $HClO_4$-pyrophosphate, 70% alcohol and ether. The discs were dried and the radioactivity was measured in a Packard liquid scintillation spectrometer.

Other methods

Further details were described elsewhere (Wollgiehn and Munsche, 1972). The methods for in vivo incorporation experiments and the analytical methods have been described previously (Munsche and Wollgiehn, 1970; Wollgiehn, 1968; Wollgiehn and Ruess, 1968).

RESULTS AND DISCUSSION

Characteristics of the RNA polymerase in chloroplasts

The chloroplast RNA polymerase is strictly dependent on the presence of all four nucleoside triphosphates and Mg^{2+} (Table 1). The omission of an ATP-regenerating system and mercaptoethanol from the assay and of KCl from the homogenization medium causes a clear reduction in activity. The polymerase is inhibited by DNase and RNase. These characteristics

Table 1

Some characteristics of chloroplast RNA polymerase

System	^{14}C-ATP incorporation (cpm/assay)
Complete system	4050
Omit GTP, CTP, UTP	117
Omit PEP and pyruvate kinase	1330
Omit Mg^{2+}	270
Omit Mg^{2+}, add Mn^{2+}	670
Omit KCl*	2230
Omit mercaptoethanol	1100
Add DNase (10 μg/ml)	71
Add RNase (10 μg/ml)	400

* Omitted during chloroplast isolation.

suggest that the reaction is DNA dependent and that the product is RNA.

Figure 1 shows some characteristics of the polymerase reaction in purified chloroplasts in more detail. The ^{14}C-ATP incorporation increases linearly with time for approximately 20 minutes, however, the newly synthesized RNA is very quickly degraded by endogeneous RNase. The enzyme reaction is strongly dependent on the temperature and the pH. The optimal temperature was found to be 25 to 30 °C and the pH optimum 7·8 to 8·2. 10 mMolar Mg^{2+} concentration is necessary for optimal activity.

We have tested the polymerase activity with chloroplasts after different purification steps. A high degree of purification, i.e. a complete removal of nuclei and nuclear fragments by sucrose gradient centrifugation or other techniques, has only a small influence on the polymerase activity, because the nuclear polymerase activity is very low under our standard assay conditions, compared with the activity of the plastid polymerase. Only 10 per cent of the total polymerase activity in a 2000 $\times$ g fraction containing chloroplasts and nuclei was found in the nuclei. The reason for the limited activity of the nuclear polymerase of higher organisms is the association of the template-DNA with histones. In contrast, bacterial as well as chloroplast DNA are not associated with such proteins (Tewari and Wildman, 1969) and are therefore much more available to the enzyme.

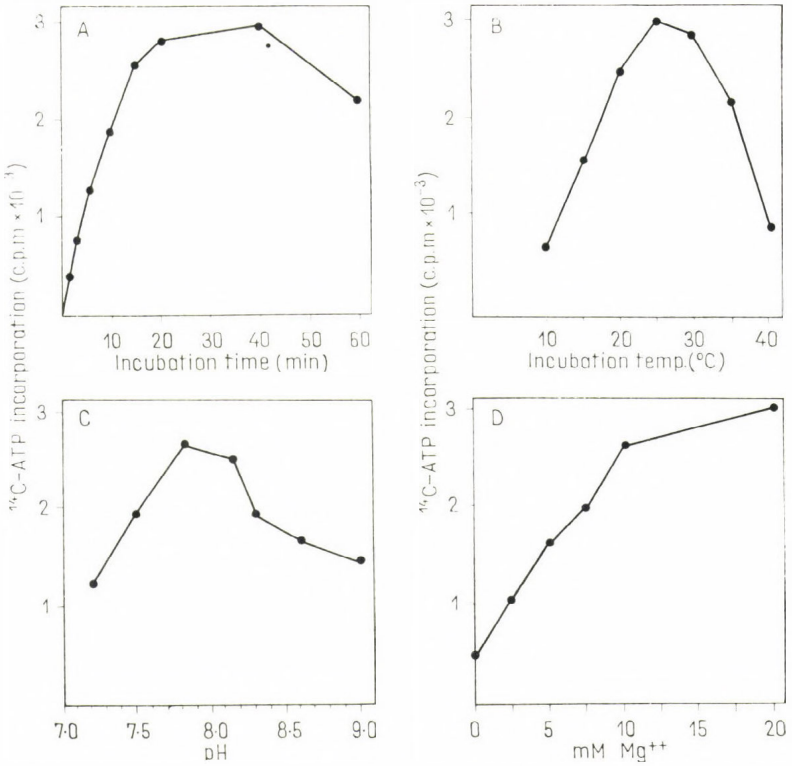

Fig. 1. Time course, temperature, pH and Mg^{2+} dependence of the RNA polymerase reaction in purified chloroplasts

In purified chloroplasts the total, or the main part of the polymerase is bound to membranes and sediments by centrifugation even after disruption of the chloroplasts by 1% Triton X 100. Nevertheless, it is probable that during isolation and purification some free polymerase is washed out. This can be explained by the fact that after addition of pure tobacco DNA the RNA synthesis is markedly stimulated in non-washed chloroplasts, but scarcely in highly purified ones.

Size of the RNA synthesized in vitro

The previous experiments with isolated chloroplasts failed to determine which types of RNA can be synthesized in isolated chloroplasts. In the experiments of Tewari and Wildman (1969, 1970) and Spencer et al. (1967, 1971) the RNA synthesized in vitro was very heterogeneous and ranged in size from 4 S to 30 S, with a major peak at about 11 S (Spencer and Whitfeld, 1967; Spencer et al., 1971). According to the interpretation of the authors the chloroplasts may be able to synthesize all three forms of RNA, i.e. transfer, ribosomal and messenger (10 S to 12 S) RNA.

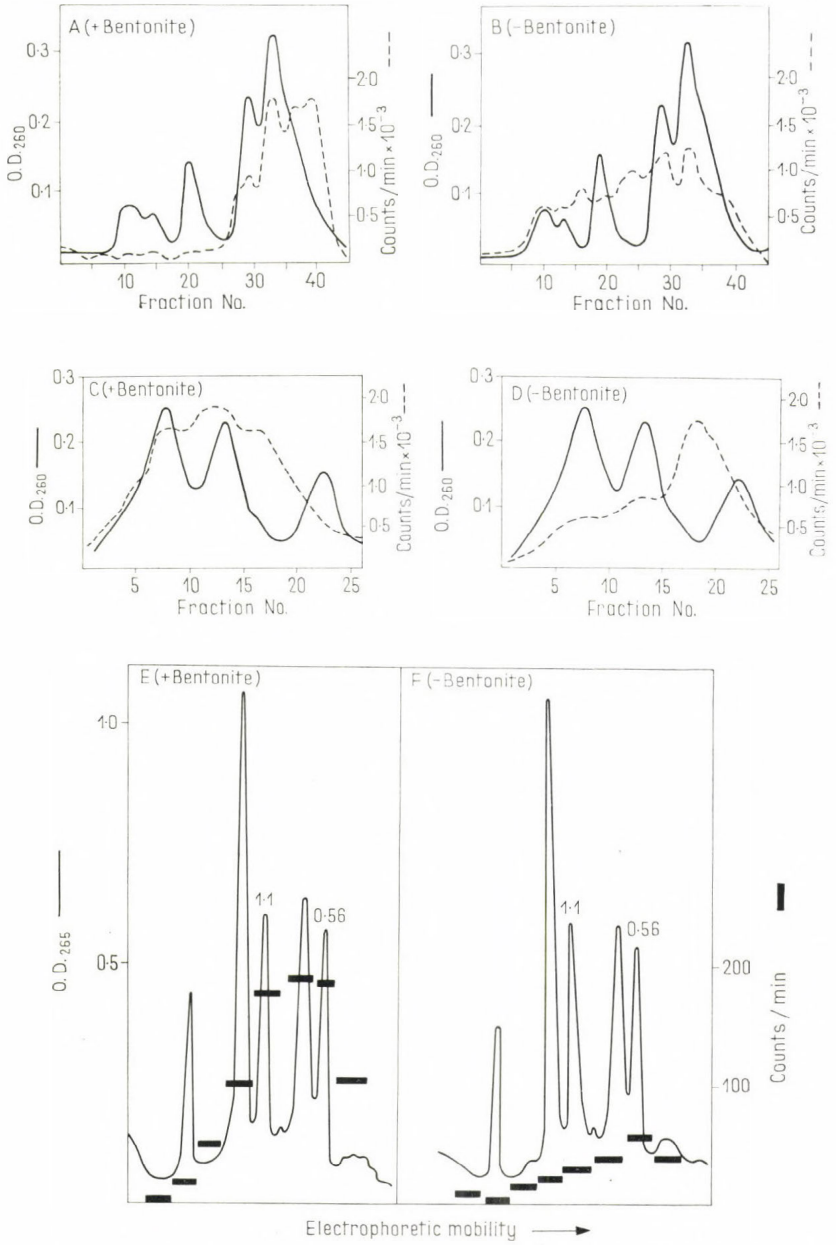

Fig. 2. MAK-column chromatography (A, B), sucrose density-gradient centrifugation (C, D) and polyacrylamide gel electrophoresis (E, F) of RNA, isolated from chloroplasts after 20 min incubation with ^{14}C-UTP. A, C, E: Incubation in presence of bentonite (1 mg/ml). B, D, F: Incubation under standard assay conditions. After incubation, leaf homogenate with non-labelled RNA as a carrier was added prior to extraction with phenol-sodium dodecylsulphate

We have also analyzed the RNA which was synthesized by isolated chloroplasts, under nearly the same assay conditions, by MAK-chromatography, sucrose-gradient centrifugation and PAA gel electrophoresis. We have obtained closely the same results (Fig. 2, B, D, F). The RNA is very heterogeneous in size with a main peak at about 10—12 S.

However, we supposed, that this analysis does not show the actually synthesized chloroplast RNA, but only the degradation products. Therefore, we added bentonite (1 mg/ml) as an RNase inhibitor to the incubation medium. Now, we have isolated from the chloroplasts a quite different radioactive product. The MAK-column analysis showed a profile of radioactivity (Fig. 2 A) which corresponded to the analysis of total RNA and chloroplast RNA, rapidly labelled in vivo (Wollgiehn and Ruess, 1968). Also the labelling pattern obtained after fractionation on the sucrose gradient shows, that the RNA is not very heterogeneous, the radioactivity is concentrated in the region of the rRNA (Fig. 2 C). Particularly convincing are the results of polyacrylamide gel electrophoresis. We could demonstrate that the isolated chloroplasts have synthesized 16 S and 23 S (1·1 and $0{\cdot}56 \times 10^6$ daltons) RNA, i.e. the characteristic chloroplast ribosomal RNA (Fig. 2 E). We were not able to show with certainty the synthesis of low molecular weight RNA in the region of transfer RNA. The very limited synthesis of tRNA in isolated chloroplasts may be explained by the fact, that only 0·4—0·7 per cent of the chloroplast DNA is complementary to tRNA (Tewari and Wildman, 1970).

It is very difficult to decide whether in isolated chloroplasts other types of RNA are also synthesized. Some of the new synthesized RNA fractions are difficult to identify: 1. the small fractions of low molecular weight RNA, 2. the high molecular weight RNA, which was eluted from the MAK column after the ribosomal RNA, and 3. the radioactive fraction in the 18 S and 25 S (1·3 and $0{\cdot}7 \times 10^6$ daltons) region of the PAA gel profile. It remains open, whether these fractions contain messenger RNA or high molecular weight ribosomal precursor RNA. It is not yet clear, whether chloroplasts synthesize also other types of rapidly labelled RNA perhaps on repetitive DNA sequences.

The action of inhibitors on the RNA synthesis in isolated chloroplasts

RNA polymerases of different origin differ in their sensitivity to inhibitors. All polymerases are inhibited by actinomycin, but only the prokaryotic and not the nuclear polymerases are sensitive to rifampicin (Hartmann et al., 1967; Wehrli et al., 1968). On the other hand, α-amanitin specifically inhibits some eukaryotic nuclear polymerases (Kedinger et al., 1970; Lindell et al., 1970).

No uniform results were obtained, by using rifampicin, on the RNA synthesis in chloroplasts, until now (Bogorad and Woodcock, 1971; Bottomley et al., 1971; Spencer et al., 1971; Surzycki, 1969; Surzycki et al., 1970), although one should assume, that in this respect, the plastid polymerase corresponds to prokaryotic polymerases.

At first we have tested the action of actinomycin, α-amanitin and rifampicin on the RNA synthesis in young tobacco leaves in vivo. The leaves were preincubated for 12 h with solutions of the inhibitors (α-amanitin and rifampicin are active only after long preincubation) and then incubated for one hour with ^{32}P. Actinomycin in high concentrations inhibits nearly

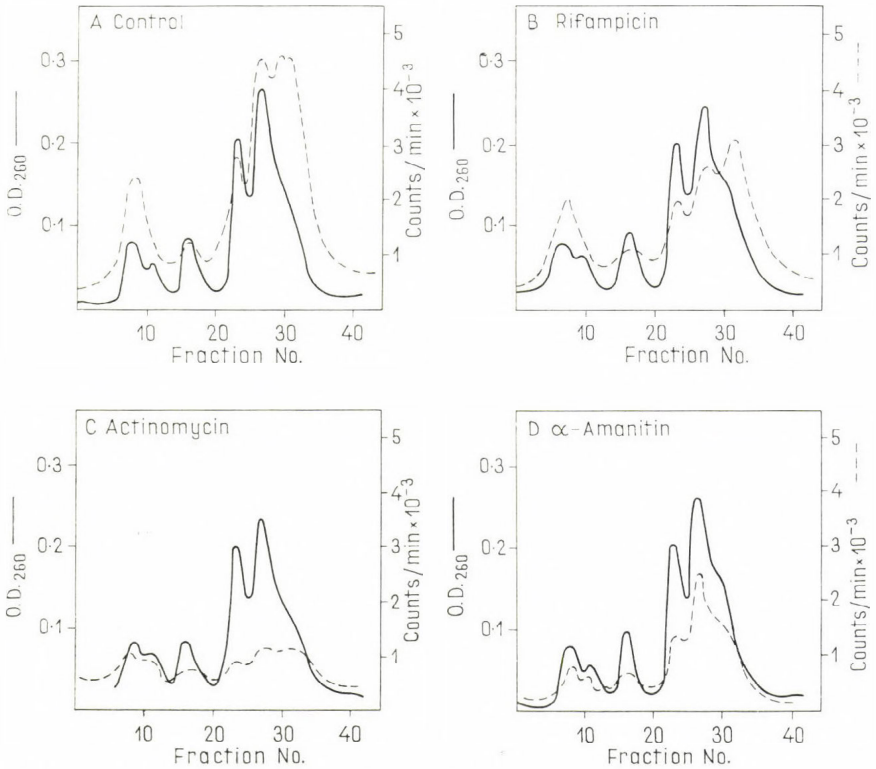

Fig. 3. The effect of rifampicin, actinomycin and α-amanitin on the RNA synthesis in tobacco leaves. The leaves were preincubated for 12 hours with solutions of the inhibitors (AD 50 μg/ml; rif. 300 μg/ml; α-am 30 μg/ml) and than incubated for 1 hour with ^{32}P. MAK column analysis of the RNA

completely the synthesis of all RNA fractions to the same degree (Fig. 3 C). Rifampicin also inhibits all RNA fractions, but even in very high concentrations (300 μg/ml) not more than by 30 per cent (Fig. 3 B). We believe that only the synthesis of chloroplast RNA is inhibited. α-amanitin inhibits the ^{32}P incorporation particularly into the high molecular AU-type RNA and not into the ribosomal RNA (Fig. 3 D).

These positive results obtained in vivo stimulated us to investigate also the effects of inhibitors on the RNA synthesis in isolated chloroplasts. The purified plastids were preincubated for 10 min with the inhibitors and than incubated under standard assay conditions with ^{14}C-ATP. Actinomycin in higher concentrations inhibits the RNA synthesis nearly completely

(Table 2). α-amanitin has no inhibitory effect in plastids even in very high concentrations. It inhibits, on the other hand, the Mn^{2+} stimulated RNA synthesis in nuclei (Bottomley et al., 1971). This result was expected, because α-amanitin inhibits RNA synthesis neither in bacteria (Jacob et al., 1970) nor in mitochondria (Tsai et al., 1971), and in nuclei it affects only the nucleoplasmic but not the nucleolar, ribosomal RNA synthesizing polymerase (Kedinger et al., 1970; Lindell et al., 1970). This corresponds to our results obtained in vivo (Fig. 3 D).

Table 2

The effect of inhibitors on the RNA synthesizing activity of isolated chloroplasts The purified chloroplasts were preincubated with the inhibitors for 10 minutes before RNA synthesis was started

Inhibitor	^{14}C-ATP incorporation as percentage of control
Control	100
Actinomycin D (50 μg/ml)	12
α-Amanitin (20 μg/ml)	100
Rifampicin (100 μg/ml)	98

Contrary to our expectation rifampicin has no inhibitory effect on RNA synthesis in purified chloroplasts under our standard assay conditions. This failure of rifampicin to inhibit plastid polymerase could thus be due to the inherent insensitivity of the enzyme. The results of the in vivo experiments contradict to this opinion. On the other hand, the plastid polymerase could be indeed sensitive to rifampicin, but we must search for more favourable experimental conditions.

The inhibition of RNA synthesis by rifampicin in bacteria is possible only if the synthesis begins with the binding of the enzyme to the DNA, because all the following steps of synthesis are insensitive to the antibiotic (Sippel and Hartmann, 1970). Chloroplasts must contain free polymerase as well as free template DNA in order to initiate new RNA chains. But DNA and also the polymerase could be lost during preparation of the chloroplasts, so that the observed RNA synthesis in vitro is only due to elongation of RNA chains which have been initiated in vivo.

We have tried to solve this problem by some different experiments. The procedure of chloroplast purification has only an insignificant influence on the action of rifampicin. We observed only in some experiments a small inhibition in non washed plastids compared to highly purified ones. Preincubation of the chloroplasts in the complete, rifampicin-containing incubation medium has no significant effect on the following RNA synthesis with ^{14}C-ATP either. Other experiments in which we have tried to release the polymerase or to stimulate the template activity of the DNA were also not highly successful.

Some results are summarized in Table 3. Sonication of the isolated plastids in the presence of rifampicin causes a significant inhibition of

RNA synthesis compared to the rifampicin-free control. Increasing the ionic strength has only a very small effect on the rifampicin action, because the template activity of chloroplast DNA does not increase at high ionic strength, contrary to that of the nuclear DNA. Dissociation of the DNA-histone complex in nuclei results in stimulation of the RNA synthesis.

It can be excluded that a deficiency in rifampicin uptake by whole chloroplasts is a reason for the lack of inhibitory effects, because even after disruption of the chloroplasts by Triton X 100 no inhibition was observed (Table 3).

Table 3

The effect of rifampicin on the RNA synthesizing activity of isolated chloroplasts after different pretreatment

Chloroplasts	Rifampicin 100 µg/ml	^{14}C-ATP incorporation as percentage of control without rifampicin
Purified chloroplasts	−	100
	+	101
Purified chloroplasts, after sonication in presence of rifampicin	−	100
	+	72
Purified chloroplasts, addition of 0·3 M $(NH_4)_2SO_4$	−	100
	+	92
Purified chloroplasts, disrupted with Triton, 100,000×g sediment	−	100
	+	101

It was possible to stimulate the polymerase activity of chloroplasts by exogeneous tobacco DNA. This stimulation was inhibited by rifampicin (Table 4). This can be explained by the mechanism of action of the antibiotic. In this experiment the RNA synthesis started with a rifampicin-sensitive formation of the complex of DNA and RNA polymerase.

A very distinct and well reproducible rifampicin effect was obtained when we isolated the chloroplasts from leaves after 12 h preincubation with rifampicin (300 µg/ml). The RNA polymerase of such chloroplasts is far less active than the enzyme of chloroplasts from water treated control leaves (Table 5).

Although we have found some distinct rifampicin effects, and we believe, that the chloroplast polymerase resembles the prokaryotic enzyme in sensitivity to the drug, the action of rifampicin on chloroplast RNA polymerase is not yet unequivocal (Bottomley et al., 1971; Spencer et al., 1971). A similar controversy exists as to the effect of rifampicin on mitochondrial RNA synthesis. Some papers report inhibition and others no inhibition of the RNA polymerase of this organelle (Shmerling, 1969; Tsai et al., 1971; Wintersberger and Wintersberger, 1969). Therefore, we believe, that

Table 4

The effect of rifampicin on the RNA synthesizing activity of isolated, non-purified chloroplasts after addition of tobacco DNA

To the chloroplasts, suspended in Tris-Mg^{2+}-SH medium, DNA and rifampicin was added. After 10 min of preincubation at 0° RNA synthesis was started

Rifampicin 100 µg/ml	DNA 100 µg/ml	^{14}C-ATP incorporation as percentage of control
−	−	100 (control)
+	−	102
−	+	153
+	+	104

Table 5

The effect of rifampicin on the RNA synthesizing activity in isolated chloroplasts

The chloroplasts were isolated from leaves, which were treated for 12 hours with a solution of rifampicin (300 µg/ml). Control leaves were treated with water

Treatment of the leaves before chloroplast isolation	^{14}C-ATP incorporation (cpm/0·1 mg chlorophyll)	Inhibition as percentage of control without rifampicin
Water	2330	100
Rifampicin	917	39

the properties of chloroplast RNA polymerase in comparison to nuclear as well as prokaryotic polymerase cannot be clearly defined until an active polymerase has been isolated from the organelles.

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RIBOSOMAL RNA SYNTHESIS IN NUCLEI OF FREELY SUSPENDED CELLS OF HIGHER PLANTS

by

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We wish to describe, at the outset, our system in a few words. We work with cell cultures of parsley tissue from the root. The cell material is homogeneous, bearing large nuclei, and behaves like embryonic tissue. The culture is free of bacterial contamination and for this and other reasons the system is suitable for tracer experiments.

During our analysis of rapidly labelled RNA by MAK chromatography we found by pulse-chase experiments the facts shown in Fig. 1. In the control (0 h) there is a RNA component eluted at higher NaCl concentration than 25 S RNA. After longer chase times this RNA disappears and the radioactivity in mature ribosomal RNA is enhanced. By this fact we are tempted to conclude that this specific, highly labelled component on MAK columns consists at least partially of rRNA precursor.

α-amanitin treatment

You can object to this assumption that this RNA has a high AMP content and not a ribosomal base composition. It seems as if in this region, in addition to the rRNA precursor, an AMP-rich RNA were eluted. Therefore, we tried to use α-amanitin as an inhibitor for the nuclear RNA synthesis outside the nucleolus. As shown in Table 1 under such conditions, when only one RNA with ribosomal base composition is synthesized, the high AMP content disappeared.

After this preparatory work it was important to look for newly synthesized ribosomal RNA, both in the ribosomes as well as in the nuclei. If you want to attribute the molecules to a certain organelle you have to extract the RNA from isolated nuclei and isolated ribosomes which is not possible in preparations full of cytoplasmic material.

METHODS

1. For the reason mentioned above it was necessary to prepare a pure nuclear fraction, if possible, from plant cells. The method is described in Fig. 2. The tests for the quality of the preparation were the microscopic picture and the DNA content in relation to RNA.

2. The ribosomes were prepared in the usual manner (Bielka et al., 1968).

3. Electrophoresis. We work with 2·1 % polyacrylamide gels. The running buffer is Tris-boric acid-EDTA, pH 8·3, after Peacock and Dingman (1968). The DNA appears in this buffer system between the two ribosomal peaks. We are able to say that the DNA is not mechanically fragmented. In E-buffer after Loening (1969) it has a lower electrophoretic mobility.

4. The tracer in all experiments was ^{32}P-orthophosphate. The pulse time was 30 min.

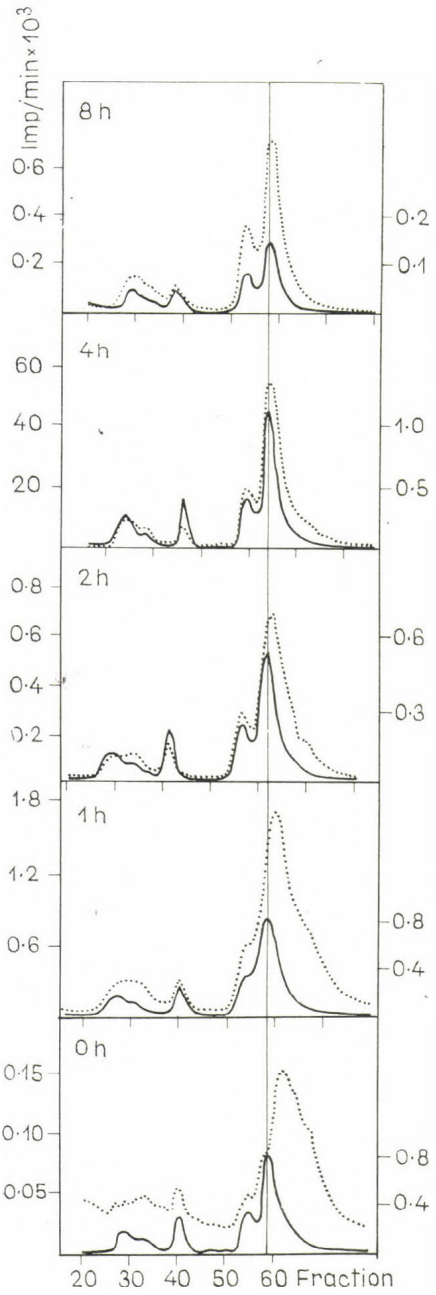

Fig. 1. MAK chromatography of total nucleic acids from parsley cells grown in a synthetic medium. The cells were labelled with ^{32}P-orthophosphate (20 μc/ml) for 30 min, chased with ^{31}P-orthophosphate for 0, 1, 2, 4, 8 h respectively. Nucleic acids were extracted with phenol-SDS at room temperature. ——— absorbance (260 nm); - - - - ^{32}P radioactivity (cpm)

RESULTS

Pulse experiments

First of all let us consider the electrophoretic pattern of nuclear nucleic acids (Fig. 3). Apart from the DNA there are two UV peaks with molecular weights of $1{\cdot}3 \times 10^6$ and $0{\cdot}7 \times 10^6$ daltons. Besides these three peaks, detectable in the UV curve only, a rapidly labelled fraction appears on the gel. Its molecular weight has been calculated to be $2{\cdot}3 \times 10^6$ daltons.

Contrary to this finding the RNA from isolated ribosomes contains no label at all (Fig. 4).

Pulse-chase experiments

To demonstrate the precursor character of the rapidly labelled fraction isolated from the nuclei we made pulse-chase experiments similar to those made on MAK-columns.

Table 1

Base composition of high molecular RNA eluted from MAK columns

Incubation with α-amanitin for 8 h. At the end of the α-amanitin incubation the cells were labelled with ^{32}P orthophosphate for 30 min. The base composition of the rapidly labelled component was determined by anion-exchange chromatography (Dowex 1×2, 200—400 mesh, Cl^- form) of the mononucleotides after alkaline hydrolysis

RNA-component	α-amanitin	CMP	AMP	GMP	UMP
Rapidly labelled RNA	—	23·5	30·0	26·0	20·5
25 S RNA	—	22·4	27·2	28·2	22·2
Rapidly labelled RNA	+ 17·2 μg/ml	20·1	26·5	28·4	25·0

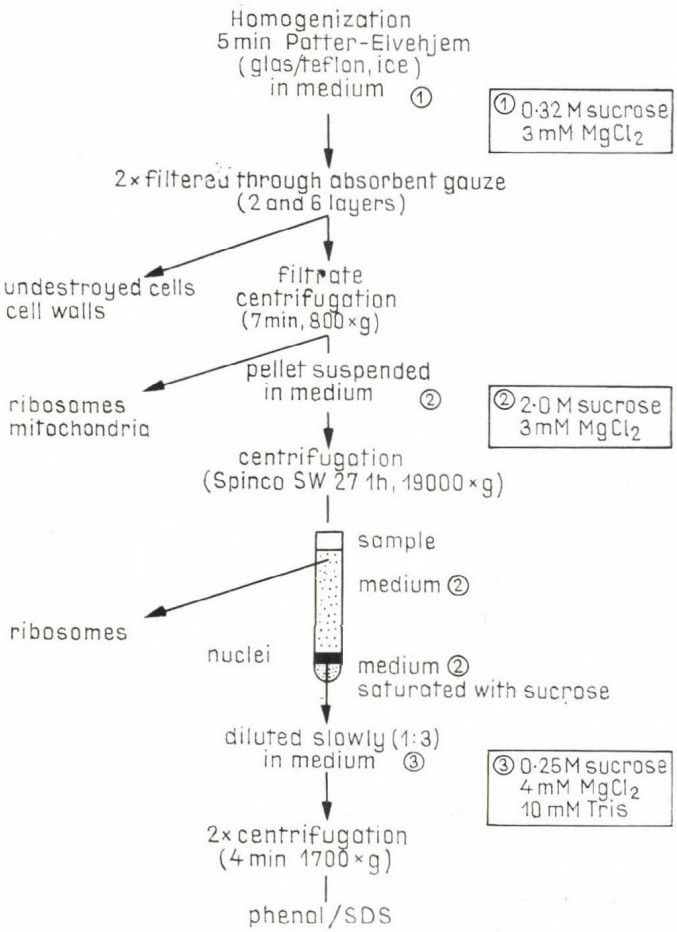

Fig. 2. Scheme of purification of nuclei from parsley cells

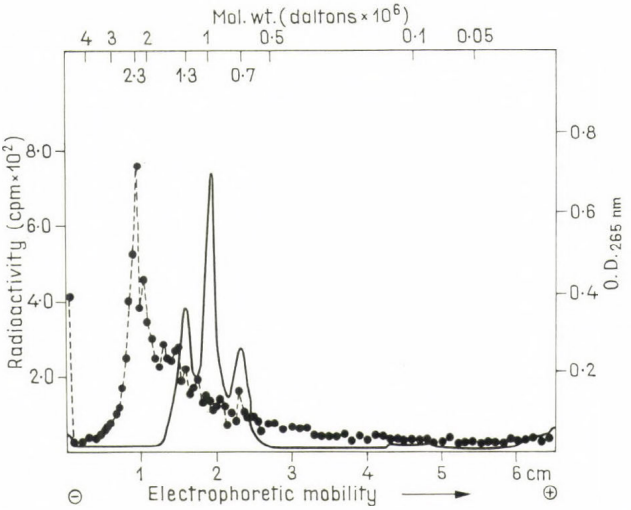

Fig. 3. Polyacrylamide gel electrophoresis of RNA from nuclei of parsley cells. After labelling the cells with ^{32}P orthophosphate (20 μc/ml) the nuclei were isolated. RNA was applied to 2·1% acrylamide gels and electrophoresed for 25 min at 5 mA/gel in Tris-boric acid-EDTA buffer (pH 8·3). After fixation in 1 molar acetic acid the gels were scanned at 265 nm. The radioactivity was determined by slicing the frozen gels into 0·5 mm sections and counting the dried gels in toluene scintillator. ——— absorbance (265 nm); ●- - -● ^{32}P radioactivity (cpm)

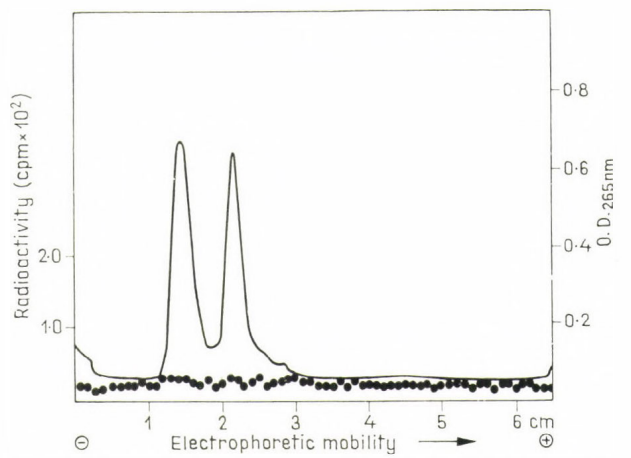

Fig. 4. Polyacrylamide gel electrophoresis of RNA from purified ribosomes of parsley cells. After labelling the cells with ^{32}P orthophosphate (20 μc/ml) the ribosomes were isolated. RNA was applied to 2·1% acrylamide gel (methods see Fig. 3). ——— absorbance (265 nm); ●- - -● ^{32}P radioactivity (cpm)

Before showing you the results we have to say something about the difficulties of chase experiments. It could be that there is a pool of phosphate in the cells which disturbs the results of such experiments. We should like to demonstrate the kinetics of ^{32}P incorporation into the total RNA of the ribosomes after various times of chase incubation (Fig. 5). During a period of 4 to 8 h chase incubation the specific activity is enhanced. Before the 4 h point there is a tendency for saturation. After treatment of the cells, at the end of ^{32}P incubation, with actinomycin D to stop the DNA dependent RNA synthesis, no enhancement in specific activity takes place. Before

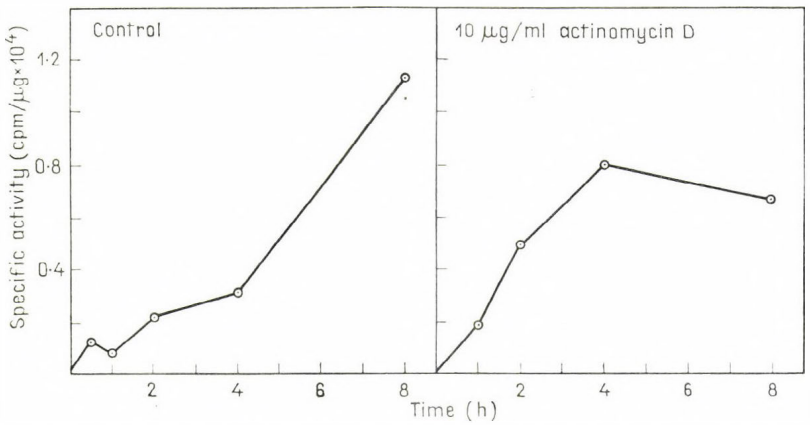

Fig. 5. Relative specific activity of RNA from ribosomes of parsley cells after labelling for 30 min with ^{32}P-orthophosphate followed by various times of chase incubation. a) control, b) with 10 μg/ml actinomycin D

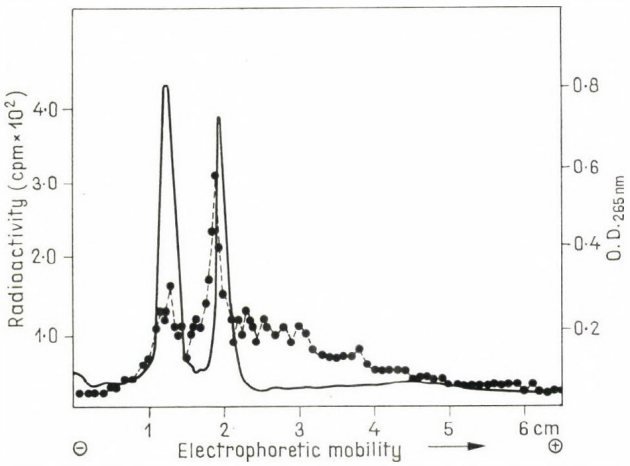

Fig. 6. Polyacrylamide gel electrophoresis of RNA from ribosomes of parsley cells. The cells were labelled with ^{32}P orthophosphate for 30 min followed by chase incubation for 1 h. ——— absorbance (265 nm); ●- - -● P radioactivity (cpm)

the 4 h period the synthesis is not levelled off. For this reason it is possible to look for the processing of rRNA before 4 h without actinomycin treatment.

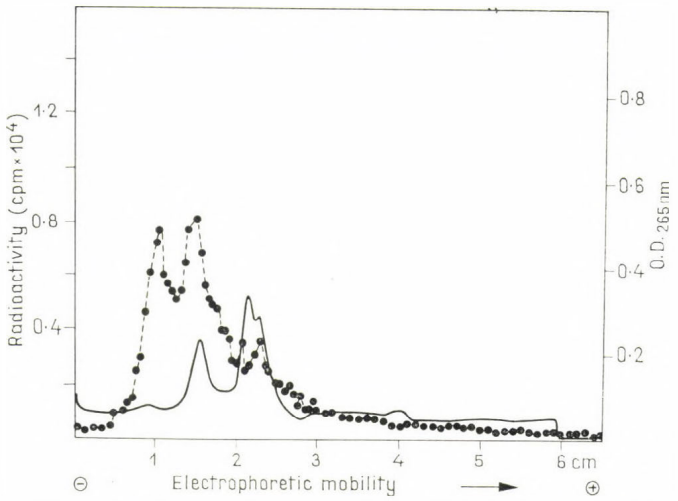

Fig. 7. Polyacrylamide gel electrophoresis of RNA from nuclei of parsley cells. The cells were labelled with ^{32}P orthophosphate for 30 min followed by chase incubation for 1 h. ——— absorbance (265 nm); ●- - -● ^{32}P radioactivity (cpm)

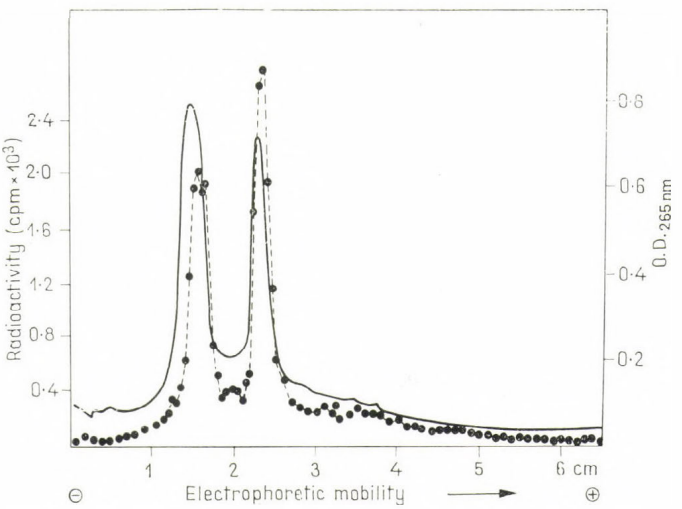

Fig. 8. Polyacrylamide gel electrophoresis of RNA from ribosomes of parsley cells. The cells were labelled with ^{32}P orthophosphate for 30 min followed by chase incubation for 4 h. ——— absorbance (265 nm); ●- - -● ^{32}P radioactivity (cpm)

The following experiment shows the distribution of radioactivity in the nuclear and ribosomal RNA.

1. (a) After 1 h chase (or even 30 min) we can detect activity in the 18 S RNA of the ribosomes; only very little activity appears in the 25 S RNA at this time (Fig. 6).

(b) At the same time the high molecular RNA is still present in the nuclei. In addition, the gel contains also radioactivity in the region of the 25 S RNA (Fig. 7).

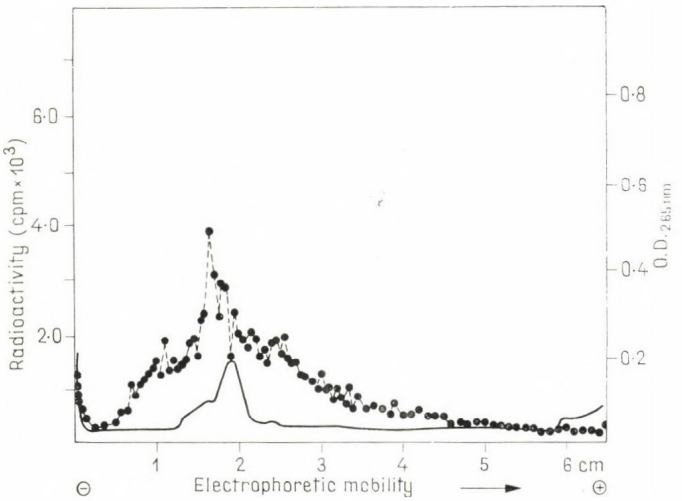

Fig. 9. Polyacrylamide gel electrophoresis of RNA from nuclei of parsley cells. The cells were labelled with ^{32}P orthophosphate for 30 min followed by chase incubation for 4 h. ——— absorbance (265 nm); ●- - -● ^{32}P radioactivity (cpm)

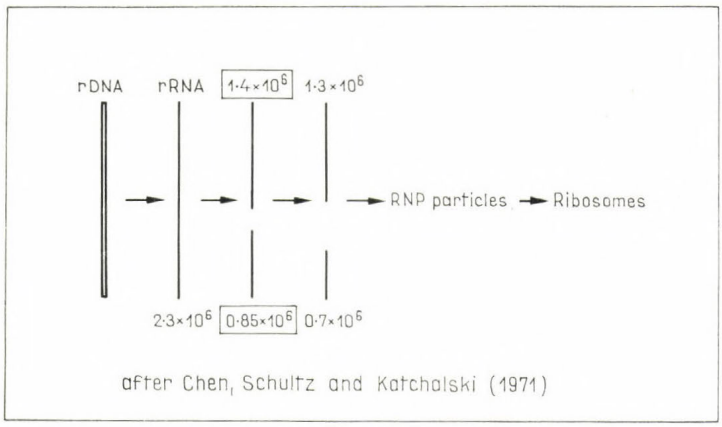

Fig. 10. Scheme of RNA processing

2. After 4 h of chase-incubation the 25 S RNA of ribosomes is also labelled (Fig. 8). In the nuclei the large molecule has disappeared. Part of the labelled 25 S RNA is still there (Fig. 9).

As shown above (Fig. 5), in our system chase experiments longer than 4 h are useless to solve our questions.

SUMMARY AND CONCLUSIONS

We can summarize our results as follows:

1. After 30 min incubation a large RNA molecule is synthesized in the nuclei. At this moment there is no newly synthesized material in the ribosomes.

2. Under chase conditions after 30 min and 1 h, respectively, we can detect new material in the 18 S RNA of the ribosomes. After 4 h (and after 2 h) the 25 S RNA appears. At the same time the large molecule disappears in the nuclei, while the 25 S RNA partly remains there.

How do these facts agree with the known schemes of ribosomal RNA processing (Fig. 10)? If we accept the pathway published by Chen et al. (1971) we should discuss the following questions. We have to look for at least two other precursor molecules (1·4 and $0{\cdot}85 \times 10^6$ daltons) and we have just started experiments in this direction. If we cannot find them we ought to analyse the $2{\cdot}3 \times 10^6$ region for other molecules similar to those recently described by Tiollais et al. (1971) for animal cells. They discuss the possibility that two distinct precursor molecules exist, one leading to the 18 S and the other to the 25 S RNA.

Furthermore, we should find a reason why the 25 S RNA appears more slowly than the 18 S RNA in the cytoplasm.

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ELECTRON MICROSCOPY OF DNA IN SUBCELLULAR ORGANELLES OF PEA SEEDLINGS

by

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Recent studies have shown that in addition to the nucleus other cell organelles, such as plastids and mitochondria, also contain DNA. We report here observations on the localization, form and size of DNA molecules, carried out during the last two years in the Institute of Biochemistry of the USSR Academy of Sciences. A significant part of the work was accomplished together with Dr. E. Mikulska of the University of Lodz.

Electron microscopic investigations of sections of isolated pea chloroplasts revealed in the finely granulated matrix electron-transparent areas which are believed to contain DNA. In these electron-transparent areas (ETA) core-like structures with radiating fine fibrils (both structures can be removed by DNase), small dense particles or aggregates of particles, some of which might represent ribosome-like particles (or polysomes?), can be seen.

A study of a good number of pea chloroplast sections showed that ETA within the matrix may differ in their number, form and size. Usually the section displays 3 to 7 ETA situated at random. In electron micrographs no membranes limiting these areas were seen and no structures connecting separate ETA in the matrix were observed either. Certainly, the study of chloroplast sections do not allow to conclude whether the entire DNA of chloroplasts is localised within these areas. It is very likely that they contain only a portion of DNA which can be seen due to a low electron density of these stroma regions; the remaining DNA may be localized in other regions of the stroma, being "free" or attached to the lamellar system of chloroplasts. The possibility is not ruled out that a high density of the chloroplast matrix does not permit us to observe this DNA in sections. It is interesting to note that the electron-transparent DNA-containing areas of pea chloroplast matrix are also the sites of the localization of starch grains.

In sections of young pea leaves dark spots in electron-transparent DNA-containing areas can be distinguished which can be regarded as the beginning of starch grain formation. In these areas fibrillar structures containing DNA as well as dense ribosome-like particles can be seen. The entirely formed starch grains fill up almost the whole ETA.

It should be noted that starch grains were never found in regions other than the ETA of pea chloroplast matrix. In view of the presence of DNA, ribosome-like particles, proteins (probably enzymes, as it was shown by other investigators) and starch in the ETA, it seems very probable that these areas are biologically very active ones in chloroplasts.

Fig. 1. Electron micrograph of pea chloroplast DNA released by osmotic shock. Rotary shadowing with Pt-Pd. ×60,000

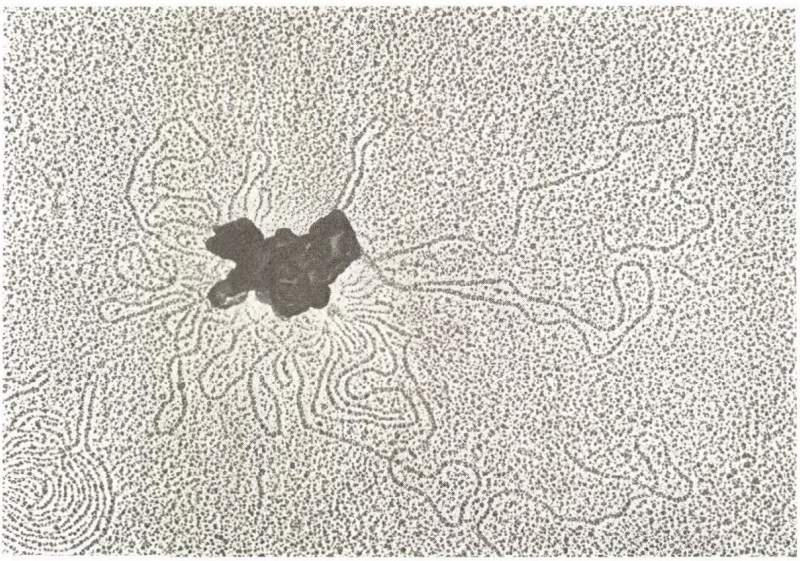


Fig. 2. DNA-membrane complex isolated from pea chloroplasts. DNA linear strands and loops of different forms and sizes can be seen. Rotary shadowing with Pt-Pd. ×44,000

Contrary to chloroplasts, mitochondria of pea seedlings contain usually only one DNA-containing ETA with hardly discernible ribosome-like particles. The ETA is localized within the central part of the mitochondrial matrix.

The problem of DNA arrangement in subcellular organelles has not yet been solved. In sections no direct connection between the DNA fibrils and membrane system has been observed.

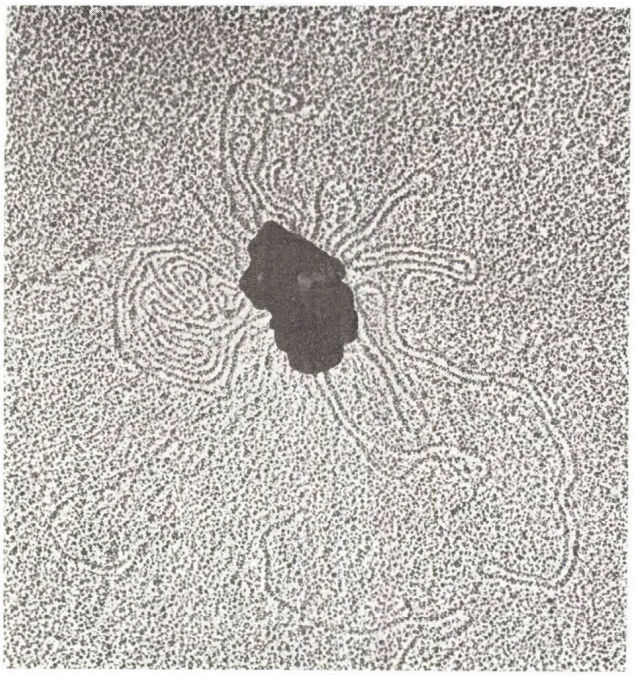


Fig. 3. The same as in Fig. 2. The regular packing of DNA strands (arrow) can be seen. Rotary shadowing with Pt-Pd. ×60,000

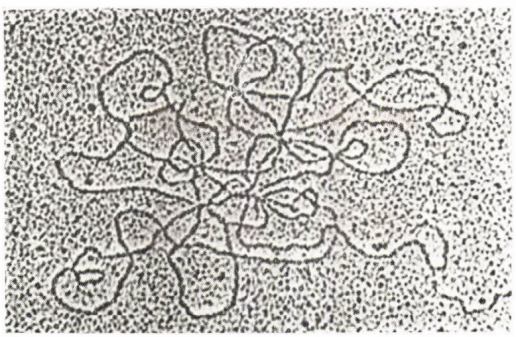


Fig. 4. Electron micrograph of a DNA molecule from pea chloroplasts. The molecule is 8·8 μ in total length. Rotary shadowing with Pt-Pd. ×55,000

Electron microscopic investigations of chloroplast preparations exposed to osmotic shock indicated that a number of DNA fibrils might be associated with chloroplast membrane fragments. However, in such preparations the possibility is not excluded that they are fragments of membranes with the stroma adhering to them. Fig. 1 shows DNA released from osmotically disrupted pea chloroplasts. Multiple loops and fibrils essentially identical in diameter, radiating irregularly from the chloroplast fragment can be seen. A similar picture was obtained by studying the DNA released from osmotically disrupted mitochondria. DNA filaments radiated from all sides of the mitochondrial membrane and there was no single site where they attached to the fragments. Sometimes, clusters of DNA filaments free from the remnants of mitochondrial fragments were observed.

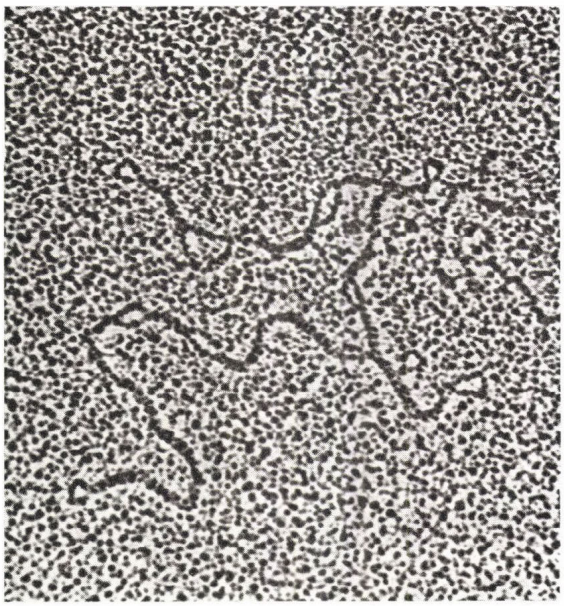
While the lysis of animal mitochondria by osmotic shock liberates predominantly DNA in the form of twisted circles, no circular DNA was released from pea mitochondria and chloroplasts.

In a more direct way the association of DNA fibrils and the membrane system of cell organelles can be demonstrated on electron micrographs of membrane fractions isolated from them. Figure 2 shows a DNA-membrane complex, isolated from pea chloroplasts. Single and double loops scattered on all sides of the chloroplast membrane fragment. In some cases certain regular packing of DNA threads was observed (Fig. 3). Even larger "displays" were released from some chloroplasts. They are similar to the "single-center" and "multi-center" displays described for bacterial cells.

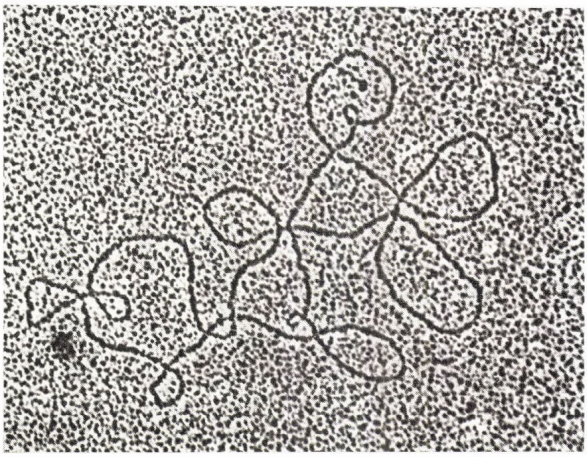
Fig. 5. Electron micrograph of a DNA molecule from pea mitochondria. The molecule is 9·0 μ in total length. Rotary shadowing with Pt-Pd. $\times$36,000

By analogy with bacteria it can be supposed that the membranes play an important role in the replication of chloroplast DNA.

It is well known that the mitochondrial DNAs of many animal species exhibit a remarkable structural similarity: they have been found to occur in the form of closed circular duplex molecules approximately 5 μ in contour length with a molecular weight of 10^7 daltons. The true size of DNA mole-



(a)



(b)

Fig. 6. Electron micrographs of DNA molecules from rat liver mitochondria. Twisted circle (a; $\times 88{,}000$) and open circular molecules (b; 5·1 μ; $\times 66{,}000$)

cules in subcellular organelles of plants is unknown. In lysates of chloroplasts and mitochondria and in purified DNA preparations isolated from these organelles linear molecules were found ranging mostly from 5 to 25 μ. This value corresponds to a molecular weight of approximately $20-22 \times 10^6$ daltons.

Representative molecules of DNA prepared from chloroplasts and mitochondria of pea seedlings are shown in Figs 4 and 5.

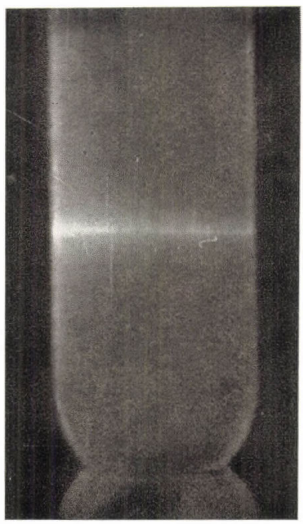


Fig. 7. Chloroplast DNA from pea seedling cells in CsCl-ethidium bromide density gradient. A photograph of tube illuminated with near ultraviolet light (365 mμ). The band contains only linear DNA

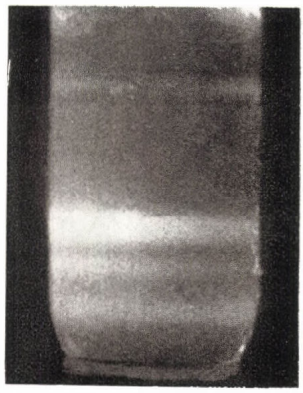


Fig. 8. Pea chloroplast DNA and mitochondrial DNA from rat liver in CsCl-ethidium bromide density gradient. The upper band contains only linear DNA. The three other bands contain different forms of circular DNA

The DNA molecules exhibit different forms: from extended linear filaments and filaments having multiple loops to rosette or flower-like configurations, most of which showed free ends. Certainly the length of DNA molecules observed in such preparations does not represent the size of the DNA molecules in situ. The measurement of the total length of the DNA extruded from pea chloroplasts by osmotic shock testified for significantly longer DNA molecules in situ. Thus, apparently, the DNA molecules in plant cell organelles are several times longer than the DNA molecules of animal mitochondria.

We have never found circular molecules in preparations obtained by osmotic shock or by lysis of subcellular organelles. Purified preparations of DNA isolated from chloroplasts and mitochondria of pea seedlings

displayed only linear molecules while those from mitochondria of rat liver exhibited closed and open forms of DNA, as shown in Fig. 6.

The linear form of DNA molecules in subcellular organelles of pea seedlings was confirmed by means of the CsCl-EthBr density gradient centrifugation. The DNA from pea chloroplasts and mitochondria as well as the DNA from the nuclear fraction occupies the same position in the CsCl-EthBr gradient. It forms a single band at a density of 1·57—1·58 g/cm^3 (Fig. 7). Different forms of circular DNA from rat liver mitochondria locate in the more dense region of the gradient with a density of 1·62—1·66 (Fig. 8).

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RIBOSOMES OF PEA SEEDS

by

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At present it is well known, that seed germination is connected with the activation of the protein-synthesizing system. Not much is known about the ribosomes in plant seeds at the stage of dormancy and after imbibition with water. We studied some properties of the ribosomes isolated from dry pea seeds and from the cotyledons of germinated pea seeds.

It was shown, that the ribosomes are not attached to membranes of the endoplasmic reticulum. Free ribosomes were found in the cotyledon cells of both the dry and germinated pea seeds. This phenomenon may be connected with the absence of protein synthesis for translocation or storage at this time. We could not find ribosomal subunits in the cell extracts of dry seeds. However, the addition of the ionic detergent DOC (0·7—1·0%) to cell extracts or isolated ribosomes in the presence of Mg^{2+} ions (0·01 M) resulted in a dissociation of ribosomes into two subunits (Fig. 1). Triton had no similar effect. The reason of such an effect of DOC on pea ribosomes is not clear at present. But this fact should be kept in mind during the investigation of naturally occurring ribosomal subunits in cell extracts in the presence of the ionic detergent DOC.

Pea seed ribosomes belong to the 80S type, they have a sedimentation constant of 79 Svedberg units, an RNA/protein ratio of 1·0, and a buoyant density on CsCl of 1·54—1·55 g/cm³ (Table 1). No variations were found in the buoyant density of ribosomes isolated from different tissues of ger-

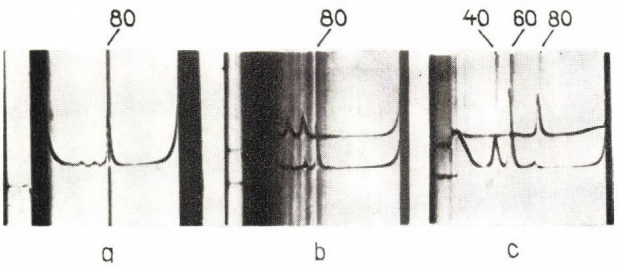

Fig. 1. Analytical ultracentrifugation sedimentation patterns of ribosomes of dry pea cotyledons: (a) 20,000 g supernatant ("cell extract"); (b) "cell extracts" after addition of detergents DOC (0·5%) and Triton (1%); (c) ribosomes before and after addition of DOC. Buffer: Tris-HCl 0·05 M, KCl 0·025 M, $MgSO_4$ 0·01 M, pH 7·6

minated seeds, and of ribosomes from dormant and germinated seeds (Table 2). It seems that the activation of ribosomes after imbibition of seeds is not followed by changes in protein content. Ribosomes from dry seeds are capable of complete and reversible dissociation into 60S and 40S subunits after 20 min incubation in 0·5 M KCl with 0·01 M Mg^{2+} (Fig. 2). In cotyledons after germination and in seedlings there are populations of ribo-

Table 1

Effect of Triton X 100 and DOC on the buoyant density in CsCl of the ribosomes and their subunits from dry pea seeds

Detergent	80S	60S	40S
No detergent	1·544—1·550	1·555	1·530
Triton, 1%	1·544—1·550	1·560	1·523
DOC, 0·5%	1·548—1·552	1·560	1·520
DOC, 1%	1·548—1·555	1·558	1·524

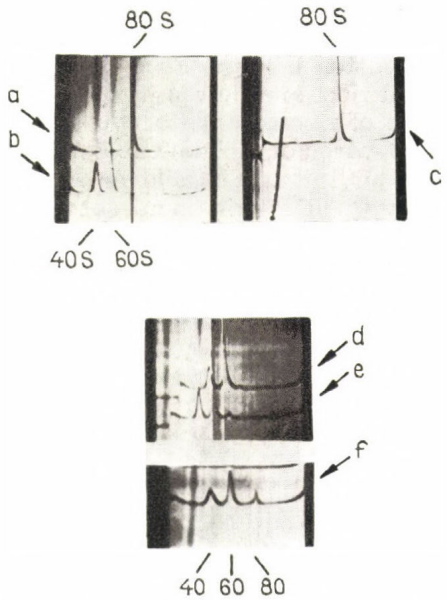

Fig. 2. Analytical ultracentrifugation sedimentation patterns of the dissociation of ribosomes from dry and germinated seeds. (a) ribosomes from dry pea seeds; (b) dissociated ribosomes from dry pea seeds; (c) reassociated ribosomes; (d) ribosomes from dry seeds; (e) ribosomes from cotyledons of germinating seeds; (f) ribosomes from shoots + roots of germinating seeds

somes resistant to dissociation under the conditions mentioned above. Perhaps the high rate of dissociation is connected with the absence of protein synthesis by the ribosomal system of dry plant seeds. Therefore, the ribosomes from dry pea seeds represent a good object for investigating the properties of plant ribosomal subunits. The subunits of pea seed ribosomes,

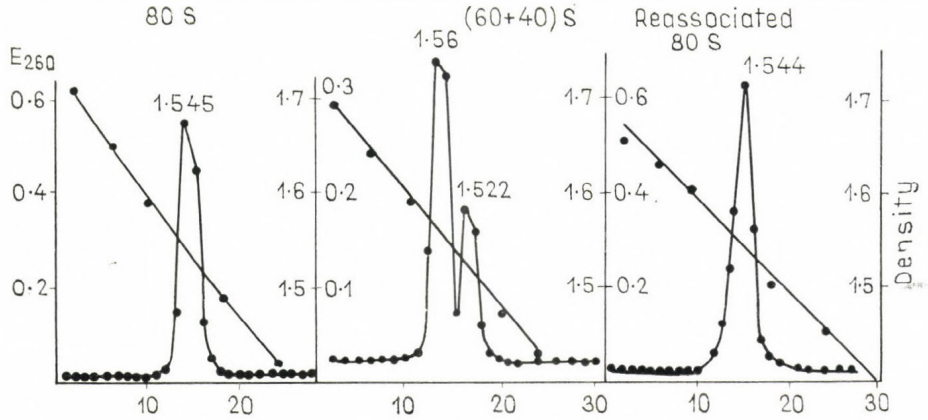

Fig. 3. Profiles of CsCl density gradient centrifugation of ribosomes and their subunits

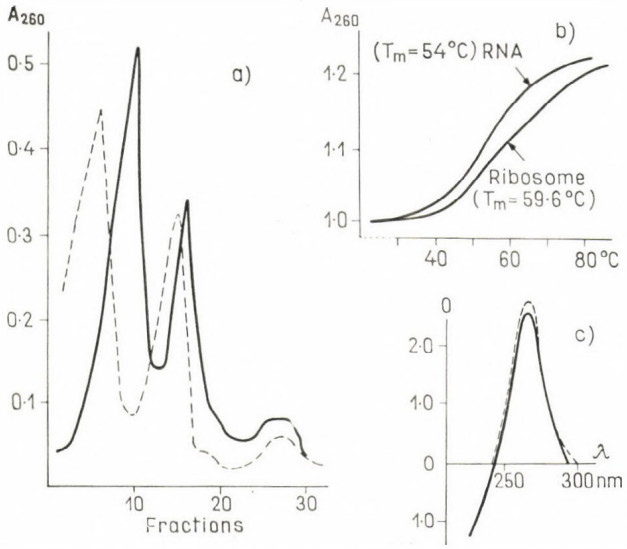

Fig. 4. Properties of rRNA from dry pea seeds:(a) Sedimentation in sucrose gradient after treatment of ribosomes of pea seeds (- - - -), and Krebs ascites tumor cells (———) with SDS. (b) Dependence of optical density of ribosomes and RNA of dry pea seeds on the temperature (after dialysis for 12 h in Tris-HCl buffer 0·01 M, $MgSO_4$ 10^{-4} M, pH 7·6). (c) Curves of circular dichroism of ribosomes and RNA

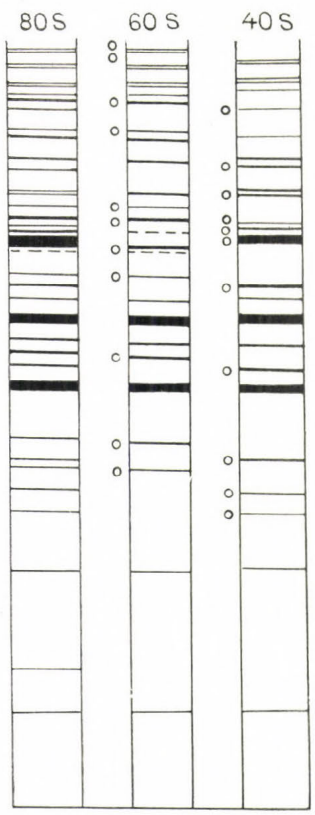

Fig. 5. Proteins of ribosomes and their subunits from dry pea seeds in polyacrylamide gel electrophoresis (scheme)

formed in 0·5 M KCl in the presence of 0·01 M Mg^{2+}, and the original 80S ribosomes differ in their buoyant density in CsCl. The large subunit has a density of 1·558—1·560 g/cm³ and its protein content is equal to 48 %. The buoyant density of the smaller subunit is 1·520—1·524 g/cm³ (Fig. 3) and its protein content is as high as 54 %. The dissociation of 80S ribosomes was not followed by a release or loss of proteins from the ribosomes or their subunits, because the protein content of intact ribosomes is equal to the sum of protein contents of their two subunits. The density of reassociated 80S ribosomes is equal to the density of original ribosomes. The results obtained indicate that there are substantial variations among the plant and animal 80S ribosomes. On the other hand, no changes were found in the buoyant density of ribosomes and their subunits during germination. Ribosomes from dry pea seeds contain 2 types of ribosomal RNA. The heavy component sediments somewhat slower than the heavy rRNA of animal ribosomes. The determination of hypochromicity and assays of circular dichroism showed that the secondary structure of RNA is similar within the ribosomes and in the free state. The melting temperature of RNA in ribosomes is higher than that of free RNA (59·6 °C and 54·0 °C). This indicates the stabilization of double-stranded regions of RNA in ribosomes by the ribosomal proteins (Fig. 4).

The proteins of 80S ribosomes of pea seeds are represented by a heterogeneous mixture and can be resolved by polyacrylamide gel electrophoresis into 39 components. The comparison of the proteins of the large and small ribosomal

Table 2

Buoyant density in CsCl of the ribosomes and their subunits from pea

Source of ribosome	80S	60S	40S	80S reassociated
Dry pea seeds	1·548±0·002	1·558±0·003	1·524±0·004	1·546
Cotyledons of germinating seeds	1·545±0·002	1·560±0·002	1·521±0·003	1·546
Seedlings	1·546±0·002	1·560±0·001	1·520±0·002	1·542

subunits has shown a certain degree of specificity: the proteins of the 60S as well as of the 40S particles are separated into 27 bands; at least 11 of these bands belong either to the large or to the small subunits; 16 bands seems to be common (Fig. 5).

Thus, the ribosomes in cotyledons of mature pea seeds retain all the major characteristics of native ribosomes, they have no essential defects and, evidently, can guarantee protein synthesis during germination.

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SITES OF SYNTHESIS OF CHLOROPLAST PROTEINS

by

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INTRODUCTION

Chloroplasts as the unique organelles of green plant cells contain a considerable amount of protein which is necessary to fulfil the specific functions of the organelle. It is well known that many of these proteins are formed by light-induced synthesis and that they increase in content parallel to chlorophyll formation, thylakoid membrane assembly and evolution of photosynthetic O_2. It is further without doubt that chloroplasts contain the machinery for protein biosynthesis and that they are able to use it *in vivo* and *in vitro* (Kirk and Tilney-Bassett, 1967; Smillie and Scott, 1969; Schiff, 1970; Parthier and Wollgiehn, 1966).

On the other hand, it has become increasingly evident that the biogenesis of the organelle is not only controlled by the plastid DNA,* although its capacity of genetic information may be sufficient to code all chloroplast-specific RNAs and proteins (Kirk and Tilney-Bassett, 1967; Wildman, 1971). We have to assume an intricate interrelation between the two genetic systems of the plant cells, the prokaryote-like system in the chloroplasts and mitochondria and that of the eukaryotic nucleo-cytoplasm (Parthier, 1970).

The aim of several laboratories, including ours, is the determination of the sites of synthesis or genetic control of chloroplast-specific components. This aim can be reached mainly by two approaches, the use of mutant strains (gives evidence for the site of genetic control) and the use of specific inhibitors of RNA and protein synthesis (determines the synthetic site). For protein synthesis, the second approach bases on the different sensitivities of cytoplasmic and chloroplast ribosomes to certain antibiotics, preferably CHI and CAP (Kirk and Tilney-Bassett, 1967; Smillie and Scott, 1969; Schiff, 1970; Parthier and Wollgiehn, 1966; Wildman, 1971; Parthier 1970; Smillie et al., 1971).

Table 1 is a survey on the sites of control and synthesis of certain chloroplast proteins. For some proteins, e.g. RuDP-carboxylase, the results from different plants are fairly uniform, but differences exist for other enzymes of the reductive pentose phosphate cycle or components of the photosynthetic electron transport chain. Meagre evidence is present particularly

* Abbreviations: DNA and RNA, deoxy- and ribonucleic acid; tRNA, transfer RNA; a-tRNA, aminoacyl-tRNA; CHI, cycloheximide; CAP, chloramphenicol; NA, nalidixic acid; RuDP, ribulose-1,5-diphosphate.

Table 1

The sites of genetic control or intracellular synthesis of chloroplast specific enzymes and other chloroplast proteins. N = nucleolus; P = plastid

Enzyme of protein	Source	Sites of		References
		control (mutants)	synthesis (inhibitors)	
RuDP-carboxylase	*Phaseolus*		P	Ireland et al., 1971
RuDP-carboxylase	*Euglena*	P	P	Schiff, 1970; Smillie et al., 1967
RuDP-carboxylase	*Chlamydomonas*	N	P	Surzycki et al., 1970
(NADP)-GAPDH	*Phaseolus*		P	Ireland et al., 1971
(NADP)-GAPDH	*Euglena*	N	P, N	Smillie et al., 1967; Schiff, 1970
Fructose-1,6-DP-Aldolase	*Pisum*	N		Anderson and Levin, 1970
Phosphoribulokinase	*Phaseolus*		P	Schiff, 1970
Phosphoribulokinase	*Chlamydomonas*	N		Armstrong et al., 1971
Transketolase	*Phaseolus*		N	Schiff, 1970
Triosephosphate isomerase	*Phaseolus*		N	Schiff, 1970
Nitrite reductase	*Zea*		P	Smillie and Scott, 1969
5-aminolevulate-DH	*Euglena*		P	Smillie and Scott, 1969
Thylakoid proteins	*Chlamydomonas*		P + N	Eytan and Ohad, 1970; Hoober, 1970; Hoober et al., 1969
Fd-NADP reductase	*Eugl., Chlamydom.*		P, N	Smillie et al., 1967; Armstrong et al., 1971
Cytochrome 552	*Eugl., Chlamydom.*		P, P + N	Smillie et al., 1967; Armstrong et al., 1971
Cytochrome 561	*Eugl., Chlamydom.*		P	Smillie et al., 1967; Armstrong et al., 1971
Plastocyanin	*Chlamydomonas*	N		Surzycki et al., 1970
DNA polymerase	*Chlamydomonas*		N	Surzycki et al., 1970
RNA polymerase	*Chlamydomonas*		N	Surzycki et al., 1970
Ile-tRNA synthetase	*Euglena*	P		Reger et al., 1970
Phe-tRNA synthetase	*Euglena*	N		Reger et al., 1970
Ribosomal proteins	*Chlamydomonas*		N	Surzycki et al., 1970

for two groups: the thylakoid-protein complex and the enzymes involved in the realization of the genetic information, enzymes which catalyze reactions of RNA and protein synthesis.

We have focussed our attention to the site of synthesis of thylakoid proteins and to the a-tRNA synthetases which catalyze the activation and specific transfer of amino acids onto the cognate tRNAs. Some of the enzymes have been shown to be present in chloroplasts of *Phaseolus* (Burk-

ard et al., 1970) and *Euglena* (Reger et al., 1970). We used CHI as a specific inhibitor of protein synthesis on 80S cytoplasmic ribosomes and CAP as the corresponding inhibitor for 70S chloroplast ribosome activity. Since CAP inhibits protein synthesis in plant cells in much higher concentrations than CHI, we also included nalidixic acid, a specific inhibitor of chloroplast replication in *Euglena* (Lyman, 1967). This substance blocks DNA replication in bacteria, if RNA and protein synthesis is allowed to proceed (Deitz et al., 1966). NA can be used in much lower concentrations than CAP to obtain the same effect. The organisms, *Euglena* cells, were grown in the presence of these inhibitors for at least 5 generations.

MATERIALS AND METHODS

Euglena gracilis, Z strain, was mixotrophically grown as described previously (Meissner et al., 1971). Dark-grown bleached cells were inoculated (10^5 cells/ml) in normal medium or medium containing inhibitors: CAP 1·5 mg/ml, NA 5 to 40 μg/ml or CHI 1 to 2 μg/ml.

Apoplastic mutants (correctly, they do contain very few small, proplastid-like organelles) were obtained after U.V. irradiation or NA-treatment. The colorless strains were selected from agar plate colonies. — *Anacystis nidulans* was grown at 40 °C, *E. coli* at 33 °C.

All preparations were obtained from late log-phase cells (3 days old). The preparation of crude enzyme and tRNA fractions, enzyme assays and the determination of chlorophyll, protein, tRNA and cell number have been described earlier (Meissner, et al., 1971; Parthier, 1971). Chloroplasts were isolated either by 10 sec. ultrasonic treatment or glass-bead disrupture of the cells in 0·05 M Tris-HCl buffer (pH 7·8), containing 20 per cent sucrose, 0·01 M $MgCl_2$, 0·006 M KCl and 0·005 M mercaptoethanol. The separation of the chloroplast fractions was carried out on 25 to 60 per cent discontinuous sucrose gradients, centrifuged 20 min at 8,000 g. The fractionation of thylakoid proteins on polyacrylamide gel electrophoresis has been described elsewhere (Heinze, 1961). The a-tRNA synthetases from the crude enzyme preparation were fractionated on 20 × 1·5 cm columns of hydroxylapatite prepared according to Tiselius et al. (1956). The gradient was 0·01 M to 0·3 M potassium phosphate, pH 7·5, including 0·001 M $MgCl_2$, 0·0005 M mercaptoethanol and 10 per cent glycerol.

RESULTS AND DISCUSSION

Effects of inhibitors on cell division, chlorophyll, total protein and RuDP-carboxylase synthesis

The different effects of CAP and NA on one side and CHI on the other upon cell division, protein and chlorophyll contents of *Euglena* cultures are demonstrated in Fig. 1 A—C. While CAP and NA do not appreciably affect cell number and total cell protein, CHI inhibits very strongly at concentrations as low as 1 μg/ml. Doses higher than 5 μg/ml are lethal under our growth conditions. After a certain time, protein synthesis and cell multiplication begin slowly to recover from CHI inhibition. The increase

of chlorophyll synthesis and RuDP-carboxylase activity (Fig. 1 D) indicates that CHI does not inhibit chloroplast-specific processes. On the contrary, with low inhibitor concentrations chlorophyll content and enzyme activity exhibit values significantly higher than in untreated cells (see also Smillie et al., 1971). Electron microscopic observations provide evidence that the chloroplasts of CHI-treated cells are not only higher in number and membrane content than chloroplasts from untreated cells, but also bizarre deformations of the organelle shape occur. These are indications that in sublethal doses the antibiotic causes a miscontrol of chloroplast biogenesis obviously by preventing the formation of cytoplasmic components necessary for the normal chloroplast development.

Both CAP and NA inhibit the synthesis of chlorophyll and RuDP-carboxylase to a high extent, dependent on the time of presence of the inhibitors in the cultures before illumination.

These results prove the drug-specificity on long-term inhibition of protein synthesis proceeding either in chloroplasts or in the cytoplasm. It is unlikely, but cannot be excluded *a priori*, that secondary effects others than on nucleic acid or protein synthesis are responsible for the drug actions.

We have checked, therefore, also short-time effects of CAP and CHI on protein synthesis of *Euglena* cells. The proteins of untreated or antibiotic-treated cells were 15 min pulse-labelled with ^{14}C-leucine, and the

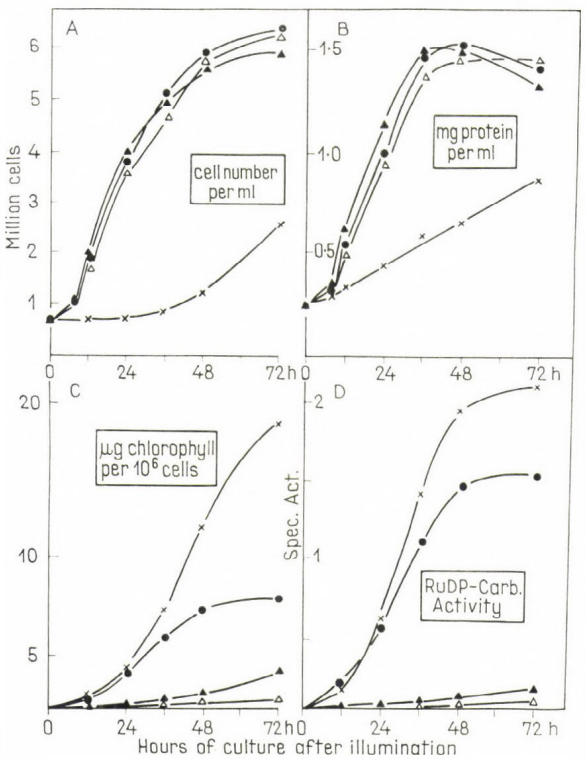

Fig. 1. Effects of CAP, NA and CHI on cell division (A), total protein (B), chlorophyll (C) and RuDP-carboxylase synthesis (D) of *Euglena gracilis* cultures after induction of chloroplast formation by illumination. The antibiotics were added with the start of illumination. Concentrations: CAP, 1·5 mg/ml; NA, 40 µg/ml; CHI, 1 µg/ml. Cells were counted with an electronic particle counter, determination of protein and chlorophyll content as described earlier (Meissner et al., 1971; Parthier, 1971), RuDP-carboxylase activity according to Keller and Huffaker (1967)

rates of incorporation were determined after separation of the cell fractions. The results in Table 2 indicate complete inhibition of ^{14}C incorporation into cytoplasmic proteins by CHI but no inhibition by CAP. The leucine incorporation into chloroplast proteins was suppressed to 50 per cent by both inhibitors.

Table 2

Effect of chloramphenicol and cycloheximide on the ^{14}C-leucine pulse incorporation into proteins of chloroplast and cytoplasm fractions of Euglena cells

Fraction	Control spec. act.	5×10^{-3} M CAP		2×10^{-5} M CHI	
		spec. act.	% inh.	spec. act.	% inh.
Whole chloroplasts	350	200	43	170	51
Chloroplast lamellae	320	163	50	170	47
Soluble stroma extract	450	400	10	42	90
Cytoplasm fraction	1,650	1,620	0	30	98

After disruption of the whole chloroplasts by osmotic shock or sonication and after separation of the soluble stroma proteins from the green thylakoid proteins we found the inhibition caused by CAP mainly restricted on the synthesis of lamellar proteins. It is unknown whether the 10% inhibition of ^{14}C incorporation into stroma proteins represent the inhibition of RuDP-carboxylase formation (Fig. 1 D). CHI clearly inhibits leucine incorporation into stroma proteins suggesting their synthesis on cytoplasmic ribosomes. However, we cannot exclude a contamination of the stroma fraction by cytoplasmic proteins.

Effects of inhibitors on thylakoid protein formation

The reduced ^{14}C-content of the thylakoid proteins caused both by CAP and CHI treatments, indicating the synthesis of these proteins on chloroplast and cytoplasmic ribosomes, prompted us to fractionate the proteins on polyacrylamide gel electrophoresis. *Euglena* chloroplast thylakoid proteins can be separated into 14 individual bands. If the proteins are analyzed from cells grown 3 days in the presence of CAP or NA, at least 4 bands (Nos 4, 6, 9, 10) are missing (Fig. 2). These 4 bands are assumed to be synthesized by the genetic apparatus of the plastids.

Labelling experiments should confirm this suggestion. Dark-grown *Euglena* cells were illuminated for 8 h to induce chloroplast formation and were then treated with appropriate concentrations of CAP and ^{3}H-leucine or CHI and ^{14}C-leucine, respectively, for further 8 h.

The results are demonstrated in Fig. 3 as the ^{14}C : ^{3}H ratios of the single protein bands after co-electrophoresis of the two preparations. Considerably higher ratios were found in bands Nos 4 and 6. This suggests a strong inhibition by CAP of the ^{3}H-leucine incorporation. Bands Nos 9 and 10, dominantly increasing during the greening process, possess low ^{14}C : ^{3}H

ratios. Consequently, their synthesis should proceed at least partially on cytoplasmic ribosomes or, alternatively, these proteins are synthesized under the control of cytoplasm-specific components. In *Chlamydomonas* chloroplasts these proteins are apparently built up by individual proteins, both synthesized inside and outside the organelles (Eytan and Ohad, 1970; Hoober, 1970; Hoober et al., 1969).

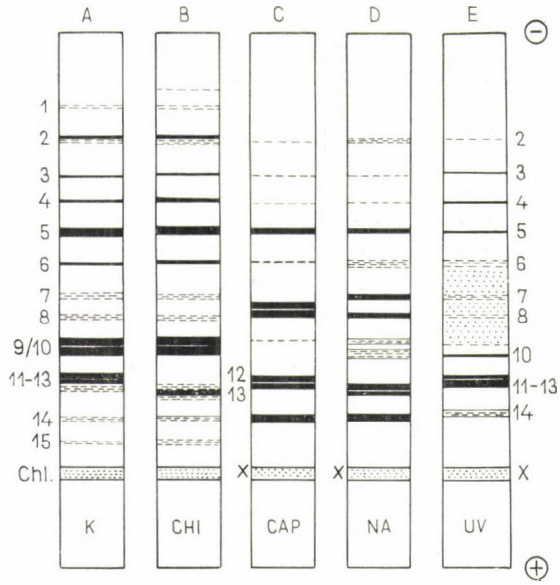

Fig. 2. Effects of CHI, CAP and NA on the formation of chloroplast thylakoid proteins fractionated by polyacrylamide gel electrophoresis (Heinze, 1971). The antibiotics were added at the start of illumination of dark-grown *Euglena* cultures in the concentrations indicated in Fig. 1. Cells were harvested after 3 days; the chloroplast were isolated as described in Materials and Methods

Effects of inhibitors on aminoacyl-tRNA synthetases

We selected the a-tRNA synthetases for the study of the synthetic site of stroma proteins for 4 reasons:

a) Chloroplasts are able to synthesize only 4 or 5 amino acids *via* photosynthesis (Kirk and Tilney-Bassett, 1967). It seems interesting whether these amino acids are attached to tRNA by chloroplast-specific synthetases and, reversely, whether the amino acids synthesized in the cytoplasm enter the chloroplasts as tRNA-bound amino acids or as free compounds which need chloroplast tRNAs for their polymerisation to proteins;
b) a-tRNA synthetases play a bottle-neck role in protein synthesis;
c) High class-specificity between the enzymes and their cognate tRNAs exist. It should facilitate the determination or the localization of the sites of enzyme synthesis;
d) Very little is known in this respect.

Unlike thylakoid proteins these enzymes are easily washed out from chloroplasts during aqueous isolation of the organelles. It was also nearly impossible to prepare the proplastid-like organelles of CAP- or NA-treated cells in sufficient amounts and purity. Therefore, we looked for another

approach which exploits the fact of the high specificity of the enzymes to their cognate tRNAs. If we presume different rates of a-tRNA formation between the reaction of enzymes from green *Euglena* cells with prokaryotic (*E. coli* or *Anacystis*) tRNA and the reaction of enzymes from apoplastic *Euglena* mutants with prokaryotic tRNA, we may ascribe such differences to chloroplast-specific synthetases in the crude enzyme fraction. Similar considerations can be made for the tRNAs.

This assumption was found to be partially correct for prokaryotic enzymes charging eukaryotic tRNAs: Of 14 amino acids tested 8 were accepted in absolute class-specific manner (Table 3). The components for the formation of the other 6 a-tRNAs appear to behave non-specifically. In arg-tRNA formation the heterologous combination with *Anacystis* enzymes is even much more active than the homologous one.

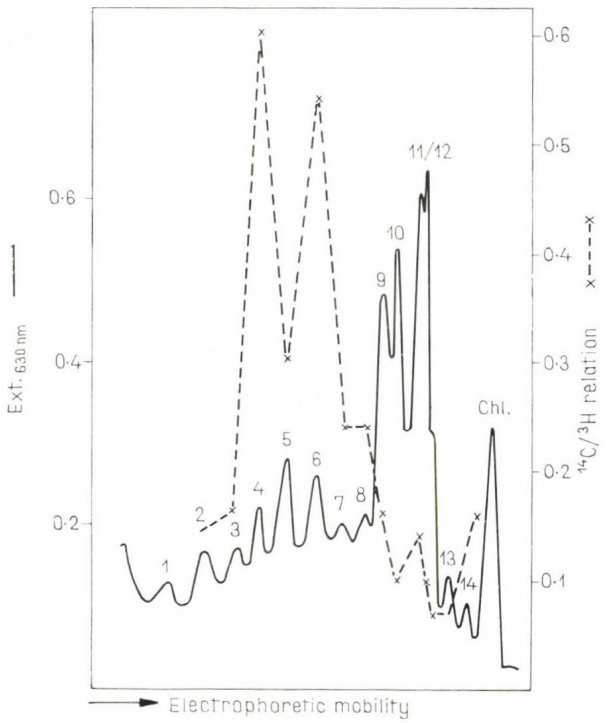

Fig. 3. Polyacrylamide gel electropherogram of chloroplast thylakoid proteins from 8 h light-induced *Euglena* cells. To one culture (5×10^9 cells) 2 mg/ml CAP and 150 μc ^{3}H (4, 5)-leucine (spec. act. 3,000 mc/mmole), to another culture 8 μg/ml CHI and 300 μc ^{14}C-(U)-leucine (spec. act. 80 mc/mmole) were added and incubated under growth conditions for further 8 h. CAP was added 5 h. before, CHI was added simultaneously with the administration of radioactivity. The protein bands were obtained by co-electrophoresis, sliced and determined for radioactivity (Heinze, 1971)

Table 3

Attachment of ^{14}C-labelled amino acids to eukaryotic tRNA from green Euglena cells (G), from apoplastic Euglena mutants (M), and from rat liver cytoplasm (R) by means of prokaryotic aminoacyl-tRNA synthetases (Anacystis nidulans, E. coli). Homologous system: Enzymes and tRNA from Anacystis nidulans and E. coli, respectively

Amino acid	Per cent of the homologous system					
	Enzyme source					
	Anacystis			*E. coli*		
tRNA	G	M	R	G	M	R
ala	—	—	—	27	28	45
arg	900	1300	1200	65	75	50
gly	12	0	0	10	0	0
his	100	0	0	60	0	0
ile	30	0	0	20	0	0
leu	25	0	0	35	0	0
lys	90	60	75	40	10	10
met	50	50		100	90	
phe	60	0	0	15	0	0
pro	60	0	0	15	0	0
ser	30	0	0	10	0	0
thr	—	—	—	75	20	15
tyr	80	0	0	20	0	0
val	65	60	15	40	40	40

The reverse combinations, aminoacylation by eukaryotic enzymes and prokaryotic tRNAs, were far less unambiguous (Table 4). Except glycine- and tyrosine-specific components no strict class-specificity exists. For a number of amino acids, however, the enzymes from green *Euglena* cells show a significantly higher percentage of charging prokaryotic tRNAs than the crude enzymes from *Euglena* mutants. The enzymes specific for these amino acids (arg, gly, ile, leu, phe, val) have been chosen for the study of their sites of synthesis. They are first of all supposed to contain chloroplast-specific components, i.e. enzymes with prokaryotic charging specificity.

At first, we studied the existence of these a-tRNA synthetases in isolated chloroplasts (Table 5). 4 of 6 chloroplast-associated enzymes can charge prokaryotic (*E. coli*) tRNA to the same extent as tRNA from the homologous green cells. This result does not only indicate the penetration of exogenous tRNA into chloroplasts but also that chloroplast enzymes aminoacylate bacterial tRNAs. More surprising, however, is the equally high a-tRNA formation with eukaryotic tRNA from the U.V. mutant of *Euglena*. We can explain these data as the existence of certain synthetases in the organelles which are able to charge cytoplasmic tRNA. Similar findings were reported by Burkard et al. (1970) with *Phaseolus* chloroplasts. Another explanation is that chloroplast enzymes, far better than *E. coli* or *Anacystis*

Table 4

Attachment of ^{14}C-labelled amino acids to prokaryotic tRNA (Anacystis, E. coli) by means of eukaryotic aminoacyl-tRNA synthetases (Euglena: green cells, apoplastic mutants; rat liver cytoplasm). A: Anacystis tRNA; E: E. coli tRNA. Homologous systems: Enzymes and tRNA from the eukaryotic cells

Amino Acid	Per cent of the homologous system					
	Enzyme source					
	Green Euglena		Euglena mutant		Rat liver	
tRNA	A	E	A	E	A	E
ala	12	20	18	18	50	100
arg	20	45	<5	10	<5	0
gly	10	<5	0	0	0	0
his	—	—	—	—	0	0
ile	20	20	<5	15	35	45
leu	25	15	<5	<5	0	0
lys	50	45	35	30	10	25
met	15	<10	<5	0		
phe	15	20	<10	15	0	0
pro	50	25	50	25	0	<5
ser	15	40	<10	30	<5	<5
thr	20	12	55	10	40	40
tyr	0	0	0	0	0	0
val	130	50	15	60	0	<10

enzymes, can charge eukaryotic *Euglena* tRNA. The objection of contamination of the enzyme fraction by cytoplasmic enzymes and of the tRNA fraction by mitochondrial tRNA cannot be rejected but they should cause only minor effects.

The effect of CAP, NA or CHI on the enzyme formation in cells grown in the presence of the drugs are presented in Table 6. The a-tRNA formations catalyzed by the mixed systems are compared with those of the homologous *Euglena* system from non-treated cells.

The enzyme activities from CAP-grown cells, specific for leu, phe and gly, show 40 to 70% inhibition not only in combination with the prokaryotic tRNA but also with the eukaryotic tRNA fractions prepared from green or dark-grown *Euglena* and mutants. The enzymes specific for arg and val, however, charge prokaryotic tRNA to a lower extent than the reference enzymes do (corresponds to the percentage of heterologous charging, see Table 3). Arg- and val-tRNA synthesis with eukaryotic tRNA are not decreased. NA-treatment gives similar results with the exception of gly-tRNA synthetase.

Taken into consideration the specific actions of these antibiotics on the synthesis of macromolecules in chloroplasts, we may conclude from our results that the chloroplast synthetases charging eukaryotic tRNAs with leu, phe and gly may be synthesized by the genetic machinery of the chloro-

Table 5

Aminoacylation of tRNA with aminoacyl-tRNA synthetases from chloroplasts and cytoplasm of mixotroph Euglena gracilis

Results are given as per cent of the homologous system (with tRNA of green cells)

Enzyme source	tRNA source	leu	phe	arg	val	gly	ile
Cytoplasm (100,000×g supernatant)	Prokaryotic (*E.coli*)	25	23	27	32	5	10
Purified chloroplasts (2×sucrose gradient)	Prokaryotic (*E.coli*)	105	150	110	95	31	54
	Eukaryotic (*Euglena, mutant*)	100	95	120	110	100	60

Table 6

Aminoacyl-tRNA formation with enzymes of Euglena cells which have been grown 3 days in light under the influence of antibiotics. Reference = enzymes and tRNA of Euglena grown without inhibitors

Enzyme source	tRNA source	Per cent of reference system				
		leu	phe	arg	val	gly
CAP-grown cells	*Anacystis*	29	45	45	43	66
	Euglena green	30	52	88	87	61
	Euglena bleached	33	48	100	105	64
	Euglena mutant	35	45	97	110	66
NA-grown cells	*Anacystis*	36	48	65	58	49
	Euglena green	39	58	100	94	105
	Euglena bleached	39	59	97	105	100
	Euglena mutant	45	55	100	125	100
CHI-grown cells	*Anacystis*	100	100	100	95	100
	Euglena green	100	120	100	85	—
	Euglena bleached	100	110	100	42	100
	Euglena mutant	100	100	95	33	85

plast, but plastid enzymes specific for val and arg should be synthesized in the cytoplasm. The data from CHI-grown cells support this suggestion at least partially since no inhibition was observed with the enzyme species for leu, phe, arg, and gly even in combination with prokaryotic tRNA, but there is a severe decrease of val-tRNA formation with tRNA from chloroplast-free cells.

We fractionated the crude synthetase preparations by hydroxylapatite chromatography in order to differentiate the organelle-specific enzymes from those of the cytoplasm. The elution pattern of leu-specific enzymes of normal grown *Euglena* cells is shown in Fig. 4. Tested with homologous

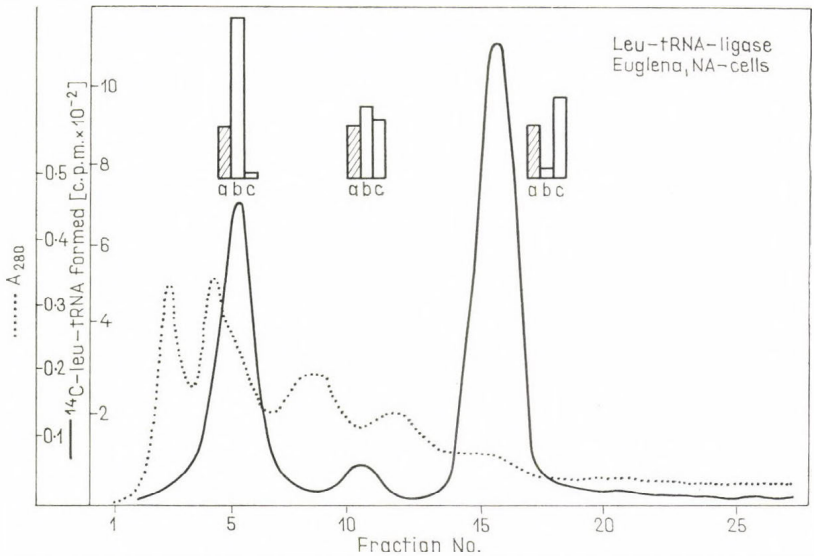

Fig. 4. Hydroxylapatite chromatography of *Euglena* leucyl-tRNA synthetases. Crude soluble enzyme fraction from light (mixotroph) grown cells were chromatographed for 20 h at +4° as described in Material and Methods. The enzyme assay was described previously (Meissner et al., 1971; Parthier, 1971). The tRNA fractions used were prepared from light grown cells (a), from prokaryotic *(E. coli* or *Anacystis)* cells (b) or from U. V. mutants of *Euglena* (c)

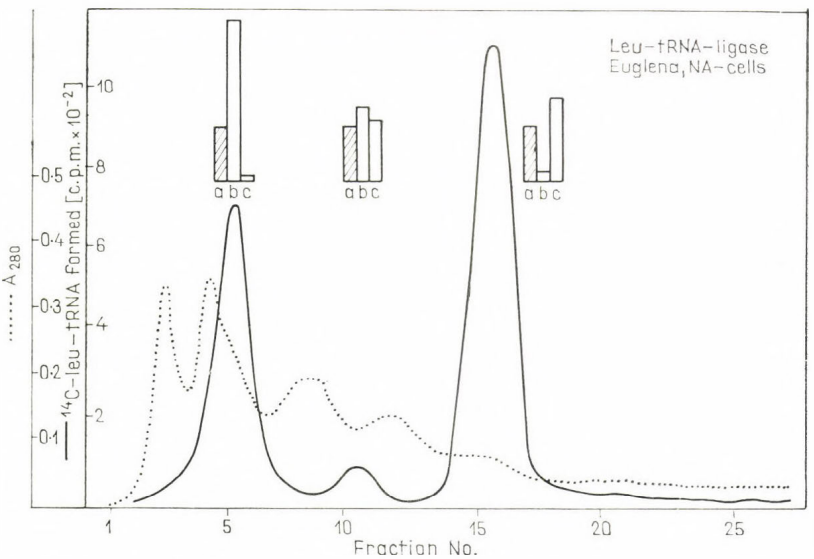

Fig. 5. Hydroxylapatite chromatography of leucyl-tRNA synthetases from *Euglena* cells grown for 3 days in 40 μg/ml NA. Other details as in Fig. 4

tRNA we obtained 3 (sometimes 4) peaks of different activity. The specificity of these fractions to the cognate tRNA was studied by combining the fractions with prokaryotic or eukaryotic tRNA (from apoplastic

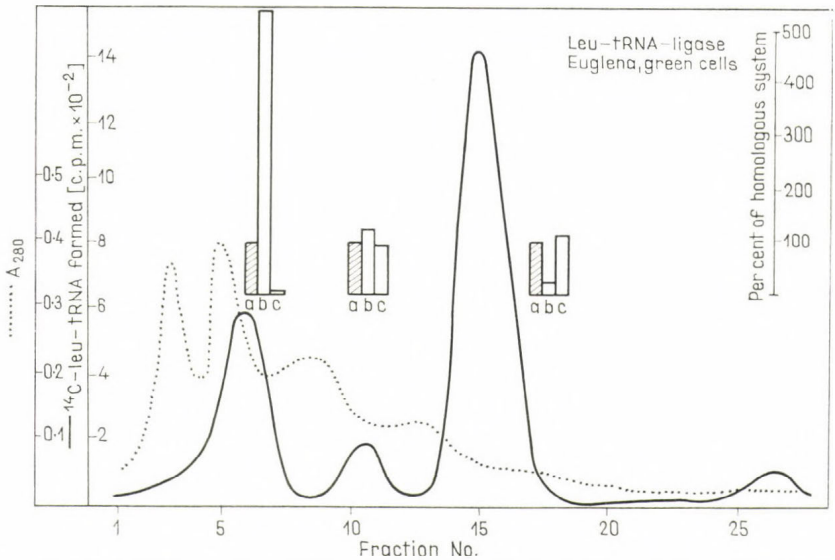

Fig. 6. Hydroxylapatite chromatography of leucyl-tRNA synthetases from *Euglena* U. V. mutants. Other details as in Fig. 4

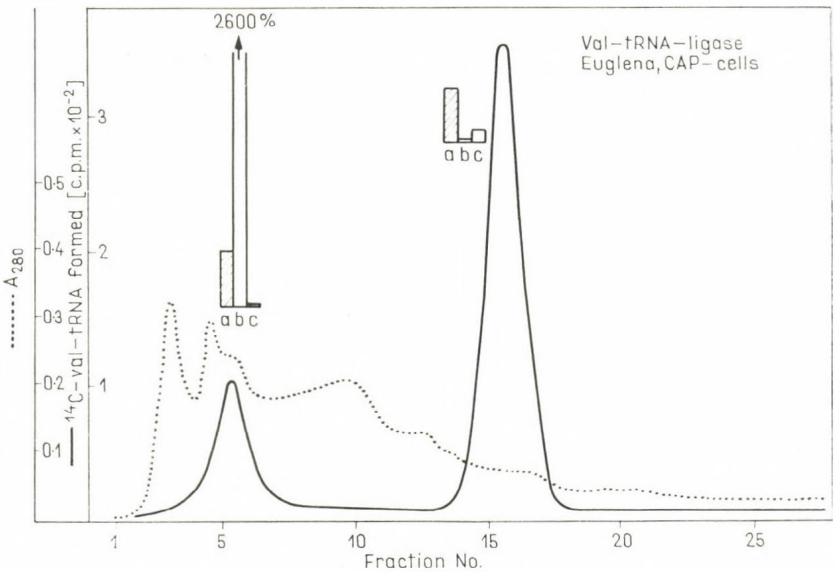

Fig. 7. Hydroxylapatite chromatography of valyl-tRNA synthetases from *Euglena* cells grown for 3 days in 1·5 mg/ml CAP. Other details as in Fig. 4

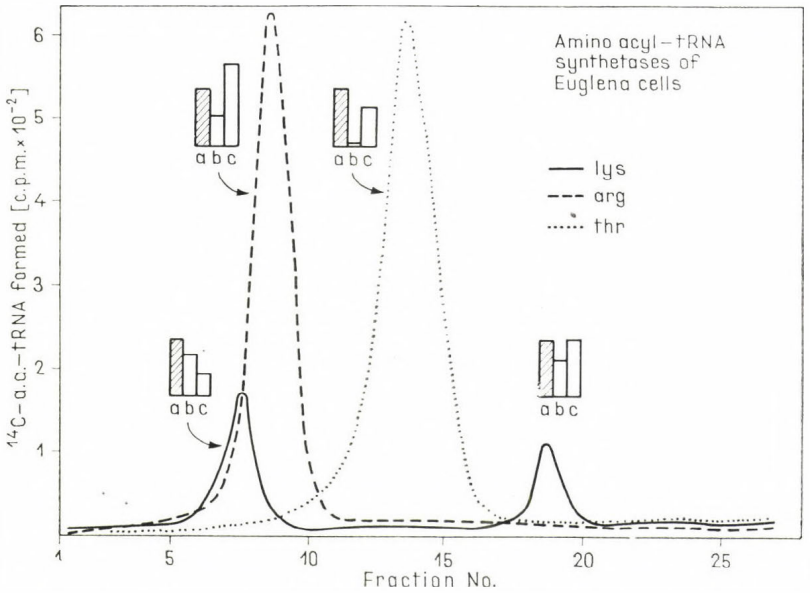

Fig. 8. Hydroxylapatite chromatography of aminoacyl-tRNA synthetases, specific for lysine, arginine and threonine, from light grown (green) *Euglena* cells. Other details as in Fig. 4

mutants). The charging rates are compared with the homologous combination as the reference system (Fig. 4, columns).

By this method we recognize the first peak as a synthetase with high affinity to prokaryotic tRNA, but the enzyme of the third peak only weakly catalyzes leu-tRNA formation with prokaryotic tRNA.

The crude synthetase preparations from cells treated with CAP or NA should, therefore, contain a diminished activity in the first peak for prokaryotic tRNA. This is, however, not the case (Fig. 5). Even the enzyme preparation of the U.V. mutant strain of *Euglena* shows the same chromatographic and charging behaviour (Fig. 6). Likewise, no difference, as compared to the green cells, was obtained for the phe- und val-specific enzymes from CAP-grown cells (Fig. 7), although the specificity of the two peaks to the tRNAs from different sources is nearly absolute. It is interesting that the two peaks with lys-tRNA synthetase activity do not show tRNA specificity (Fig. 8).

We hesitate to draw final conclusions from our experiments at this time. Of the 14 protein bands separated from the thylakoid protein, four fractions do not appear after CAP- or NA-treatment of the *Euglena* cultures (Fig. 2). ^{14}C-leucine incorporation is clearly inhibited in 2 of them after CAP-treatment (Fig. 3). Most of them, however, seem to be formed more or less by joint synthesis on chloroplast and cytoplasmic ribosomes. — Apparently chloroplasts contain a-tRNA synthetases which charge both prokaryotic and eukaryotic tRNAs (Table 5). Our results suggest that at least some chloroplast-associated enzymes are formed on cytoplasmic ribosomes (Table 6).

Finally, another point is worth to be mentioned. *Euglena* cells deficient in or lacking chloroplasts contain always many or large mitochondria. Therefore, we cannot exclude that the heterologous aminoacylation of prokaryotic tRNA by enzymes from the drug-treated cells is due to mitochondrial components. We need more information on the specificity between the components from plastids and mitochondria of *Euglena* cells. This statement is connected with the observation that CAP or NA inhibit the biogenesis of chloroplasts but not of mitochondria.

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ISOLATION AND PARTIAL CHARACTERIZATION OF CHLOROPLAST AND CYTOPLASMIC RIBOSOMES AND RIBOSOMAL SUBUNITS FROM WHEAT

by

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Ribosome preparations from 4.5 day wheat *(Triticum vulgare)* seedlings contain monoribosomes of 80S and 69S along with dimers (117S) and trimers (153S) of the 80S species. The 80S (cytoplasmic) and 69S (chloroplast) ribosomes have been separated into purified fractions by sucrose gradient centrifugation in a zonal rotor. When the purified 80S and 69S ribosomes were centrifuged through sucrose gradients containing high KCl-to-$MgCl_2$ they dissociated into subunits of 61S and 42S (80S) and 49S and 31S (69S). The large and small subunits of both cytoplasmic and chloroplast ribosomes were separated from each other and purified by zonal centrifugation. Upon mixing the purified subunits in the presence of $MgCl_2$ they reassociated to yield the parent species.

INTRODUCTION

Leaves of higher plants contain two classes of ribosomes characterized by sedimentation coefficients of 80S and 70S (Lyttleton, 1962; Clark et al. 1964; Boardman et al., 1968; Stutz and Noll, 1967; Odintsova et al., 1967; Arglebe and Hall, 1968; Gualerzi and Cammarano, 1969; Attardi and Amaldi, 1970). These are believed to exist in the cytoplasm and the chloroplasts respectively. The 80S plant ribosomes resemble cytoplasmic animal ribosomes chemically as well as physically (Marcus and Feely, 1965; Boulter, 1970; Allende, 1969; Spencer and Wildman, 1964; Allende and Bravo, 1966) while the 70S chloroplast ribosomes are similar to those of bacteria (Lyttleton, 1962; Boardman et al., 1968; Boulter, 1970; Spencer and Wildman, 1964; Ellis, 1969; Sissakian et al., 1965).

Both cytoplasmic and chloroplast ribosomes have been found in wheat (Bamji and Jagendorf, 1965; Mehta et al., 1968). While cytoplasmic ribosomes isolated from wheat germ have been extensively characterized physically (Allende and Bravo, 1966; Wolfe et al., 1968; Wolfe and Kay, 1967) and those from leaves (both cytoplasmic and chloroplast) have been investigated (Mehta et al., 1968; Hadziyev and Zalik, 1970; Marcus and Feely, 1966), relatively little is known about either the subunit structure or the protein composition of wheat leaf ribosomes. Chloroplast ribosomes of wheat (Hadziyev and Zalik, 1970) and other plants (Boardman et al., 1968; Attardi and Amaldi, 1970) have been reported to incorporate amino acids into protein at a rate 10—20 times faster than cytoplasmic ribosomes from the same plants.

This paper reports the first part of an investigation into the similarities and differences between cytoplasmic and chloroplast wheat ribosomes. Such knowledge should add to an understanding of why the two wheat ribosome species incorporate amino acids into protein at such diverse rates.

MATERIALS AND METHODS

Tricine [N-tris (hydroxymethyl) methylglycine] was from Calbiochem, Triton X-100 was from the Hartman-Leddon Company, Philadelphia, Pa., bentonite (U.S.P.) was from Fisher Scientific Company and all other chemicals were reagent grade.

Isolation of total ribosomes from wheat leaves

Seedlings of wheat (*Triticum vulgare* Vill. cv. *Manitou*) were grown in sterilized soil (3 : 2 : 1 mixture of soil, peat and sand) in a growth chamber at 21°, 55% relative humidity and continuous illumination of 1500 ft-c. The leaves were harvested 4·5 days after planting. All isolation steps were carried out at 4°. The plants were allowed to stand 15 min in the cold and were then cut and immediately shredded in a stainless steel vegetable juicer (Acme Superior Juicerator, Acme Juicer Mfg. Co., Sierra Madre, California). The expressed juice was collected in an equal volume of buffer I (0·7 M sucrose, 100 mM tricine, adjusted to pH 7·5 with KOH, 5 mM $MgCl_2$, 50 mM KCl, and 5 mM 2-mercaptoethanol) containing 8% (v/v) Triton X-100 and 1·2 mg/ml bentonite. Tricine buffer was used after preliminary experiments with *E. coli* showed it gave more satisfactory results than tris. The resulting dark green suspension was stirred for 10 min and centrifuged at 25,000 ×g for 10 min to remove debris. The supernatant was then centrifuged at 340,000 ×g for 1 h to pellet the ribosomes.

The ribosomes were suspended in 25 ml of buffer II (10 mM tricine, pH 7·5, 5 mM $MgCl_2$, 50 mM KCl, 5 mM 2-mercaptoethanol) containing 2% (v/v) Triton X-100. When Triton X-100 was not included in this step the final preparation was slightly green. After stirring 10 min the suspension was centrifuged at 25,000 ×g for 10 min. The resulting clear, green solution was carefully layered over 25 ml of buffer II containing 1 M sucrose. After centrifugation at 340,000 ×g for 2 h, the resulting pellets were suspended in 10 ml of buffer III (4 mM $MgCl_2$, 1 mM tricine, pH 7·5). An aliquot of the preparation was then diluted and the absorbance at 280 nm, 260 nm and 235 nm was measured to ascertain the purity of the preparation. The 260/280 absorption ratio was between 1·9 and 2·0 with a 235/260 ratio of 1·68 to 1·78. This method of extraction normally yielded around 2,800 A_{260} units of ribosomes from 400 gm of wheat leaves.

Separation of total ribosome preparation into 69S and 80S fractions

The method of Anderson and Rutenberg (1967) was used to make a 7% (w/v) to 30% (w/v) convex sucrose gradient and the separation was carried out in a Ti-14 zonal rotor (Beckman Instruments). The convexity of the gradient inside the rotor was confirmed using dye indicators. A 125 ml mixing chamber was filled with 7% (w/v) sucrose in 10 mM tricine buffer (pH 7·5) containing 10 mM $MgCl_2$ and 4 mM 2-mercaptoethanol. A reservoir of 550 ml of 30% (w/v) sucrose in the same buffer was connected so as to siphon into the mixing chamber as the mixed solution was pumped into

the rotor. After the 550 ml gradient was in, the rotor was filled with a cushion of 35% (w/v) sucrose in buffer. Ten ml of total ribosome preparation containing up to 1,800 A_{260} units of material in 3% (w/v) sucrose was layered over the gradient, followed by 50 ml of buffer overlay. After centrifugation at 47,000 rpm for 2·5 h at 25°, the gradient was pushed out of the rotor with 40% (w/v) sucrose, through a 1 cm flow cell and the absorption was monitored at 280 nm or 290 nm. The eluate corresponding to the peaks was collected and the ribosomes pelleted by centrifugation at 340,000 ×g for 2 h at 4°.

Determination of sedimentation constants by ultracentrifugation

For direct determination of ribosome and subunit sedimentation constants, sedimentation runs were carried out on a Spinco model E analytical ultracentrifuge, with schlieren optics, at 20° and 39·460 rpm.

Analysis of ribosome and ribosomal subunit sedimentation coefficients on linear sucrose gradients

For determination of the sedimentation coefficients of various ribosome and subunit fractions linear gradients of 5% (w/v) to 20% (w/v) sucrose in appropriate buffer were poured into 1·59 ×10·16 cm nitrocellulose centrifuge tubes. For whole ribosomes buffer III was used, while for subunit analysis various buffers were necessary depending upon the species under investigation. Aliquots of 100 µl containing about 2 A_{260} units of material were carefully layered on the gradients and centrifuged at 26,000 rpm (126,000 ×g), 25°, for 4·5 h in a Beckman SW-27 rotor. After centrifugation the tubes were emptied from the bottom at a constant rate and the contents monitored at 260 nm.

The sedimentation coefficients of unknown species were determined by the method of Martin and Ames (1961), using as standards the sedi-

Table 1

Sedimentation coefficients of weat leaf ribosomes and ribosomal subunits
Sedimentation runs were made at 20 °C, at 39,640 rpm, in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics

Ribosome or subunit	S_{20}	Number of determinations
Cytoplasmic dimer (117S)	116·7±1·0*	19
Cytoplasmic monomer (80S)	79·8±0·7	31
Cytoplasmic large subunit (61S)	60·8±0·6	25
Cytoplasmic small subunit A (49S)	48·9±0·4	27
Cytoplasmic small subunit B (42S)	41·8±0·7	7
Chloroplast monomer (69S)	69·0±0·7	7

* Standard error of mean.

mentation coefficients of wheat ribosomes and ribosomal subunits as determined by analtytical ultracentrifugation (Table 1). A plot of S_{20} vs mobility of known species on the gradient gave a straight line up to S_{20}

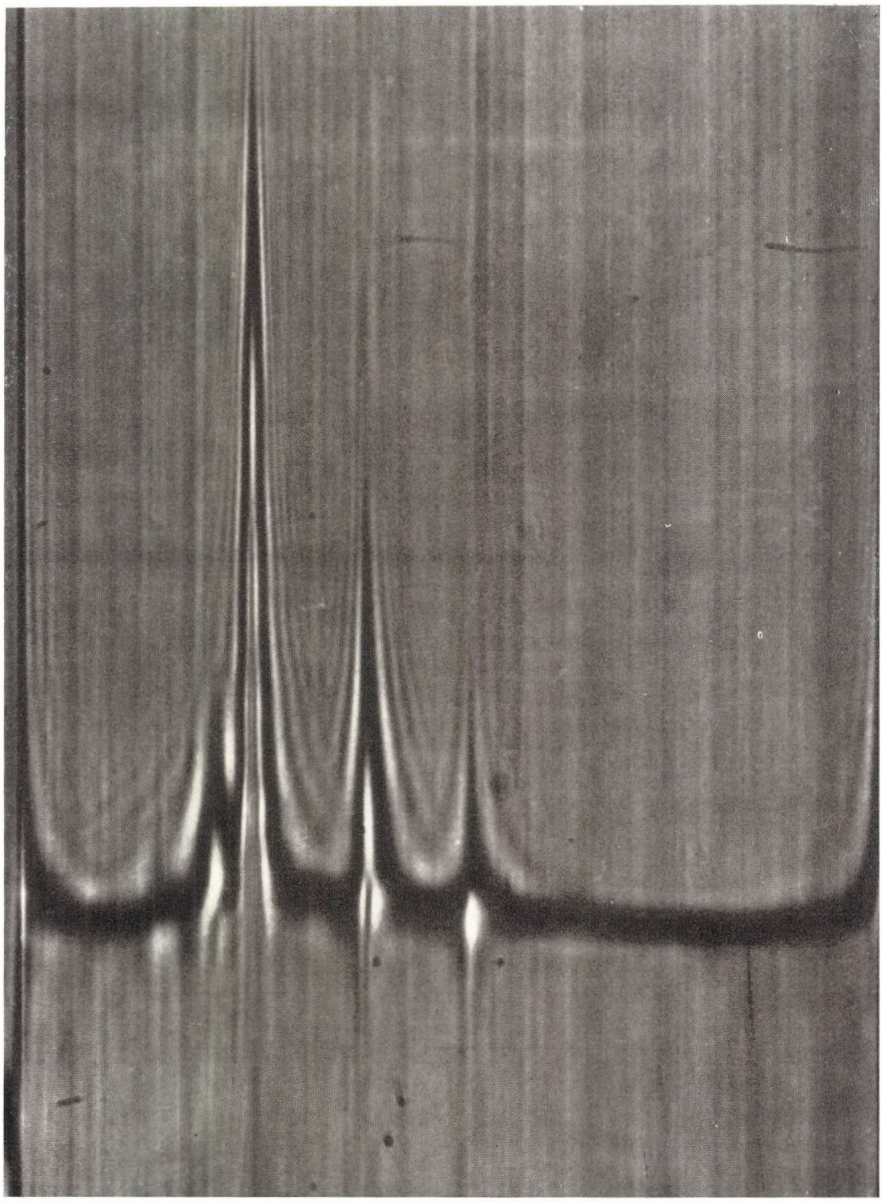

Fig. 1. Analytical ultracentrifugation pattern of total ribosome preparation from leaves of 4·5 day Manitou wheat seedlings. Ribosomes were in mM tricine (pH 7·5) and 4 mM $MgCl_2$. Picture taken 4 min after reaching speed of 39,460 rpm. Temperature 20 °C. Direction of centrifugation from left to right

of 70S but fell off somewhat above this point. When assayed by this method *E. coli* ribosomes and subunits (a gift from Dr. T. Tamaoki) showed values of 70S, 50S and 30S.

RESULTS AND DISCUSSION

Ribosome species isolated from wheat leaves

When total ribosomes were isolated from wheat leaves and examined in the analytical ultracentrifuge four major species were normally detected (Fig. 1). These displayed sedimentation coefficients of 69S, 80S, 117S, and 153S. S values were measured at 20° and were not corrected for buffer viscosity. The amounts of 117S and 153S material varied in different preparations and they probably represented dimers and trimers of the 80S cytoplasmic ribosome monomers. The 69S peak, assumed to be of chloroplast origin, was normally about one tenth as large as the 80S peak.

Separation of the 69S and 80S ribosomes

Since the object of this research was to compare the cytoplasmic and chloroplast ribosomes of wheat seedlings it was imperative that pure samples of the 69S and 80S ribosomes be obtained. Pure chloroplast ribosomes have been obtained from several plant sources by purifying chloroplasts and subsequently extracting the ribosomes from them. While we were able to obtain relatively pure preparations of the 70S ribosomes from spinach

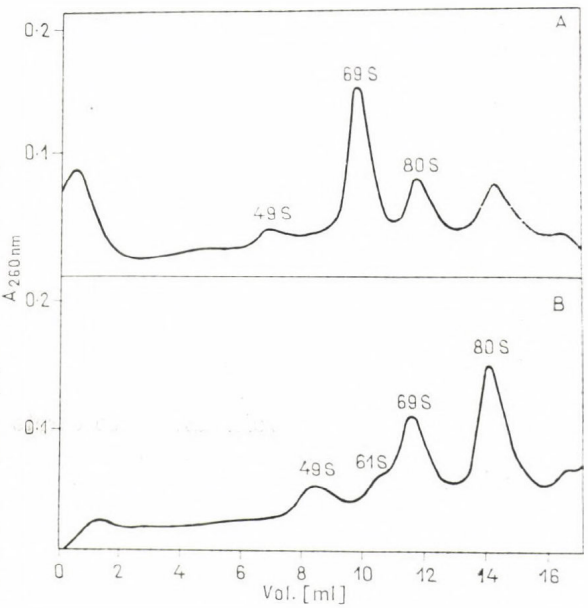

Fig. 2. Sucrose gradient analysis of ribosomes extracted from isolated chloroplasts of bean (A) and wheat (B). Chloroplasts were isolated and their ribosomes extracted by the method of Stutz and Noll (1967). Extracted ribosomes (1 A_{260} unit in 100 μl) were layered over a linear 5% (w/v) to 20% (w/v) sucrose gradient in 1 mM tricine buffer (pH 7·5) containing 4 mM $MgCl_2$. Centrifugation was for 3·5 h (A) or 4·25 h (B), at 25 °C, 26,000 rpm (126,000 ×g), in a Beckman SW-27 rotor (small buckets). After centrifugation the tubes were emptied at a constant rate and monitored with a 1 cm flow cell at 260 nm

and bean plants utilizing the method of Stutz and Noll (1967), the procedure did not give satisfactory results when applied to wheat leaves (Fig. 2). Not only was the ribosome yield from wheat chloroplasts very low but the preparation contained more 80S than 69S ribosomes.

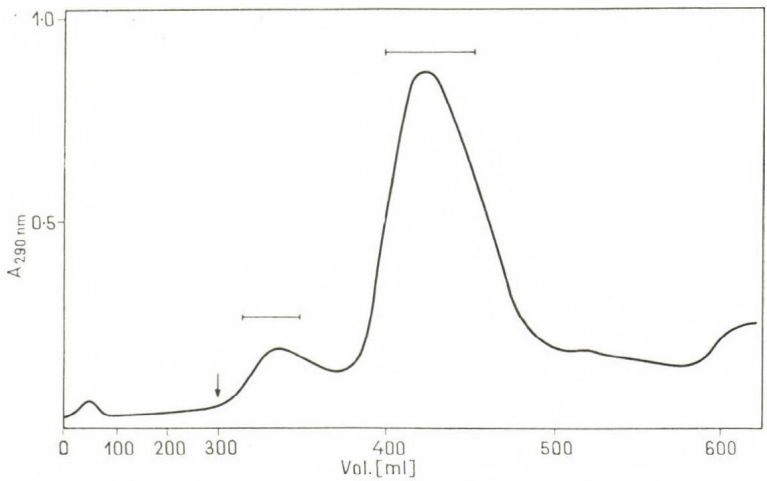

Fig. 3. Zonal sucrose gradient separation of the 69S and 80S wheat leaf ribosomes. Ten ml of ribosome preparation (105 A_{260} units/ml) in 3% (w/v) sucrose and buffer III were applied to a 550 ml convex 7% (w/v) to 30% (w/v) sucrose gradient in 10 mM tricine-10 mM $MgCl_2$ buffer (pH 7·5) over a cushion of 35% (w/v) sucrose in the same buffer. After application of a 50 ml buffer overlay, the gradient was centrifuged 2·5 h at 25 °C and 47,000 rpm. The gradient was then pushed from the rotor with 40% (w/v) sucrose, monitored at 290 nm, and the denoted fractions were collected. To accomodate the results into a single figure a change in scale was employed in the graph at the point indicated by an arrow

As it was impractical to obtain sufficient pure 69S ribosomes by extracting purified chloroplasts, 69S and 80S ribosomes from whole leaf material were extracted together and the resulting mixture was separated into chloroplast and cytoplasmic ribosome fractions by centrifugation through a sucrose gradient in a zonal rotor. Ribosomes were extracted from 200 gm wheat leaves and layered over a 550 ml convex 7% (w/v) to 30% (w/v) sucrose gradient in a Ti-14 zonal rotor. After centrifugation (2·5 h, 47,000 rpm) the preparation had separated into two distinct peaks (Fig. 2). While the zonal centrifugation step affected a good separation of the two species, it also led to the loss of some ribosomes. Gradient separations containing large amounts of material were routinely scanned at 290 nm instead of 260 nm to avoid having to dilute the samples for reading. In all cases the 290/260 ratio was 0·28.

The indicated fractions of the two peaks shown in Fig. 3 were pooled, pelleted by centrifugation, and analyzed by centrifugation on linear 5% (w/v) to 20% (w/v) sucrose gradients (Fig. 4). The more slowly sedimenting peak from the zonal separation contained exclusively material of 69S while the major peak contained pure 80S ribosomes. These purified 69S and

80S preparations both exhibited 260/280 absorbance ratios of 2·00 and 260/235 ratios of 1·74 (averages of 11 preparations). The purified 80S ribosomes isolated by this method were capable of incorporating ^{14}C phenylalanine into protein in the presence of Poly-U.

Cytoplasmic ribosomes were also obtained free of 69S chloroplast ribosome contamination when the isolation procedure was carried out with 80S dissociation buffer substituted for buffers I and II. When the resulting preparation was dissolved in buffer III and analyzed (Fig. 5), no evidence of 69S contamination was observed. Although ribosomes isolated in this way did not appear to contain chloroplast ribosomes and dissociated in the manner of 80S cytoplasmic ribosomes in the presence of 80S dissociation buffer, all the experiments being reported concerning dissociation of ribosomes and the isolation of subunits were conducted with zonal-purified ribosomes.

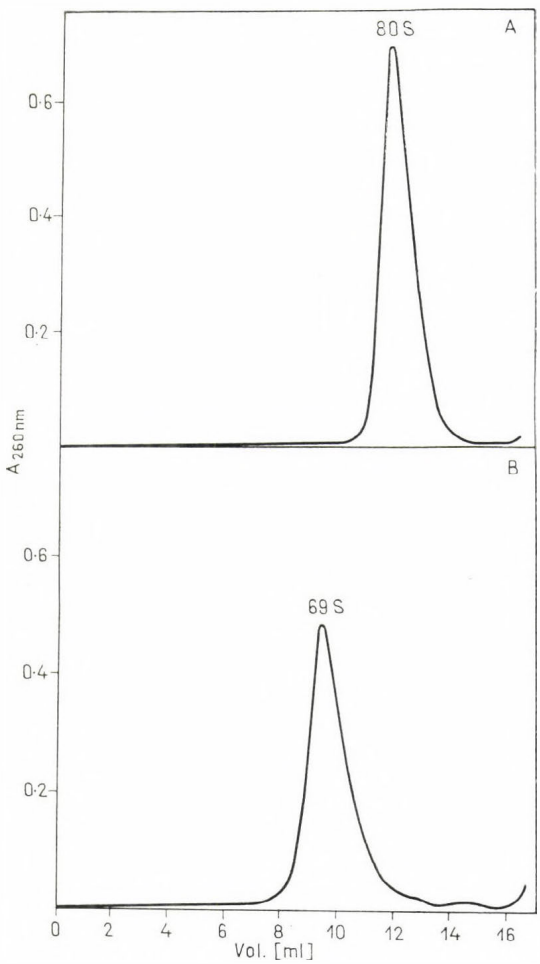

Fig. 4. Sucrose gradient analysis of 80S and 69S wheat ribosomes purified by zonal centrifugation. The ribosomes from each peak fraction of Fig. 3 were pelleted, suspended, and applied to a 5% (w/v) to 20% (w/v) linear sucrose gradient for analysis by the method of Fig. 2. A-material from the large peak of Fig. 3. B-material from the smaller peak of Fig. 3

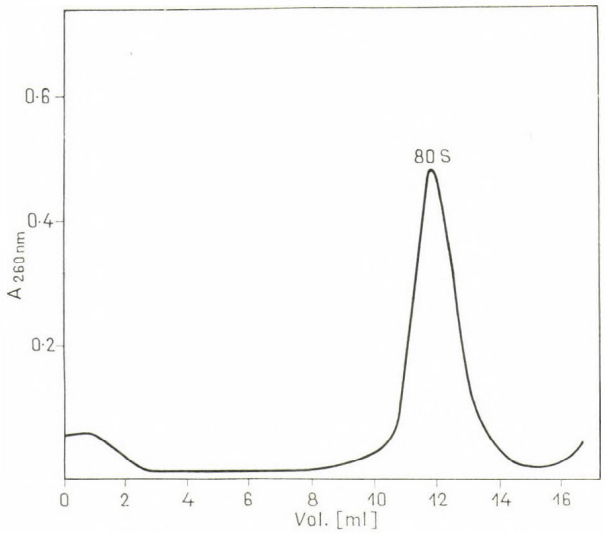

Fig. 5. Ribosomes isolated from wheat leaves using 80S dissociation buffer. Ribosomes were isolated from wheat leaves in the usual manner except that 80S dissociation buffer was used in place of buffers I and II. After isolation the ribosomes were suspended in buffer III. Two A_{260} units of the preparation were layered over a linear 5% (w/v) to 20% (w/v) sucrose gradient, centrifuged at 26,000 rpm for 3·5 h at 25 °C and analyzed as in Fig. 2

Dissociation of 69S and 80S ribosomes

Zonal-purified 69S and 80S ribosomes were dissociated into subunits in the presence of low-$MgCl_2$, high-KCl buffers. When 80S ribosomes were sedimented through a 5% (w/v) to 20% (w/v) sucrose gradient in 10 mM tricine, pH 7·5, 1 mM $MgCl_2$, 100 mM KCl, and 5 mM 2-mercaptoethanol (80S dissociation buffer) they dissociated into a mixture of 42S, 49S, 61S, and 80S species (Fig. 6A). Undissociated 80S monomers isolated from this dissociation buffer and subjected to a second treatment with the same buffer did not break down further, indicating they were probably pieces of broken polysomes, containing bound messenger RNA and thus resistant to dissociation. The relative amounts of 42S and 49S subunits in different preparations were variable, while the ratio of 61S subunit concentration to the sum of the concentrations of 42S and 49S subunits was approximately constant, suggesting that the 80S ribosome may break into a large 61S subunit and a small subunit which can appear as either a 42S or a 49S species. Evidence that the 42S and 49S peaks may be manifestations of a single component is provided later.

Chloroplast (69S) ribosomes dissociated into 31S and 49S subunits (Fig. 6B) upon sedimentation through 20 mM tricine buffer (pH 7·5), containing 400 mM KCl and 10 mM $MgCl_2$ (69S dissociation buffer). There was no evidence for two forms of the smaller subunit in chloroplast ribosomes.

Determination of S values by analytical ultracentrifugation

The sedimentation coefficients of wheat ribosomes and subunits are given in Table 1. These were used as standards in determining sedimentation coefficients by the methods of Martin and Ames (1961).

Purification of ribosomal subunits

Isolation of the small and large subunits of both 80S and 69S zonal-purified ribosomes was achieved by utilization of convex 7% (w/v) to 30% (w/v) sucrose gradients in 80S and 69S dissociation buffers in a Ti-14 zonal rotor (Fig. 7). It was impossible to separate the 42S and 49S species present in dissociated 80S ribosomes and since the two seemed very similar they were collected together. When the fractions containing the 42S and 49S subunits were mixed and the material pelleted by centrifugation and then resuspended and analyzed by centrifugation through a linear 5% (w/v) to 30% (w/v) sucrose gradient in either *a)* 80S dissociating buffer or *b)* 10 mM tricine buffer, pH 7·5, the results shown in Fig. 8 were obtained. These show that while two species were apparent in 80S dissociation buffer there was only one peak discernable when the same preparation was centrifuged through the buffer devoid of metal chlorides and mercaptoethanol. When ribosomal subunit proteins were extracted from the two halves of the twinned peak of Fig. 7A with 66% acetic acid (method of Waller and Harris, 1961, as modified by Hardy et al. 1969) and analyzed by polyacrylamide gel electrophoresis (Reisfeld et al., 1962) at pH 4·5 42S and 49S

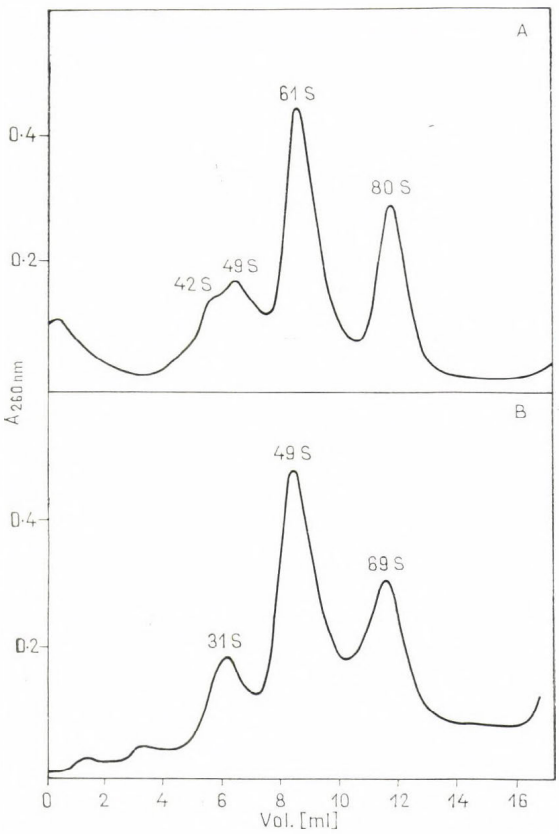

Fig. 6. Dissociation of 80S (A) and 69S (B) ribosomes by low-$MgCl_2$, high-KCl buffers. Purified 80S or 69S ribosomes were layered over 5% (w/v) to 20% (w/v) linear sucrose gradients in dissociating buffers, centrifuged and assayed as in Fig. 2, except the 80S gradient was centrifuged for only 3·5 h. The composition of the dissociating buffers used was: for 80S, 10 mM tricine, pH 7·5, 1 mM $MgCl_2$, 100 mM KCl, and 5 mM 2-mercaptoethanol: for 69S, 20 mM tricine, pH 7·5, 400 mM KCl, and 10 mM $MgCl_2$

species contained virtually identical proteins. The RNA of the 42S, 49S and 60S peaks was examined after extraction by a variation of the phenol method of Peacock and Dingman (1967) or without deproteinization after addition of sodium lauryl sulfate. Electrophoresis of these preparations on composite 0·5% agarose, 2·75% acrylamide gel (Peacock and Dingman, 1967) indicated that the RNA from the 42S and 49S subunits was similar, but they were different from the 60S subunit RNA. Since the 49S subunit contained proteins and RNA similar to the 42S one and had the same S value in the absence of $MgCl_2$, it is possible that the two subunits possess identical protein and RNA components and differ only in their secondary or tertiary structure. This would be similar to the results reported by Ceccarini (1970) for the slime mold *Dictyostelium purpureum*.

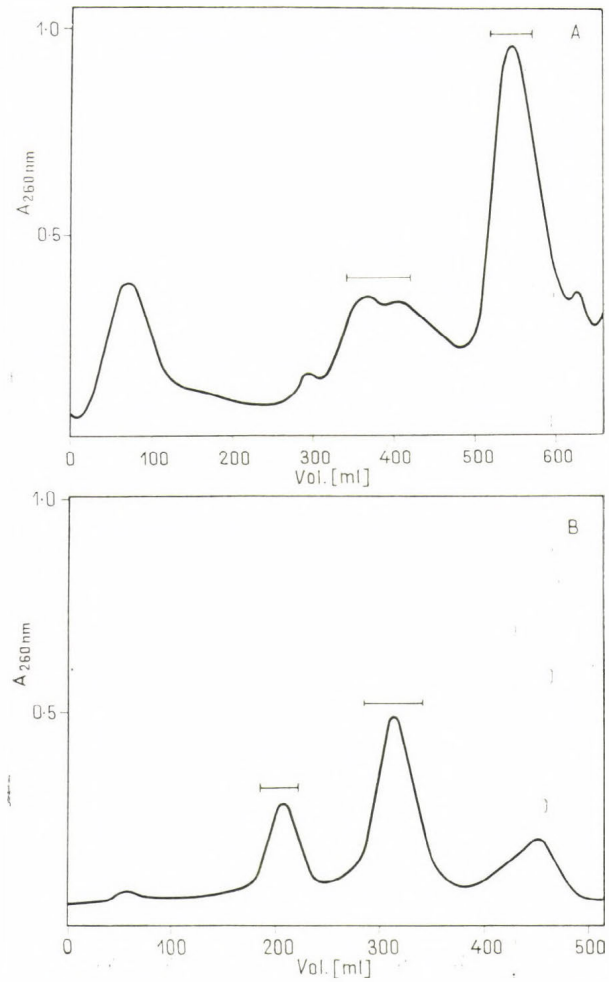

Fig. 7. Separation of dissociated 80S (A) and 69S (B) ribosomes into subunit fractions by zonal sucrose gradient centrifugation. A. Zonal-purified 80S cytoplasmic ribosomes (358 A_{260} units in 10 ml of 80S dissociation buffer containing 3% (w/v) sucrose) were layered over a convex 7% (w/v) sucrose gradient in the same buffer, with a 50 ml overlay of buffer. After a 3·5 h centrifugation at 47,000 rpm, 25 °C, the gradient was pushed from the rotor with 40% (w/v) sucrose solution, scanned at 260 nm, and the indicated fractions were collected for analysis of the peaks on linear gradients. B. Zonal-purified 69S ch oroplast ribosomes (160 A_{260} units) were treated as in A except that 69S dissociation buffer was used and centrifugation was for 2·75 h

Sucrose gradient analysis of subunits

After concentration of the subunit fractions from Fig. 7. by centrifugation, each fraction was analyzed for purity by centrifugation through a 5% (w/v) to 20% (w/v) linear sucrose gradient in the buffer utilized to dissociate the parent ribosomes. The subunits could not be analyzed in standard buffer III because, with the exception of the 49S subunit of the 69S ribosome, they aggregated and precipitated in the presence of 4 mM $MgCl_2$ and 1 mM tricine. The large subunits of both chloroplast and cytoplasmic ribosomes were stable and were easily isolated in the pure state (Figs 9A and D). The small subunits from each species, however, seemed less stable. As is seen from Figs 8A, 8B and 9B there was some 63S material present in the purified cytoplasmic ribosome small subunit 42S preparations. This 63S material was more prevalent when the subunits were in the presence of $MgCl_2$ (Fig. 8A) than in its absence (Figs 8B, 9B). A small amount of material with the same sedimentation constant was also seen when 42S and 61S

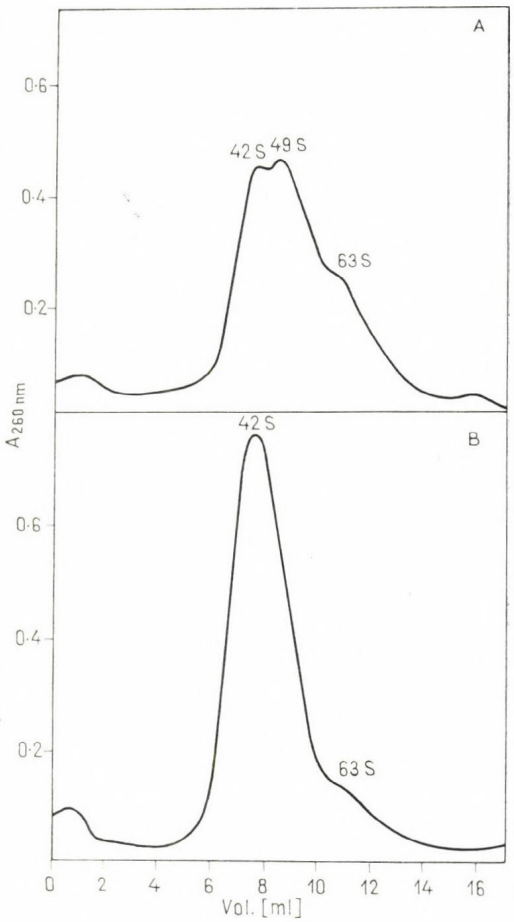

Fig. 8. Effect of removal of $MgCl_2$, KCl, and 2-mercaptoethanol on the smaller subunits of 80S ribosomes. Aliquots of 100 ml, containing 2 A_{260} units of material obtained from the small twinned peak of Fig. 7A, were layered over 5% (w/v) to 20% (w/v) linear sucrose gradients centrifuged and analyzed as in Fig. 2. The buffers used were: A — 80S dissociation buffer and B — 10 mM tricine, pH 7·5

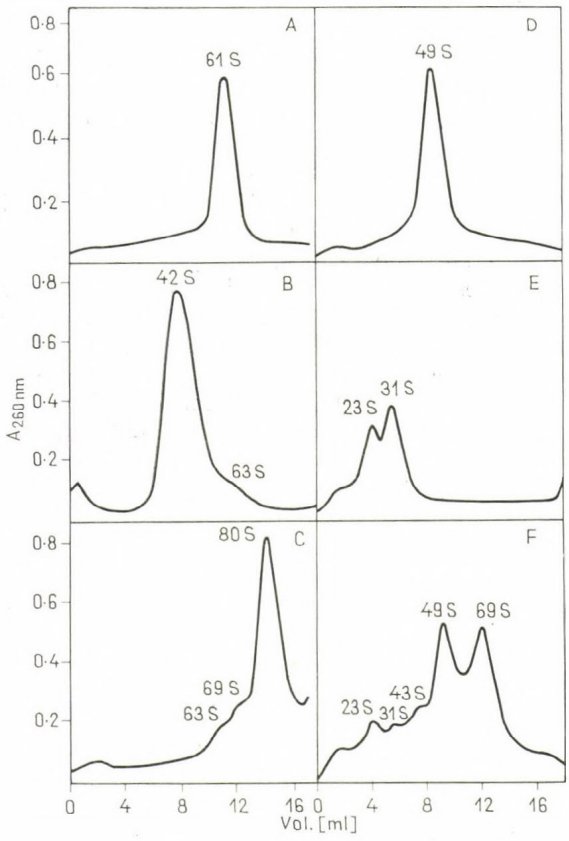

Fig. 9. Sucrose gradient analysis of zonal-purified 80S (Fig. 7A) and 69S (Fig. 7B) ribosome subunits and the products of recombination experiments. Approximately 2 A_{260} units of ribosome subunits or mixtures in 100 μl were layered over linear 5% (w/v) to 20% (w/v) sucrose gradients and centrifuged for 4·25 h at 26,000 rpm (126,000 × g) and 25 °C in a SW-27 rotor. The tube contents were assayed as in Fig. 2. Samples applied and buffers used were: A — large subunit from 80S ribosomes in 80S dissociation buffer, B — small 80S ribosome subunits in 10 mM tricine, pH 7.5, C — mixture of equal amounts of small and large 80S ribosome subunits in buffer III, D and E — large and small subunits respectively of 69S ribosomes in 69S dissociation buffer, and F — mixture of large and small 69S ribosome subunits in buffer III

subunits were mixed and assayed for reconstitution (Fig. 9C). The fact that this component had a slightly higher S value than the large subunit of cytoplasmic ribosomes and that it tended to disappear in the absence of $MgCl_2$, while the 61S subunit was stable in the absence of $MgCl_2$, indicated that it was not due to contamination of the smaller subunit preparation by large subunits. That the 61S and 63S particles were different was also indicated by the observation that the 63S material was still present in the reconstitution experiment where sucrose gradient analysis was conducted in buffer III, containing 4 mM $MgCl_2$. Under these conditions the 61S large subunit precipitated. Since the 63S peak appeared in preparations which contained only small subunits of the cytoplasmic ribosomes it seems possible that it represented dimers of the 42S subunit. This would also fit with the observation that in the presence of 4 mM $MgCl_2$ the 42S subunit apparently formed large polymers and precipitated. Figure 9E shows that the small subunit of the chloroplast ribosomes was not stable in the presence of the high-KCl buffer used to dissociate the ribosomes. While the major peak corresponded to the 31S small subunit found in the dissociation analysis (Fig. 6B) there was a relatively large amount of 23S contaminating

material present. It seems probable that this was some breakdown product which formed during the period when the subunits were being concentrated and prepared for analysis.

Reassociation of subunits

Small and large subunits from cytoplasmic ribosomes were mixed together, incubated 10 min at 25° and analyzed for reassociation in buffer III. Chloroplast subunits were studied in a like manner. In each case 1 A_{260} unit of each subunit was added. When 42S and 61S subunits were mixed they readily combined (Fig. 9C). Over 90% of the observed material appeared as an 80S peak. Small amounts of 63S and 69S material were also present after incubation. The 63S peak which has already been discussed may represent dimerized 42S subunits. The 69S peak may reflect an intermediate in 80S formation. On the other hand it could be either a dimer of the 61S subunit (though no evidence for dimerization of the purified 61S subunits was ever seen) or it could even reflect the formation of a 42S subunit trimer. It seems unlikely that this 69S peak was due to the formation of chloroplast monomers from chloroplast ribosome subunit contamination because 80S monomers utilized for the formation of the subunits were first purified by zonal gradient centrifugation and the subunits were also purified by the same method. Neither the monomers nor subunits showed any contamination by chloroplast ribonucleoproteins. Although the 42S subunit was added in excess it did not show in the analysis because of its tendency to precipitate in the analysis buffer.

The results obtained with chloroplast subunits were not as clear as those from the cytoplasmic system. It is seen from Fig. 9F that mixing of 49S and 31S subunits from chloroplast ribosomes led to formation of a large amount of 69S monomer. However, the pattern was complicated by the presence of 23S, 31S, 43S and 49S peaks. The 23S peak was probably due to the contaminating material seen in the 31S subunit preparation. The 31S and 49S peaks probably represented subunits which had not reassociated. These 31S subunits may have been altered since they did not reassociate and because zonal-purified normal 31S subunits precipitated in buffer III gradients. The identity of the 43S component is unknown, though it could possibly have arisen from dimerization of the 31S subunit. Gualerzi and Cammarano (1969) have reported an identical situation with spinach where they found that 30S subunits of chloroplast ribosomes formed dimers of 43S.

This study has shown that the two species of wheat ribosomes could be dissociated into their respective subunits. Upon mixing under suitable conditions, these reassociated in the classical manner to yield the original monoribosome species. Together with other inherent differences between wheat chloroplast and cytoplasmic ribosomes their different dissociation and reassociation requirements suggest that the diverse rates of *in vitro* amino acid incorporation reported for them (Hadziyev and Zalik, 1970) could in part be a reflection of the media used in those studies.

The methods described in this paper have made it possible to isolate wheat ribosomes and subunits in sufficient quantities for detailed physical, chemical and biochemical characterization.

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RIBOSOME SPECIFICITY OF PROTEIN SYNTHESIS IN VITRO

by

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It is now well established that the ribosomes of all organisms may be divided in two classes. One class encompasses the ribosomes present in the cytoplasm of eukaryotic organisms (ribosomes of the 80S type) while the other class is more heterogeneous and includes the ribosomes from prokaryotic organisms, from mitochondria and chloroplasts (ribosomes of the 70S type) (Wittmann, 1970). The sedimentation coefficient *per se* does not appear to provide always a clear-cut criterion for assigning ribosomes to one of the two classes. More reliable information may be obtained by determining the sedimentation coefficient of ribosomal subunits or the size and structure of ribosomal RNA's and ribosomal proteins. Reliable indications may also be obtained by assaying the functionality of ribosomes for protein synthesis *in vitro* (Ciferri and Parisi, 1970). Indeed it has been shown that ribosomes of two types respond to specific transfer factors (Perani et al., 1971; Richter and Lipmann, 1970) Further within each group of ribosomes, transfer factors are freely exchangeable. On the contrary, exchanges between the two groups of ribosome-specific transfer factors do not result in active protein synthesizing systems (Table 1). Such results were first obtained in the case of the reactions for peptide chain elongation as assayed by the polyphenylalanine-synthesizing system but now they have been extended also to the reactions for peptide chain initiation (Sala and Küntzel, 1970; Sala et al., 1970). As summarized in Table 2, not only are initiation factors freely exchangeable between bacteria but factors of bacterial origin may catalyze the reactions for the initiation of protein synthesis on ribosomes from mitochondria and chloroplasts. Such results are in agreement with the previous findings indicating that formyl-methionyl -tRNA is the peptide chain initiator in bacteria, blue-green algae, mitochondria and chloroplasts. No information is available concerning the ribosome specificity of the factors responsible for peptide chain termination but it may be fairly safely predicted that the same type of specificity is operational also for these reactions. Hence a paradigm of the specificity of protein synthesis such as that of Table 3 may be accepted rather confidently.

Three questions remain, however, to be answered. First, where is located the genetic information for the transfer factors catalyzing protein synthesis in the organelles of eukaryotic cells: is it in the cell nucleus as it is the case of some chloroplast and mitochondrial enzymes or is it in the organelle DNA?

Second, how many sets of transfer factors are present in photosynthetic eukaryotes which contain three distinct systems for protein synthesis (in

Table 1

Ribosome specificity of elongation factors

Ribosome from	Polymerizing enzymes or transfer factors from								
	Mammals		Insects		Higher plants		Microorganisms		
	Liver	Human cells	*Tenebrio molitor*	*D. melanogaster*	Wheat embryos	Castor bean seedlings	*Saccharomyces* spp.	*E. coli*	*Bacillus* spp.
MAMMALS									
Liver	+	+	+		+	+	+	−	
Human cells	+	+					+	−	
INSECTS									
Tenebrio molitor	+		+					−	
Drosophila melanogaster				+			+	−	
HIGHER PLANTS									
Wheat embryos	+				+			−	
Castor bean seedlings	+					+	+	−	−
MICROORGANISMS									
Saccharomyces spp.	+	+				+	+	−	−
Escherichia coli	−	−	−		−	−	−	+	+
Bacillus spp.						−	−	+	+

+, active system; −, lack of activity.
References to the original papers in Ciferri and Parisi (1970).

Table 2

Ribosome specificity of initiation factors

Washed ribosomes from	None	Initiation factors from	
		E. coli	*B. subtilis*
	$\mu\mu$moles of fMet-pyromycin/mg of ribosome		
E. coli	0·4	88	112
B. subtilis	0·9	68	43
Neurospora crassa mitochondria	1·5	25	—
Neurospora crassa cytoplasm	1·3	2·7	—
Euglena gracilis chloroplasts	0·9	49	—
Nostoc sp.	0·8	44	—

Data recalculated from Sala and Küntzel (1970); Sala et al. (1970).

the cytoplasm, in chloroplasts, in mitochondria). Are there just two sets of factors, one for the ribosomes of the 80S type (cytoplasm) and one for the two groups of ribosomes of the 70S type (chloroplasts and mitochondria)? Alternatively, each organelle may be endowed with its own set of transfer

Table 3

Ribosome specificity of protein synthesis

Ribosomes from	Factors from			
	Prokaryotes	Eukaryotes		
		Cytoplasm	Mitochondria	Chloroplasts
Prokaryotes	+	−	+	+
Eukaryotes				
Cytoplasm	−	+	−	−
Mitochondria	+	−	+	+
Chloroplasts	+	−	+	+

+, active system; −, lack of activity.

Table 4

Polyphenylalanine synthesis by preparations from a wild type strain of yeast (DM) and a petite mutant (DM_1)

Ribosomes	Polymerizing enzymes from strain	Phenylalanine incorporated ($\mu\mu$moles/assay)
E. coli	DM	4·5
(70 S)	DM_1	6·7
S. cerevisiae	DM	13·6
(80 S)	DM_1	11·1

Data from Parisi and Cella (1971).

factors in which case any higher plant should have three separate sets of transfer factors.

The third question concerns the differences and the similarities among transfer factors of different origin but acting on the same type of ribosome. In other words, one would like to know if, for instance, the transfer factors from yeast mitochondria, known to catalyze protein synthesis on mitochondrial and *E. coli* ribosomes but not on cytoplasmic ribosomes, are structurally more similar to *E. coli* transfer factors than to the factors from yeast that catalyze protein synthesis on cytoplasmic ribosomes.

The answer to the first question is provided in part by experiments from our laboratory (Parisi and Cella, 1971) that may be summarized in Table 4. Transfer factors of the 70S type are present also in a "petite" mutant of yeast devoid of mitochondria and mitochondrial DNA. Hence the genetic information for at least the transfer factors responsible for peptide chain elongation in mitochondria is located on the nuclear DNA and not on the mitochondrial DNA. Further, experiments with selective inhibitors (chloramphenicol, cycloheximide) demonstrated that the genetic information for

such factors is translated in the cytoplasm and not in the mitochondria. Thus mitochondrial protein synthesis appears to be controlled by the nucleus at the transcriptional and translational levels.

As yet no definite answer may be given to the second question concerning how many sets of transfer factors are present in photosynthetic eukaryotes. Preliminary evidences obtained in our laboratory in the case of light- and dark-grown cells of *Chlorella vulgaris* as well as mutant strains of the same organism seem to indicate that there appear to exist distinct transfer factors for the three types of ribosomes (cytoplasmic, mitochondrial, chloroplastic).

A few data are now available concerning the structural relationships of transfer factors from different organisms. We have purified to a considerable extent one type of elongation factor (the transfer factors G) present in the achloric alga *Prototheca zopfii* and to a lesser extent in yeast. By gel filtration it has been found that the factors active on 70S ribosomes have a molecular weight of approximately 80,000 as found for *E. coli* transfer factor G. On the other hand, the factors from the yeast and the alga acting on 80S ribosomes show a molecular weight of approximately 140,000. In addition studies on the sensitivity to selected antibiotics (fusidic acid, sporangiomycin, diphtheria toxin) indicate that the factors responsible for protein synthesis in cell organelles of eukaryotic organisms are more similar to those present in prokaryotic organisms than to those present in the cytoplasm of eukaryotes. It remains to be seen whether this similarity in the function and the gross structure is reflected in a similarity in the finer structure of the protein (e.g. in the amino acid sequence). The data of Gordon et al. (1969) on the cross-reaction between antisera prepared against *E. coli* transfer factor G and cell-free preparations from different bacteria seem to argue that any homology, if ever present, between diverging groups of prokaryotes (for instance gram-positive and gram-negative bacteria) has been lost in the course of evolution. One wonders, however, whether mitochondrial and chloroplastic transfer factors are still somewhat related to to-day's prokaryotes or if any relation exists between the factors from the two types of organelles or even between those from the organelles and those from the cytoplasm.

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TIME COURSE AND STABILITY OF LIGHT-INDUCED POLYSOME FORMATION IN ETIOLATED BEAN LEAVES

by

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Exposure of dark-grown bean seedlings to white light induces a conversion of monosomes to polysomes in the primary leaves. This conversion, which does not require continuous light and involves cytoplasmic ribosomes, occurs at a linear rate in the first 4 hours following light exposure. After this time, no more polysome formation is observed.

Actinomycin D, given at the time of light exposure, inhibits the light-triggered polysome formation. However, if the drug is given after the light-dependent polysome formation is completed, no variation in the polysome proportion is found, suggesting a low turnover rate for the newly formed mRNA.

INTRODUCTION

Exposure of etiolated plants to light stimulates several biochemical processes (Mohr, 1969). Where the time-course of light induction has been studied (Anderson and Boardman, 1964; Chen et al., 1967; Lange and Mohr, 1965; Mohr, 1966), a lag period of several hours has been observed. The induction of several photo-developmental processes is inhibited if the plants are treated with an inhibitor of protein synthesis at the time of light exposure (Mohr, 1969). This latter finding strongly suggests that protein synthesis is involved in most of the light-triggered developmental processes of the plant. A direct evidence of activation, by light treatment, of the protein synthesizing machinery of the plant came from the experiments of Williams and Novelli (1967) who showed that ribosomes extracted from light-treated plants were more active in protein synthesis than those from etiolated plants. Subsequently (Williams and Novelli, 1968; Pearson and Wareing, 1970; Poulson and Beevers, 1970) it was shown that the increased activity was due to a higher proportion of polyribosomes in the ribosome preparation of the light-treated plants.

This suggests that polysome formation may be an essential event in the light-triggered development of the plant and should occur and possibly be completed before the onset of induction of the developmental processes proper. The work reported in this paper was undertaken to test this hypothesis by determining the time-course of polysome formation in etiolated bean leaves after exposure of the plants to white light. The dependence of polysome formation on ex novo synthesis of mRNA and the stability of this mRNA were also studied.

MATERIALS AND METHODS

Phaseolus vulgaris, L., cv. Nero Bobis, was grown on wood dust in the dark at 25 °C for 6 days. Light treatment was carried out with fluorescent and incandescent lamps.

Preparation of polysomes. The primary leaves were harvested and immediately frozen in liquid nitrogen. The leaves were ground with a pestle in a prechilled mortar. The fine powder was then homogenized in a Potter homogenizer with 2 ml of Buffer A per gram of leaves. The homogenate was centrifuged at 5000 g for 10 min and at 20,000 g for 15 min. The supernatant, after addition of 1/10 volume 20% (v/v) Triton X—100, was layered over 4 ml of 1 M sucrose containing 10 mM Tris-HCl, pH 7·6, 5 mM Mg-acetate and 40 mM KCl, and centrifuged at 49,000 rpm for 200 min in a Spinco rotor 50. The pellet was resuspended in Buffer B (0·25 ml/gram of leaves). The purity of the ribosomal preparations was routinely checked by their UV spectrum. The spectral ratios were as follows: $E_{260}/E_{235} =$ $= 1{\cdot}60—1{\cdot}67$; $E_{260}/E_{280} = 1{\cdot}8—1{\cdot}9$.

Sucrose density gradient centrifugation. 0·1—0·2 ml (8 E_{260}) of polysome solution were layered on a 10—37% (w/v) sucrose solution made up in 5 mM Tris-HCl, pH 7·6, 5 mM-acetate, and 40 mM KCl in 5-ml tubes of the Spinco SW-50 rotor. They were centrifuged at 49,000 rpm for 30 min. The gradient was collected dropwise with the aid of a peristaltic pump. 44—47 fractions were normally obtained. Each was diluted with 1 ml of water and the absorbancy at 260 nm read in an Optica R 4 spectrophotometer. The polysome proportion of the total ribosomes was calculated by planimetrically integrating the pertinent areas of the sedimentation profiles.

In vitro amino acid incorporation was performed as described previously (Stout et al., 1967).

Buffers. Buffer A contains: 10% sucrose, 40 mM Tris-HCl, pH 7·6, 5 mM Mg-acetate, 40 mM KCl. Buffer B contains: 10 mM Tris-HCl, pH 7·6, 5 mM Mg-acetate, 40 mM KCl. The pH of the buffers was always determined at 20 °C.

RESULTS AND DISCUSSION

Typical sedimentation profiles of ribosomes extracted from etiolated and illuminated plants are shown in Fig. 1. There is clearly a higher proportion of faster-sedimenting particles in the ribosome preparation from illuminated leaves than from etiolated ones. The polysomic nature of the material sedimenting faster than the monosome peak was verified by incubation with trace amounts of RNase. The faster sedimenting peaks disappeared and were recovered in the monosome region of the gradient. The light-treatment, within the period used in these experiments, did not alter the net ribosomal content of the leaves. In fact, the yields of ribosomes from etiolated and illuminated leaves were consistently found equal (Table 1).

In order to check that the difference in the amount of polysomes found in the dark and illluminated leaves reflects a physiological situation and is not due to an extraction artifact, some "polysome protectants" were tried. Cycloheximide (100 μg/ml) was added to the grinding medium to block a

possible ribosome run-off from the polysomic structure. However, no effect of this drug was observed in either dark or light-treated leaves. Addition of diethylpyrocarbonate, an inhibitor of RNase, resulted in a lower recovery of ribosomes and in considerable unspecific aggregation of the particles. To check whether the lower proportion of polysomes in the preparations from etiolated leaves was due to a higher RNase activity, the polysome proportion of ribosomes prepared from a mixture of equal amounts of dark and light treated leaves was determined (Table 1). In several such experiments the polysome proportion of "dark + light" leaves was always found to be the average of that found in the dark and light treated leaves assayed separately.

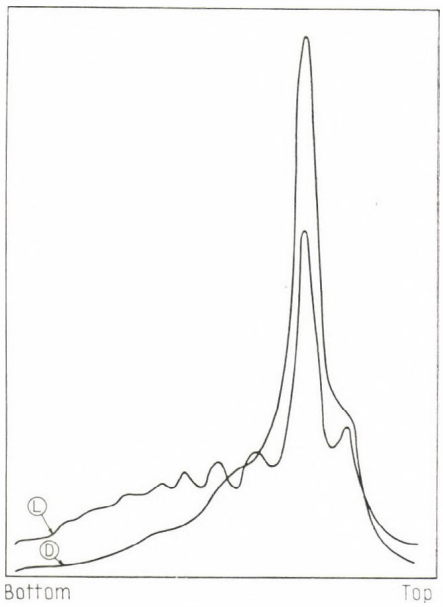

Fig. 1. Sedimentation profiles of ribosomes extracted from etiolated leaves (D) and leaves illuminated for 6 h at 2,000 lux (L)

The time-course of polysome formation is reported in Fig. 2 which shows the polysome proportion of leaves exposed to light for different periods. Values from three different experiments are plotted. These results clearly support the hypothesis, advanced in the introduction, that the light-induced increase in the polysome proportion of the leaf is an early event essentially completed within 4 h. Continuous illumination during these four hours, however.

Table 1

Additive recovery of polysomes from a mixture of etiolated and illuminated leaves

	Etiolated leaves	Illuminated leaves	Etiolated + illuminated leaves
Ribosomal yield	8·8±0·25	8·3±0·24	8·0±0·59
100 P/P + M (experimental)	32	56	43
100 P/P + M (theoretical)	—	—	44

1. Ribosomal yield is expressed as E_{260} per gram fr. wt. of leaves.
2. Polysome proportion in the ribosomal preparations is defined as: $\frac{\text{polysomes} \times 100}{\text{polysomes} + \text{monosomes}}$.
3. The theoretical polysome proportion is calculated from the formula: $P_{(e+i)} = \frac{P_e Y_e + P_i Y_i}{2}$ where P is the polysome proportion as defined in 2), Y is the ribosomal yield as defined in 1), and the subscripts *i* and *e* refer to etiolated and illuminated respectively

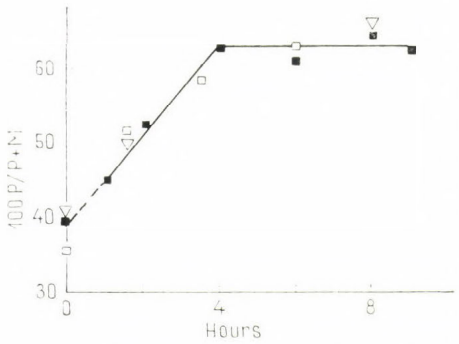

Fig. 2. Time course of light-stimulated polysome formation. The different symbols refer to different experiments. (P = polysomes; M = monosomes)

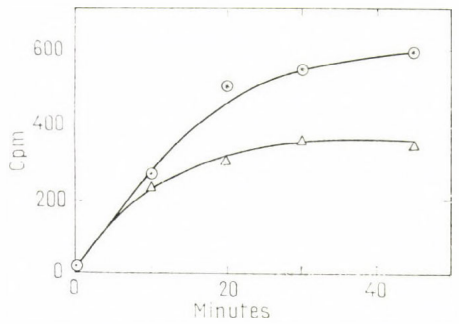

Fig. 3. Effect of cycloheximide and chloramphenicol on C-14-leucine incorporation by ribosomes extracted from illuminated leaves. The drugs concentration was 300 μg/ml. Control (○); Chloramphenicol (✳); Cycloheximide (△)

is not needed. Table 2 shows that the same proportion of polysomes is reached if the plants are exposed to light for 30 min and then transferred to the dark or if they are illuminated continuously for 6 h. The plants were routinely illuminated with a light intensity of 2,000 lux at the level of the leaves. However, the same results were obtained with light intensities ranging from 1,000 to 6,000 lux. This indicates that the photochemical process is saturated at 1,000 lux within 30 min.

Exposure of etiolated plants to light stimulates the protein synthesizing activity of subsequently isolated plastids (Dumm and Marguiles, 1970). To check whether the increase in the polysome proportion of our cytoplasmic ribosome preparation was due to contaminating plastid ribosomes the following experiments were performed. First, the ribosomal RNAs were extracted from the polysome preparation and their sedimentation characteristics analyzed in a density gradient. Only two sharp peaks were noted, thus excluding the possibility of heavy contamination of plastid ribosomes. Secondly, the amino acid-incorporating activity of the polysome preparation was tested for sensitivity to chloramphenicol and cycloheximide (Vasquez and Monro, 1968). Chloramphenicol (300 μg/ml), a known inhibitor of protein synthesis supported by 70S (plastid) ribosomes, showed no inhibition, while cycloheximide, which is known to affect 80S type but not 70S type ribosomes, inhibited, even though only partially, the incorporation. In addition, emetine, which affects 80S but not 70S type ribosomes, strongly inhibited the amino acid-incorporation (Fig. 3).

Several light-induced processes are inhibited if the plants are given an inhibitor of RNA synthesis at the time of light exposure. (Mohr, 1969; Lange and Mohr, 1965). Stout et al. (1967) have reported an increase in the soluble RNA polymerase of corn seedlings following light treatment. These findings suggest that RNA synthesis may be the early event in the chain of developmental processes induced by light. The following step would be the formation of polysomes accompanied by an increase in the synthesis of specific proteins. If this is the case, inhibition of RNA synthesis

Table 2

Effect of returning light-treated seedlings to dark on polysome formation

Treatment: minutes			100 P/P + M
Light	Dark	Total	
Zero	360	360	33
360	Zero	360	61
90	270	360	61
30	330	360	62

Table 3

Inhibition by actinomycin D of light-stimulated polysome formation

Exp. No.	Act. D concentration (μg/ml)	Light treatment (hours)	Percent inhibition
1	75	6	98
2	5	6	84
3	5	9	86

at the time of light exposure should also inhibit polysome formation. Indeed, Table 3 shows that if actinomycin D is given to the plants at the time of light exposure, the light-dependent polysome formation is strongly inhibited, even at low drug concentrations. However, if the plants which have been illuminated for 4 h are incubated in the presence of actinomycin D for 4 additional h, no variation in the polysome proportion is observed, thus indicating that the light-dependent newly synthesized mRNA is stable. This is more clearly shown in Fig. 4. In this experiment the drug was added to the plants at different times during the light treatment and all samples were then harvested at the 8th h of illumination. The plot of polysome proportion versus time of addition of the inhibitor yields a curve similar to the time-course of polysome formation, with a linear increase in the first 4 h followed by a plateau. This can only be explained if (i) the addition of actinomycin D inhibits further synthesis of mRNA and thus polysome formation; (ii)

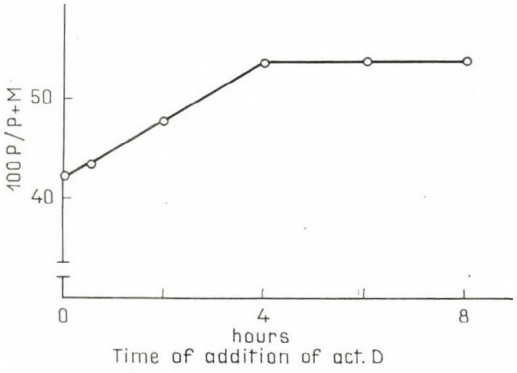

Fig. 4. Effect of addition of actinomycin D at different times during the light-stimulated polysome formation

the newly synthesized mRNA is stable and thus no polysome degradation occurs.

The experiments reported in this paper and those of the literature indicate that the light-triggered polysome formation is an early event essentially completed within 4 h; that it involves cytoplasmic ribosomes; that it does not require continuous light; that it depends on *ex novo* synthesis of mRNA which appears to be stable.

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ISOLATION AND PARTIAL CHARACTERISATION OF ENDOSPERM NUCLEI FROM IMMATURE BARLEY

by

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There are few convenient methods available for the isolation of nuclei from higher plants. In general most difficulties have arisen from the choice of tissue from which the nuclei are to be separated. The initial problem has involved breakage of the mechanically resistant cell walls without damaging the nuclei. Further problems involve the separation of a pure nuclear fraction from a cell suspension containing chloroplasts and amyloplasts at least some of which overlap the nuclei in sedimentation properties.

The unique properties of immature cereal endosperm should, at least in theory, eliminate these difficulties. Thus, during the period immediately after fertilisation rapid division of the initial triploid endosperm nucleus takes place without any accompanying cell wall formation (Yampolsky, 1957). The nuclei are closely packed, the only other structures present being undifferentiated spherical bodies or proplastids (Buttrose, 1963). It has been estimated (Thomson and Johnston, 1945) that in the barley grain at 3 days after anthesis 200—520 endosperm nuclei may be present, distributed throughout the peripheral layer of cytoplasm.

The two row barley, *Hordeum distichum* (L.) Lam. cv. *Maris Baldric*, was used and was grown in season on the School of Agriculture farms and during the rest of the year under lights in the greenhouse.

100 grains were dissected out by hand and individually squashed in an ice cold mortar in 2 mls of M sorbitol containing 30% glycerol and 0·001 M Ca^{2+} The mixture was filtered through muslin onto 2 mls of 1·8 M sorbitol, containing 30% glycerol and 0·001 M Ca^{2+} in a 5 ml polypropylene centrifuge tube. Thus two layers were formed. This was centrifuged for 20 min at 200 ×g and the nuclei were found by microscopical examination to be present in the lower layer. The top layer and interface were discarded and the lower layer transferred to another 5 ml centrifuge tube. The slight pellet was also discarded. The nuclear suspension was then centrifuged at 200 ×g for 2 h followed by 1 min at 1000 ×g in order to sediment the nuclei. The nuclei are extremely fragile and rupture under the slightest mechanical stress. They are severely damaged by high centrifugal speeds and acceleration, by dilution and even by the impact of a cover slip on a thin suspension. Hence it is difficult to obtain a uniform focal plane for phase contrast of a large number.

The isolated nuclei are mostly oval in shape with a granular surface and a rather indistinct outline (Fig. 1). There are at least 2—3 prominent nucleoli per nucleus. The DNA : RNA : protein ratio for the nuclei is 7 : 16 : 77. The low DNA value was expected since in sections of the intact grain stained

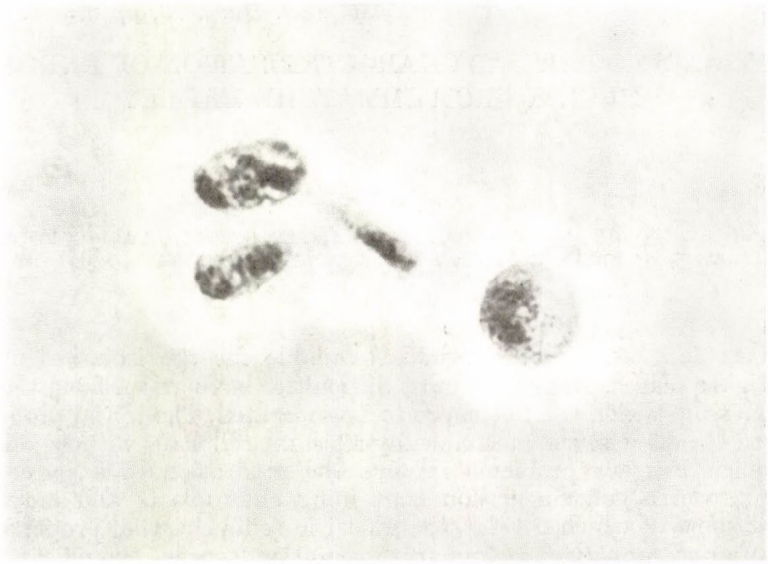

Fig. 1. Phase contrast micrograph of barley endosperm nuclei. Instrumental magnification $\times 700$

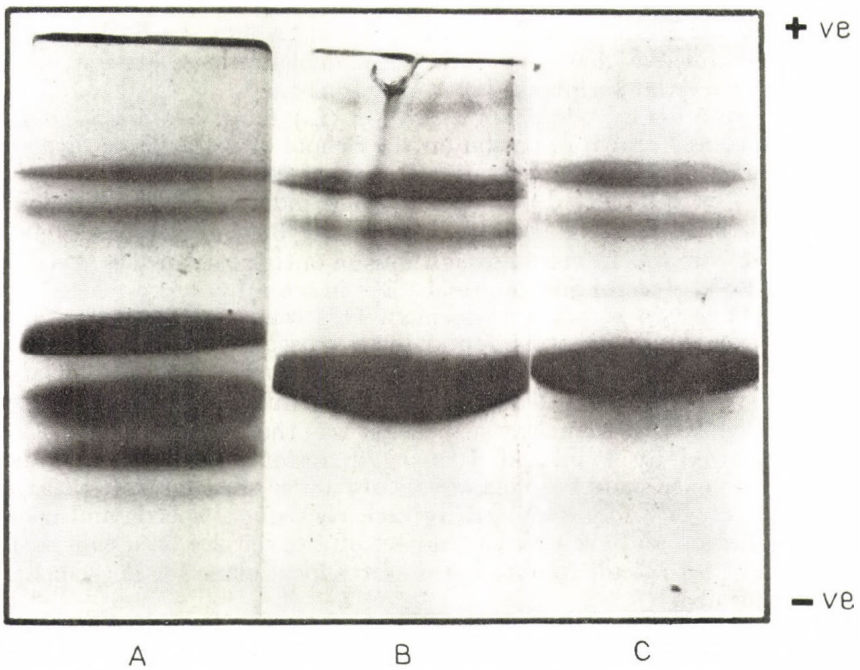

Fig. 2. Acrylamide gel electropherogram of (A) calf thymus histone (B) nuclear histone extract and (C) intact cell histone extract

with Feulgen the endosperm nuclei have a very low colour intensity. Those in the tissues surrounding the endosperm have a much higher colour intensity.

The nuclei were metabolically active and were associated with the characteristic enzymes RNA polymerase and NAD pyrophosphorylase.

Nuclear histones were extracted by the method of Johns (1967) and compared with those extracted from intact endosperm cells and a control histone preparation from calf thymus (Fig. 2). Intact tissue (C) gave four components with a mobility pattern similar to that reported by Smith et al. (1970) for pea histones prepared from purified pea chromatin. In contrast the nuclear preparation (B) contained only one histone component intermediate in mobility between the first two calf thymus components (A). This histone component was the major component of those found in intact tissue. It corresponded in mobility to pea histone II as described by Smith et al. (1970). One must conclude that there appears to be only one histone present in endosperm nuclei at this stage of development and that the other three components in the extract of whole cells are either cytoplasmic basic proteins, possibly ribosomal or, since this preparation almost certainly includes some non-endosperm tissue, histones derived from embryo or seed coat nuclei.

While a multiple histone pattern has been shown to be characteristic of chromatins isolated from whole tissue (Fambrough, et al., 1968) no results were recorded for a homogeneous nuclear preparation at the same developmental stage. Since the isolated nuclei are probably at, or even past, their peak of synthetic activity the presence of only one histone may be related to their metabolic decline.

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RESPONSE TO OBLIGATE PARASITE INFECTION OF NUCLEIC ACID METABOLISM IN HOST CHLOROPLAST

by

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INTRODUCTION

Infection of plants by parasite produces a wide range of physiological changes such as an enhancement of respiration, activation or inactivation of photosynthesis and the accumulation of abnormal metabolites. Toxins or metabolites of pathogenic microorganisms have been sought as the trigger for these physiological changes in some kinds of plant diseases.

Similar physiological changes are also observed in plants infected by obligate parasites (Allen, 1942; Bushnell and Allen, 1962; Farkas and Király, 1955), although in these cases the major trigger may not be such a drastic toxin as has been encountered in the perthophytic diseases. Nevertheless, some substance(s) must play a role in eliciting these physiological changes in the host.

A marked increase in soluble carbohydrates is one of the characteristic physiological changes associated with the early stage of powdery mildew infection (Allen, 1942; Frič, 1964). The significance of this phenomenon in the obligate parasitism can be studied simply from a nutritional aspect or more comprehensively from the aspect of biochemical regulation. In this communication the authors wish to elucidate how the powdery mildew infection exerts an effect on the nucleic acid synthesizing activity of the carbohydrate synthesizing organelle, chloroplast, in the early stage of infection. Changes of nucleic acid metabolism in infected whole plants have been reviewed by Heitefuss (1966).

MATERIALS AND METHOD

Erysiphe graminis f. sp. *hordei*, race 1, was used as the pathogen and a barley variety susceptible to race 1, Kobinkatagi, was used as host.

Plants grown in a growth chamber at 20 °C for 2—3 weeks under natural light were inoculated with conidia of race 1 by rubbing with a soft hair brush. The inoculated and uninoculated control plants were placed separately in indoor growth cabinets and kept at 20 °C. After an appropriate time interval, 3·9 g of leaves of the inoculated and uninoculated plants were detached and the cut ends were immersed in a solution of radioactive ortho-phosphate (700 μCi in 6 ml) for 4 hrs under constant illumination, temperature (25 °C), and humidity (60%). The leaves were pressed with a small amount of 0·2 M sucrose in 0·07 M phosphate buffer (pH 7·0) by a glass

roller on a glass plate and the resultant sap was centrifuged at 600×g for 30 min. The precipitate was then subjected to discontinuous sucrose density gradient centrifugation at 2,600×g for 30 min. Two green chloroplast bands were obtained at the boundary between 50% and 40% and in the 20% fraction. Microscopic observation indicated that the lower green fraction contained almost exclusively the so-called heavy chloroplasts while the upper fraction (20% fraction) contained the light chloroplasts together with an appreciable amount of mitochondria. The heavy chloroplasts were collected from the lower fraction and washed twice by centrifuging at 3,000×g for 10 min. These processes were carried out below 4 °C.

For quantitative estimation, the nucleic acids were extracted by a modified version of the Ogur-Rosen and Schmidt—Thannhauser—Schneider methods. For qualitative assay, the nucleic acids were extracted by the phenol-SDS method and subjected to methylated albumin-Kieselguhr (MAK) column chromatography. The radioactivity was determined by a gas flow counter and expressed as cpm per chloroplast.

RESULTS

Quantitative aspects of nucleic acid metabolisms

For a quantitative assay of nucleic acids, the incorporation of radioactive phosphorus into various fractions was measured in chloroplasts of the inoculated and uninoculated barley leaves by the modified Ogur-Rosen procedure. The results are shown in Table 1. It is evident that ^{32}P incorporation into each fraction increased after inoculation. This is best illustrated in the incorporation of ^{32}P into RNA. The radioactivity of the DNA fraction was remarkably high, taking relative amount of DNA in chloroplasts into consideration. Therefore the residue left after RNA extraction was subjected to alkaline digestion. It was then found that the high radioactivity of the DNA fraction can be attributed to RNA unextracted by cold perchloric acid.

Table 1

*Radioactive phosphorus incorporation into various fractions of chloroplasts of barley leaves as affected by powdery mildew infection**

Fraction	Radioactivity (cpm×10^6) per chloroplast		
	Healthy	Diseased**	Ratio (%)
Whole chloroplasts	2,031	2,409	118·6
Lipid fraction	377	451	119·8
Acid soluble fraction	276	395	143·4
RNA	394	644	163·3
DNA	405	376	92·8
Extraction recovery (%)	72	72	

* Extraction was done by a modified version of the Ogur-Rosen method.
** 12 h after inoculation.

A similar experiment was done by employing a modified version of the Schmidt—Thannhauser—Schneider method and the results are shown in Table 2. It is apparent that powdery mildew infection gives rise to a marked increase in ^{32}P incorporation into various fractions, especially the nucleic

Table 2

*Infection-induced increase of ^{32}P incorporation into the nucleic acids of barley leaf chloroplasts**

Fraction	Radioactivity (cpm×10^6) per chloroplast		
	Healthy	Diseased**	Ratio (%)
Whole chloroplasts	3,681	5,361	145·6
Ethanol soluble	1,007	1,683	166·7
0·1% PCA soluble	1,146	1,191	104·0
Lipid	26	46	174·3
Acid soluble	698	874	125·3
RNA	537	1,701	316·4
DNA	34	143	420·5
Extraction recovery (%)	94	105	

* The extraction procedure was a modified Schmidt—Thannhauser—Schneider method.
** 24 h after inoculation.

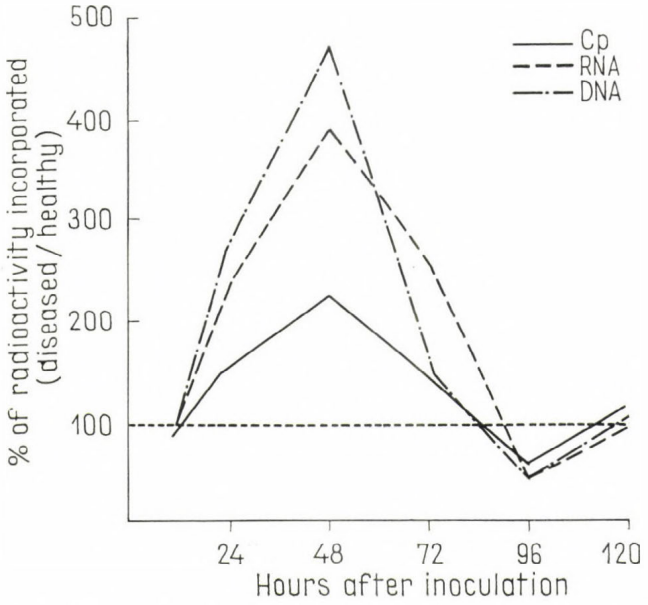

Fig. 1. Dynamic change of ^{32}P incorporation into the chloroplast nucleic acids of barley leaves during the early stage of powdery mildew infection. Cp represents total ^{32}P incorporation by the chloroplast

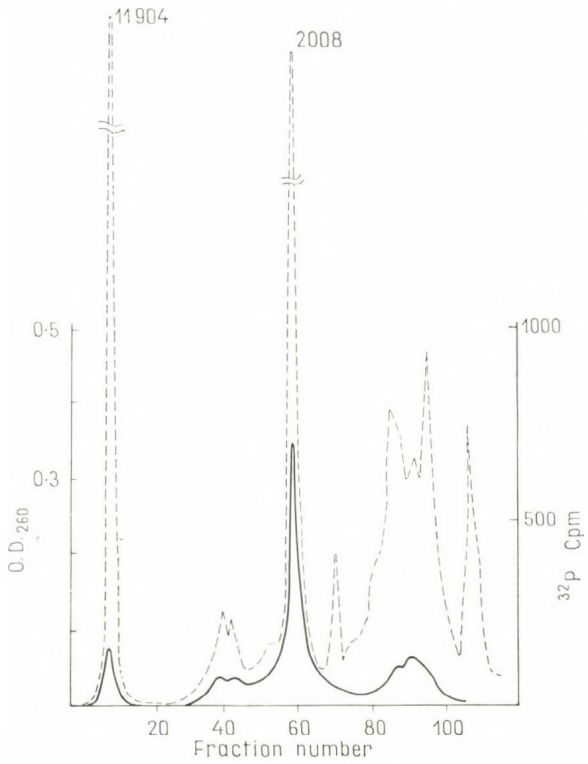

Fig. 2. Elution profile of chloroplast nucleic acids from a MAK column. Radioactive nucleic acids were extracted by the phenol-SDS method from chloroplasts isolated from leaves fed with ^{32}P. A certain amount of cold chloroplast nucleic acid was added to the preparation which was then subjected to MAK column chromatography

acid fractions. Even though the radioactivity inthe DNA fraction decreased after alkaline digestion (cf. Table 1), the net incorporation after infection increased in this fraction as well.

The ratio of incorporation into chloroplasts of diseased leaves to that of healthy ones was plotted in Fig. 1. The dotted line represents the level of incorporation into chloroplasts of the healthy control. It is apparent that the RNA synthesis in chloroplasts was activated within 24 h after inoculation and reached a maximum at about 48 h. The incorporation was estimated to be about 4 times as high as in the healthy control at this stage. The rate of RNA synthesis then decreased to the normal level by about 4 days after inoculation. This corresponds to the flecking stage.

Qualitative assay of DNA synthesis in chloroplasts

It was shown in Fig. 1 that the radioactivity in the DNA fraction was also higher in chloroplasts of the inoculated leaves. It seemed interesting to find out whether or not this increased incorporation represents a real activation of DNA synthesis. As a primary approach to this problem, nucleic acids extracted from chloroplasts of healthy leaves were fractionated on a MAK column. The elution profile of nucleic acids is shown in Fig. 2. Although ^{32}P incorporation into ribosomal RNA is significantly high, the radioactivity of the DNA fraction is even higher and the peak of optical density coincides with that of the radioactivity. It is assumed that the radioactive phosphorus is indeed incorporated into the DNA of chloroplasts.

To verify this assumption, the DNA fraction was subjected to digestion with deoxyribonuclease or ribonuclease and then to rechromatography on

MAK column. The result is presented in Fig. 3. Rechromatography of the DNA fraction not treated with enzyme gave a sharp peak at or around the fractions expected from the linear salt gradient. Ribonuclease treatment of the fraction did not give rise to any significant change in the elution pattern suggesting that the radioactivity is not due to RNA contamination in the DNA fraction. Treatment of the DNA fraction with deoxyribonuclease, on the contrary, resulted in a complete loss of the peak at the expected position. The peak appeared in the region of nucleic acids of small molecular weight.

It seems reasonable, therefore, to conclude that the incorporation of ^{32}P into DNA is due to de novo synthesis of DNA in chloroplasts. A more detailed analysis of DNA synthesis activated by powdery mildew infection is under way and the preliminary results suggest that the enhanced ^{32}P incorporation into DNA should be attributed to the activation of DNA synthesis.

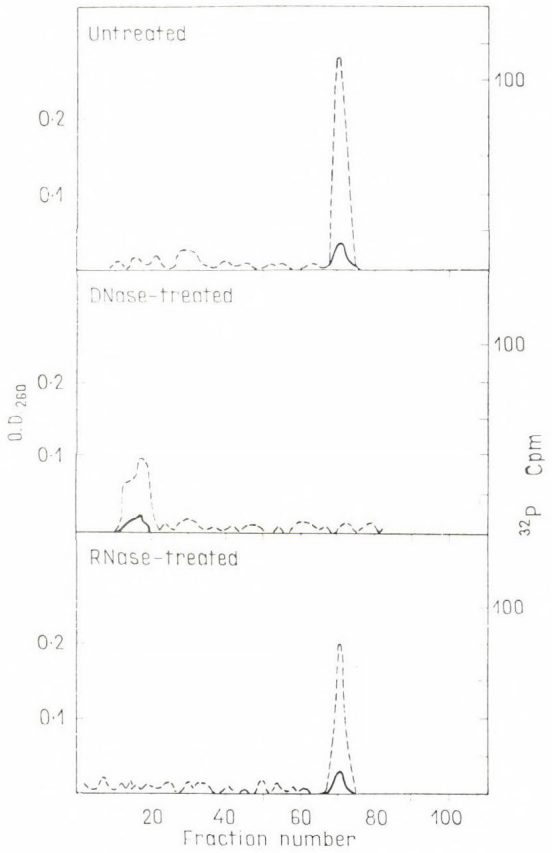

Fig. 3. MAK column chromatograms of the DNA fraction digested with ribonuclease or deoxyribonuclease. The DNA fraction in Fig. 2 was incubated at 37 °C for 1 h with 25 μg/ml of DNase or 1 μg/ml of RNase and rechromatographed on MAK columns

DISCUSSION

Powdery mildew is known to parasitize on the epidermal cells of barley leaves but causes various physiological changes in the underlying assimilatory tissues. This combination, therefore, provides the most suitable model for studying the information exchange between host and parasite.

The present report shows that RNA synthesis in barley chloroplasts was activated within 24 h after inoculation with powdery mildew and this suggests that some information to activate RNA synthesis is sent to the chloroplasts from the upper epidermal cell layer. These results also suggest

that the activation of chloroplast RNA synthesis might possibly be related to the enhanced carbohydrate synthesis in mildew infected barley leaves, perhaps through an increased synthesis of enzyme protein.

In contrast to these results, Hirai and Wildman (1969) reported that the RNA synthesis in tobacco chloroplasts was reduced when infected with tobacco mosaic virus and suggested that the RNA synthesis in chloroplasts is regulated by information from nucleus, because the viral RNA is synthesized in the nucleus.

It is not yet clear in the powdery mildew infection system whether the regulatory substance responsible for the infection-specific enhancement of RNA synthesis comes directly from the fungus or affects the host metabolism indirectly through the mediation of the host cell nucleus. The fact that DNA synthesis in chloroplasts is significant suggests that the whole of nucleic acid metabolism in chloroplasts is quite intensive.

The authors wish to express their thanks to Professor U. Hiura, Ohara Institute for Agricultural Biology, Okayama University, for his kind supply of experimental material and also for his advice during this work.

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SECTION IV

HORMONAL CONTROL OF NUCLEIC ACID AND PROTEIN SYNTHESIS

Symp. Biol. Hung. 13, pp. 285—291 (1972)

TRANSLATION CONTROL BY ABA IN COTTON SEED EMBRYOGENESIS

by

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In 1966 we reported that a great deal of the protein synthesis that occurs in the cotyledons of cotton plants during the first 3 days of germination does not require concomittant RNA synthesis (Waters and Dure, 1966). From this it appeared that this protein synthesis is directed by mRNA that exists in cotyledons of the dry seed, which implies that this mRNA is synthesized during embryogenesis.

We have subsequently followed up this observation in order to use the embryogenesis and germination of the cotton cotyledon as a tool for exploring mechanisms that regulate gene expression in tissue development.

As a first step we decided to attempt to identify some of the proteins that are synthesized *de novo* in the cotyledons during early germination which might be proteins unique to germination (not present in embryogenesis), and which are synthesized even when all detectable RNA synthesis is prevented by actinomycin D. Since during germination the stored protein bodies that are so extensive in cotyledon cells are degraded to support the germinative growth of the axis, we chose a proteolytic enzyme that hydrolyzed the trypsin substrate benzoyl arginine ethyl ester (BAEE) as a likely possibility, Further, since a great deal of stored lipid of the cotyledons must be converted to carbohydrate during germination, we selected isocitritase as another possibility. In both cases we were fortunate since both of these enzyme activities showed the characteristics we were seeking. Neither activity is present in dry seed cotyledons or in embryonic cotyledons. Their activities show up after 24 h of germination and increase until the 5th day of germination and then decay slowly thereafter in a 1st order manner. And, of more interest, both activities show up and increase in the normal fashion in cotyledons that are germinated in the presence of enough actinomycin D to prevent all detectable incorporation of radioactive precursors into RNA. Neither activity shows up in cotyledons germinated in the presence of cycloheximide. (The details of the enzyme extraction and assay procedures, the kinetics of enzyme appearance during germination and the effects of inhibitors on enzyme appearance have been published by Ihle and Dure in 1969.)

Since the *de novo* appearance of enzyme activity and its sensitivity to cycloheximide does not unequivocally prove *de novo* synthesis, we subsequently demonstrated that radioactive amino acids could be extensively incorporated into the polypeptide chains comprising the protease enzyme when the cotyledons were exposed to these amino acids during germination. This demonstration required that a protocol for purifying the protease be

evolved and that the protease be purified from cotyledons that were germinated in presence of ^{14}C amino acids. When this was done we found that the specific activity of the purified protease (CPM/mg protein) was over 30 times greater than that of the total buffer soluble protein of the cotyledon.

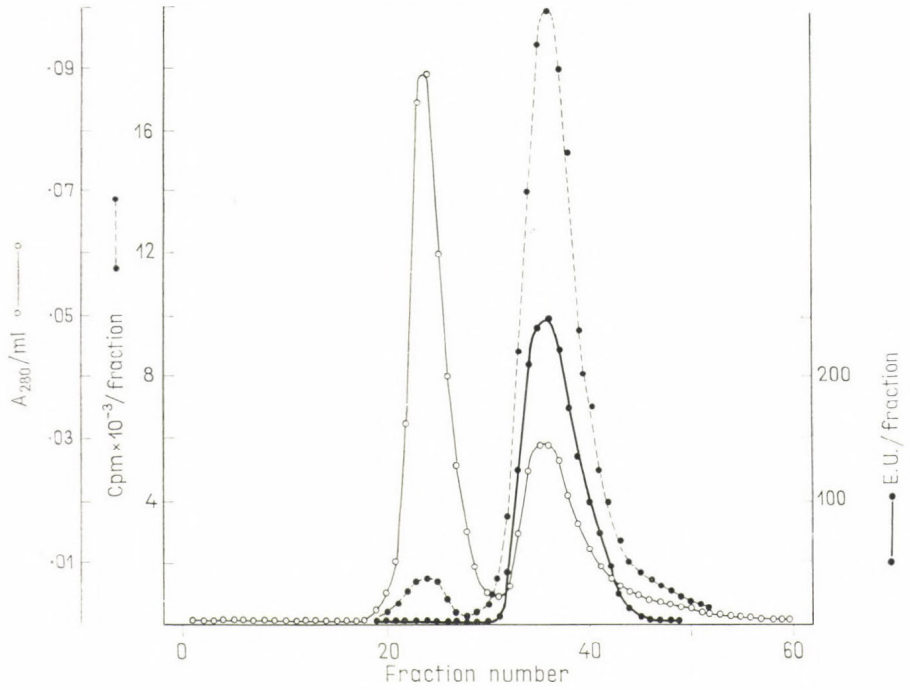

Fig. 1

Figure 1 indicates the extensive incorporation of radioactivity into the protease. This figure gives the elution profile of a G-150 Sephadex Column that constitutes the final step in the purification of the protease. Notice the greatly increased cpm/A_{280} values for the elution peak that constitutes the protease.

Since we had worked out a procedure for purifying the protease, we characterized this enzyme with respect to its catalytic properties and subunit structure. We found it to be a carboxypeptidase enzyme that utilizes the serine active site mechanism of hydrolysis and that was comprised of 3 subunits. Its carboxypeptidase activity is not stopped by proline or phenylalanine in the polypeptide chain which has made the enzyme useful in establishing the amino acid sequence of small polypeptides.

Once we had established that the carboxypeptidase (and, by analogy, isocitritase) is *de novo* synthesized during germination from preexisting mRNA, we made use of the cotton embryo's ability to germinate precociously to determine the point in embryogenesis when the mRNA for the carboxypeptidase and isocitritase are transcribed. Precocious germination refers

to the fact that immature cotton embryos will readily germinate when removed from the boll and placed on 0·8% agar or on wet filter paper or even in flasks with water and shaken on a microbiological shaker. During this precocious germination the shoot and root axes elongate, the cotyledons unfurl and green when exposed to light, and the carboxypeptidase and isocitritase activities develop in the cotyledons.

We were able to determine the time in embryogenesis at which the transcription of the mRNA for the two enzymes begins by precociously germinating successively younger embryos in the presence of actinomycin D until an embryo age was reached at which no carboxypeptidase or isocitritase activity developed. These results are presented in Table 1 which shows that embryos larger than 85 mgs will develop the two enzyme activities in their cotyledons whereas embryos smaller than this will not. Since the final wet weight reached by cotton embryos before seed dessication sets in is 125 mgs, the point at which the mRNA for these two enzymes is first demonstrable (85 mgs) represents about 60% completion of embryogenesis. Furthermore, it takes about 20 additional days inside the boll for the embryos to grow from 85 mgs to their final 125 mgs size. Since the mRNA for the two enzymes is apparently present during the last 20 days of embryogenesis, the translation of this mRNA during this period must be inhibited in some fashion. Notice in Table 1 that not only did

Table 1

Enzyme activities during precocious germination

Embryo wet weight (mgs)	Days germinated	Enzyme units/cotyledon pair			
		Carboxypeptidase		Isocitritase	
		— ACT D	+ ACT D	— ACT D	+ ACT D
110—125	3	9·33	12·48	12·5	21·06
100—110	4	5·46	13·44	9·12	20·03
85—100	4	1·33	7·11	6·46	21·32
60—85	5	2·21	*0*	5·25	*0*

actinomycin D fail to prevent the development of the enzyme activities, but actually enhanced them.

The stimulation of carboxypeptidase activity by actinomycin D is also shown in Fig. 2A which plots the development of activity against the time of precocious germination for embryos larger than 85 mgs. Figure 2A also shows that simply washing embryos in distilled water prior to placing them in germination dishes causes a stimulation in the development of carboxypeptidase activity equivalent to that caused by actinomycin D. Completely analogous results were obtained for isocitritase activity. The fact that washing embryos results in a more rapid development of enzyme activity suggests that a compound that somehow inhibits translation may be located on the cotyledon surface (presumably originating in the ovule wall and being absorbed by the cotyledons). The fact that enzyme activity does develop in time in unwashed cotyledons suggests that the compound

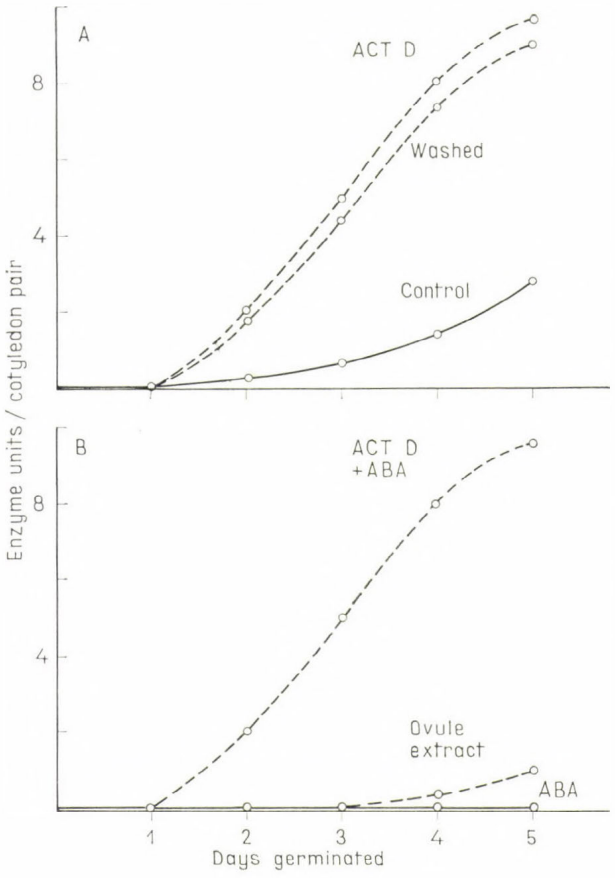

Fig. 2

can be degraded by cotyledon cells, and the fact that actinomycin D can effect the same stimulation as does washing indicates that the inhibition somehow requires RNA synthesis.

To test this possibility washed embryos larger than 85 mgs were precociously germinated in the presence ofan aqueous extract of ovule walls. The inhibitory effect of this extract on the development of carboxypeptidase activity in the cotyledons of these washed embryos is shown in Fig. 2B. Again, completely analogous results were obtained for isocitritase activity. These results prompted us to investigate the effect of abscisic acid (ABA) on carboxypeptidase and isocitritase appearance during precocious germination, since ABA was first isolated from cotton cells (Ohkuma et al., 1963) and its inhibitory action on plant development is well documented (Addicot and Lyon, 1969). Figure 2B shows that 10^{-6} M ABA completely inhibits the appearance of the carboxypeptidase during precocious germination, and also shows that if actinomycin D is supplied to the embryos along with ABA, the ABA inhibition is overcome, and carboxypeptidase activity develops as rapidly as it does in washed embryo cotyledons. Again, the development of isocitritase activity is affected in the same manner. Because of the similarity of action of ABA and the ovule extract and of the demonstrated presence of ABA in embryonic cotton seed of this age (Addicot and Lyon, 1969), we feel that one of the functions of ABA in cotton embryogenesis is to inhibit the translation of mRNA destined to be utilized during germination. This notion is attractive since the apparent source of the ABA during this stage of germination is the ovule tissue, which sclerifies and dies at the end of embryogenesis.

The mode of action of ABA here is not known, but the actinomycin D

data suggest that ABA may act on DNA to induce the synthesis of an inhibitor that prevents the translation of a specific body of mRNA or may act in concert with a rapidly turning over product of RNA synthesis to effect this translation inhibition. Oddly, ABA has no effect on the appearance of the carboxypeptidase or isocitritase in the cotyledons during the germination of embryos that have begun to dessicate or of mature seeds. This transition from sensitivity to insensitivity to ABA in mature embryos is not understood as yet. The details of these experiments involving the inhibition of translation by ABA have been published (Ihle and Dure, 1970).

Since it appears from these data that the transcription of the mRNA for the carboxypeptidase and isocitritase takes place 20 days before the end of embryogenesis, we proceeded to explore the induction of this transcription. Table 2 shows that although embryos smaller than 85 mgs do not develop either enzyme activity when precociously germinated in the presence of actinomycin D, both enzyme activities do develop in the presence of actinomycin D, if the embryos are allowed to precociously germinate for 24 h before being exposed to the inhibitor. Thus presumably, the transcription of the requisite mRNA for these two enzymes begins in cotyledons within 24 h after removal from the boll. Table 2 also shows that the 24 h period is sufficient time for embryos of different ages below 85 mgs to produce this body of mRNA and that the presence of ABA does not influence this induction of transcription. Subsequently we observed that this premature transcription could be induced in cotyledons in 24 h by simply removing the bolls from the plant.

These experiments with embryos smaller than 85 mgs imply that the transcription of the mRNA for these germination enzymes is induced by a severing of the vascular connection between the ovule and the maternal plant. This implication is strengthened by the observation that *in vivo* the funiculus connecting the ovule to the placenta breaks when the embryos reached approximately 85 mgs. This corresponds to the time in embryogenesis when the transcription of this mRNA occurs normally as indicated by the experiments with actinomycin D. Curiously, the severing of the vascular connection appears related to the synthesis of ABA in the ovule tissue, since the inhibitory action of ovule extracts on the development

Table 2

Induction of premature transcription

Embryo wet weight (mgs)		Enzyme units/cotyledon pair after 4 days precocious germination	
		Carboxypeptidase	Isocitritase
70	Control	1·3	6·40
	+ ACT D	0	0
	+ ACT D after 24 h	1·1	6·16
60	Control	1·1	2·15
	+ ACT D	0	0
	+ ACT D after 24 h	1·0	2·85
	+ ABA (+ ACT D after 24 h)	1·1	2·4

of the carboxypeptidase and isocitritase activity during precocious germination is observed only with ovule extracts obtained from ovules that contained embryos larger than 85 mgs. Finally, the breaking of the funiculus appears to stop DNA synthesis and cell division in the cotyledons as well (unpublished observations).

We feel that since the regulation of the carboxypeptidase and isocitritase activities during germination and precocious germination appears to be identical, they represent an entire class of germination enzymes whose mRNA is transcribed in late embryogenesis, but whose translation is prevented by ABA until embryogenesis is complete.

This idea seems to be born out by very recent experiments in which we have followed membrane assembly during germination. The formation of endoplasmic reticular membranes during germination can be followed by sucrose gradients and can be shown to take place during the first 3 days of normal germination and not inhibited by actinomycin D. It also takes place during precocious germination but is prevented by ABA. Its formation during precocious germination in cotyledons from embryos above 85

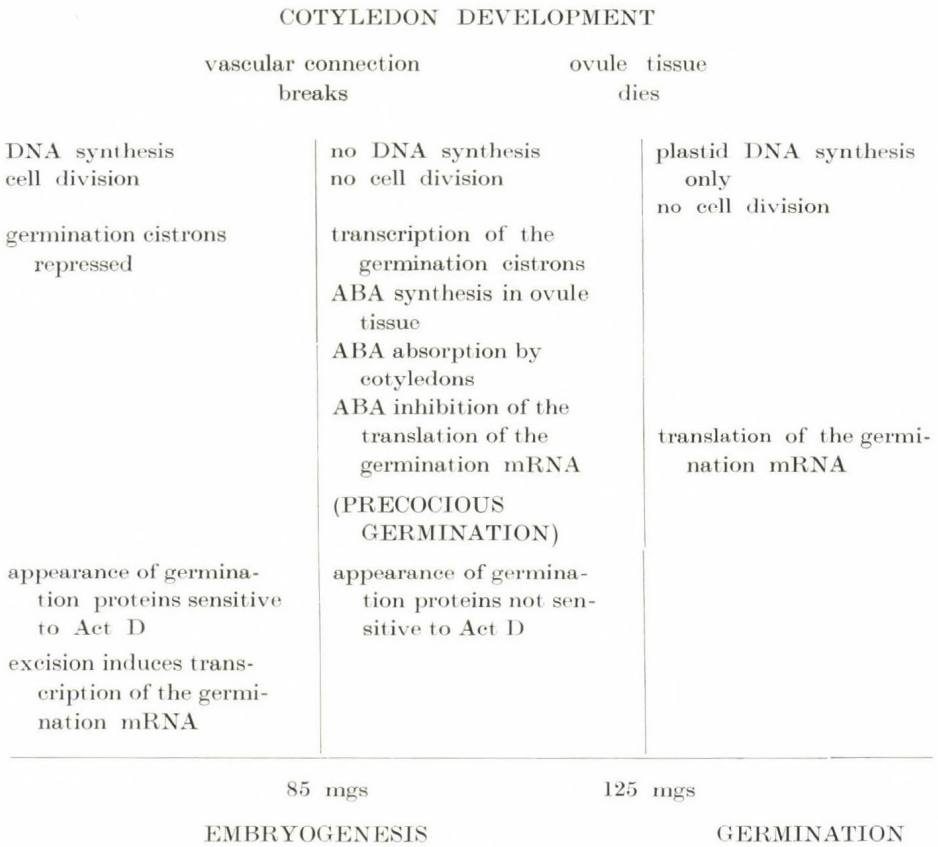

Fig. 3

mgs is *not* sensitive to actinomycin D whereas in younger cotyledons it is sensitive. This suggests that the mRNA for the enzymes and structural proteins necessary to form these membranes is present after the 85 mg stage, and that their translation into proteins is blocked by ABA as is the translation of the mRNA for the carboxypeptidase and the isocitritase. Figure 3 presents a schematic presentation of the developmental events that are suggested by the foregoing experiments.

Although any comparison of dicot and monocot embryogenesis must take into consideration the obvious differences between the two type seeds that are produced, many analogies can be found. One of the most striking analogies to us is the sensitivity of enzyme synthesis to ABA in cotton during late embryogenesis and in barley during early germination (Chrispeels and Varner, 1967) and the sensitivity to ABA of membrane formation in cotton embryogenesis and barley germination (Evins and Varner, 1971). This analogy along with many others suggests that monocots evolved an abbreviated embryogenesis by transferring some of the late developmental events of dicot embryogenesis to the early stages of monocot germination.

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HORMONAL CONTROL OF NUCLEASE LEVEL IN *AVENA* LEAF TISSUES

by

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The synthesis and/or breakdown of nucleic acids and proteins in tissues of higher plants, particularly in leaves, is known to be under hormonal control (Parthier, 1961; Wollgiehn, 1961; Osborne, 1962; Srivastava, 1967; Tavares and Kende, 1970; Khan et al., 1970). The same hormones which affect the level of nucleic acids also affect the level of nuclease activity in leaf tissues. Thus, kinetin has been shown to decrease (Srivastava and Ware, 1965; Bagi and Farkas, 1967; Udvardy et al., 1969; McHale and Dove, 1969; Sodek and Wright, 1969), abscisic acid has been shown to increase (Srivastava, 1968; De Leo and Sacher, 1970) the nuclease level in various leaf tissues. Although there is striking correlation between hormone-induced increase in nuclease level and decrease in RNA content (and vice versa), a causal relationship has not been established. It is hopeless to speculate about such a causal relationship until the point of attack of the hormones on the nuclease level is not understood. The tissues of higher plants contain several nucleolytic enzymes and, consequently, to establish a correlation between overall enzyme level and changes in nucleic acids in a tissue is meaningless.

To be able to obtain any meaningful correlation, one must identify the various nucleases in a target tissue and one must be able to detect a more specific point of attack of the hormone(s), if any. We attempted to approach the problem of hormonal regulation of nuclease level with these principles in mind. As a first step, we isolated, purified and characterized the major nucleases of the first seedling leaves of *Avena sativa* L. (Wyen et al., 1969; 1971; Udvardy et al., 1970), the test system chosen. Also, we worked out reliable methods for the quantitative assay of the individual nucleases present in *Avena* leaf extracts. Then, *Avena* leaf segments were treated with various growth regulators and assayed, at different times, for changes in the level of individual nucleases.

Figure 1 shows an example of the separation and quantitative assay of nucleases isolated from the *Avena* leaf. Proteins extracted from the leaf tissues were precipitated with $(NH_4)_2SO_4$ at 80% saturation. The precipitate was dissolved in a small amount of buffer and chromatographed on a Sephadex G—75 column. The fractions were assayed for protein content, RNase, DNase, alkaline and acid phosphodiesterase activity. Four distinct peaks of RNase, three peaks of DNase, two peaks of acid phosphodiesterase and one peak of alkaline phosphodiesterase activity emerged.

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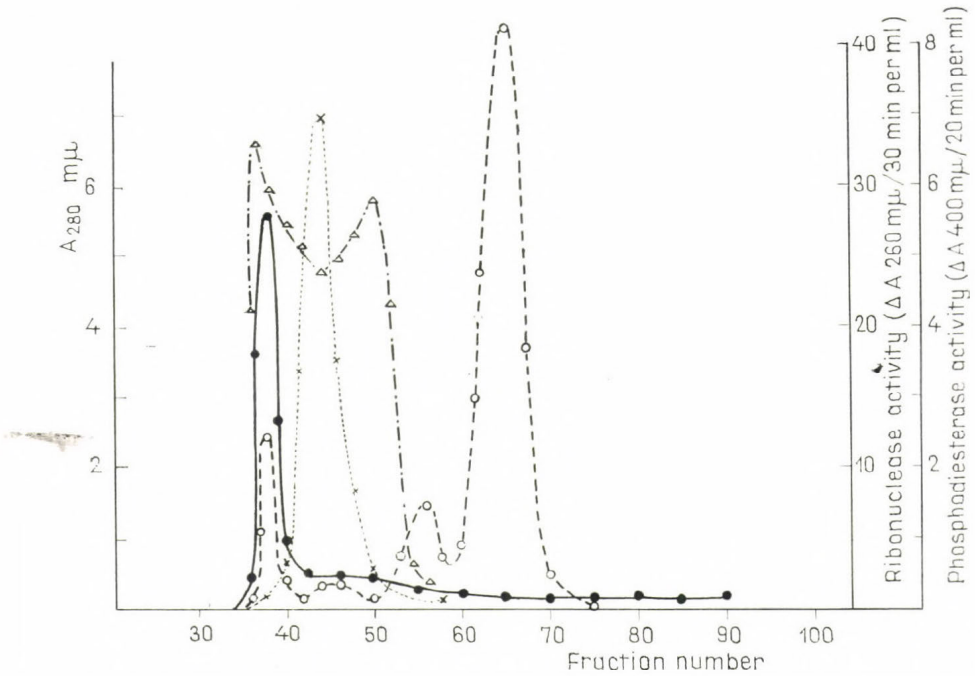

Fig. 1. Chromatography of ribonucleases and phosphodiesterases isolated from *Avena* leaves. Proteins from crude extracts centrifuged at 100,000×g for 90 min were precipitated with $(NH_4)_2SO_4$ at 80% saturation, the precipitate was dissolved in 3 ml of 0·01 M Tris-HCl buffer (pH 7·5) and applied to a Sephadex G—75 column (2·4 cm×100 cm) equilibrated with 0·01 M Tris buffer. Proteins were eluted with the same buffer and monitored at 280 mμ. The assay system for ribonuclease activity consisted of 1·5 mg yeast RNA, 100 μmoles of acetate buffer (pH 5·5) and 0·4 ml of effluent in a final volume of 2·0 ml. The incubation was carried out at 37 °C for 30 min. The increase in absorption at 260 mμ of acid soluble [0·3% $La(NO_3)_3$ in 2·5% trichloracetic acid] digestion products was measured. The assay system for phosphodiesterase activity contained 1 μmole of bis-(p-nitrophenyl) phosphate, 100 μmoles of acetate buffer (pH 5·5) or 100 μmoles of Tris-HCl buffer (pH 8·8) and 0·3 ml of effluent in a final volume of 2·0 ml. After 20 min of incubation at 37 °C, the reaction was stopped with 1 ml of 0·3 M NaOH, and increase in absorbance at 400 mμ due to the liberation of p-nitrophenol was measured. ●—●, protein; □ - - □, ribonuclease; ×. . . .×, phosphodiesterase (pH 8·8); △ - - - △, phosphodiesterase (pH 5·5)

The first peak (fractions Nos 35—40) represents a mixture of aggregated proteins. The enzymes corresponding to the other peaks, however, represent well defined nucleases which, after pooling the fractions, were purified by ion exchange chromatography and characterized. (As to the details of purification procedures and enzyme characterization refer to our earlier publications.)

We found that four nucleases can be assayed quantitatively right after Sephadex G—75 chromatography since each of the nuclease activities corresponding to these peaks represents one well defined enzyme. The phosphodiesterase activity corresponding to fractions Nos 40—50 represents an alkaline phosphodiesterase, an exonuclease producing 5'-nucleotides from

both RNA and DNA. The nuclease activity in fractions Nos 52—58 represents a sugar non-specific endonuclease which produces 5'-nucleotides from both DNA and RNA and has a relative specificity for adenine (sugar non-specific endonuclease I.) The RNase activity corresponding to fractions Nos 60—70 represents a relative purine specific endo-ribonuclease producing nucleoside 2',3'-phosphates which are slowly converted, if at all, into 3'-nucleotides. The nuclease activity in fractions Nos 46—53 corresponds to an acid phosphodiesterase. The smallest peak (fractions Nos 42—48) of nuclease activity proved to be inhomogeneous. It contained a closely related variant of the sugar non-specific nuclease (fractions Nos 52—58) and, in addition, another distinctly different sugar non-specific nuclease which has not yet been purified and characterized satisfactorily. A summary of the properties of the enzymes described is presented in Table 1.

Table 1

Properties of nucleolytic enzymes isolated from Avena leaf tissues

Enzyme	Substrate hydrolyzed	pH optimum	Mode of action	Final breakdown product	Relative specificity
Relative purine specific endo-ribonuclease	RNA	5·5	endo	2′,3′-cyclic phosphates	G > A > U > C
Sugar non-specific endonuclease I	RNA>DNA	5·5	endo	5′-nucleotides	A > G ≅ U(T) > C
Sugar non-specific endonuclease II	RNA>DNA	5·5	endo	5′-nucleotides	A > G ≅ U(T) > C
Alkaline phosphodiesterase	DNA>RNA	9·3	exo	5′-nucleotides	none
Acid phosphodiesterase	DNA>RNA	5·5	not investigated		

The effect of hormones on the level of nucleases described was tested in the following way. The first leaves of *Avena* seedlings were detached, the basal parts and tips of the leaves were removed to ensure relatively homogeneous material from a physiological point of view, and the remaining middle portions of the leaf blades were cut longitudinally into halves. One half of each leaf was floated of the surface of distilled water (control) and the other half on a solution of a growth regulator in Petri dishes. The Petri dishes were kept on a laboratory bench in diffuse day light for various periods. The leaf fragments were then extracted, and the proteins, after $(NH_4)_2SO_4$ precipitation (80% saturation), chromatographed on Sephadex G—75 as described above. The amount of various nucleases was determined from the elution profile of enzyme activity. The sums of the values of enzyme activities measured in each fraction belonging to a peak represent the total amount of the enzyme present in the tissues. Results of a typical experiments are presented in Table 2. After incubation of the tissues in 0·05 mM kinetin for 3 h the amount of the relative purine specific endonuclease decreased by about 50%. Incubation for 3 h in 0·005 mM abscisic acid

Table 2

Effect of growth regulators on the level of nucleolytic enzymes in the Avena leaf

Enzyme	Amount of nuclease[1] in control (—) and hormone treated (+) tissues						Amount of nuclease in zero-time controls[3]
	Kinetin[2] 0·05 mM			Abscisic acid[2] 0·005 mM			
	—	+	+/—	—	+	+/—	
Relative purine-specific endoribonuclease	16·8	9·0	0·53	12·6	24·6	1·95	7·1
Sugar non-specific endonuclease I	5·7	5·5	0·96	8·8	9·4	1·06	5·8
Alkaline phosphodiesterase	0·5	0·5	1·00	0·9	0·8	0·89	0·7

1. The amounts of nucleases are expressed as $\Sigma \Delta A_{260}$ (nucleases) and $\Sigma \Delta A_{400}$ values (phosphodiesterase). The figures presented represent the sum of all fractions belonging to a well defined peak of enzyme activity.

2. Three-week-old *Avena* leaf tissues were floated on water (—) or on solutions of various growth regulators (+) for 3 h before the extraction, separation, and quantitative assay of nucleases.

3. Zero-time controls consisted of leaf tissues extracted at the beginning of the incubation period.

4. All treatments were carried out in 3 to 5 replicates (separate experiments). Highly reproducible trends were obtained, although absolute figures varied from expriment to expriment. Representative examples are shown in the table.

resulted in a dramatic increase in the amount of the same enzyme. The level of the other nucleases was hardly affected by the hormone treatments.

In summary: (a) The *Avena* leaf contains a number of distinctly different nucleolytic enzymes. (b) We succeded in isolating, purifying and characterizing the major nucleolytic enzymes of the *Avena* leaf. (c) We provided evidence to show that the target of hormone action is quite specific since the overall change in nuclease level, which occurs upon hormone treatment, is due mainly, if not entirely, to a rapid change in the level of one nuclease only. (d) The action of hormones on the level of the relative purine specific ribonuclease is very rapid. Significant effects can be detected already in 1 to 2 h after treatment. (e) The response of the relative purine specific ribonuclease to hormone treatment represents an excellent system for studying the mechanism of enzyme regulation in plants and might give a clue to a better understanding of the role of the individual, distinctly different nucleases in the life of the green plant.

Naturally, it remains to be elucidated which nucleic acids are affected by the hormone-sensitive nuclease. Investigations along these lines are being carried out in our laboratory.

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ENVIRONMENTAL AND CHEMICAL CONTROL OF RNA BREAKDOWN IN LEAVES

by

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The ribonucleic acids of plant leaves are destroyed by several enzymes which are unusually sensitive to changes in environmental and metabolic conditions. Attempts to discover a regulatory mechanism for ribonuclease activity in leaves have been frustrated by the fact that, although these enzymes have been investigated in many plants, no single plant species has been studied intensively enough to permit construction of a model. Fortunately, one of the ribonucleases of plant leaves has characteristics which are sufficiently uniform from one plant species to another, that some generalizations can be made which will permit a preliminary discussion of metabolic control. Table 1 summarizes the biochemical properties of the enzyme called ribonuclease I by Reddi (1966) and Wilson (1968). Ribonuclease I hydrolyzes RNA with a preference for purine nucleotide bonds; end products of the reaction are a mixture of 2′3′cyclic nucleotides and 3′ purine nucleotides. Optimum activity occurs in the pH range from 5·0 to 5·5, with the precise pH optimum depending upon the plant source and the ion concentration of the isolation medium. Divalent ions inhibit ribonuclease I activity in crude homogenates and in some partially purified material.

Interesting changes in ribonuclease activity occur in leaves in response to stress treatments (Table 2). Udvardy et al. (1969) found that the ribonuclease I activity of *Avena* leaves increases over a 7·5 h period following leaf detachment. The increase in enzyme activity is completely inhibited by cycloheximide. A similar increase was found by Sodek and Wright (1969) in detached wheat leaves in the light; it is also dependent upon protein synthesis, and can be inhibited with chloramphenicol. Other inhibitors of protein synthesis have also been effective in blocking the early increase in ribonuclease activity in detached or damaged leaves and roots. While the use of crude homogenates in most of these studies leaves the contribution of ribonuclease I to the total ribonuclease activity open to question, the increase in ribonuclease I activity was greater than any other ribonuclease within the first 24 h following detachment of *Avena* leaves (Udvardy et al., 1969). Ribonuclease I activity also accounts for approximately 90% of the total activity of tomato leaves during the same time period (Dove, unpublished).

Ribonuclease activity of tomato leaves reaches a maximum in three to six hours after detachment (Dove, 1971). This "pulse" of activity following leaf detachment is due to mechanical damage brought about by a disturbance of the water balance of the leaf at the time of detachment. Bagi and

Table 1

Biochemical characteristics of partially purified ribonuclease[a]

Plant material[b]	Substrates	Products	pH optimum	Molecular weight	Inhibitors in enzyme assay	References
1. Corn *(Zea mays)* seedlings	RNA, with relative purine specificity; 2′3′ cyclic purine nucleotides also hydrolyzed	2′3′ cyclic nucleotides, 3′ nucleotides	5·0 in most preparations due to salt, pH 5·8 in low salt medium	23,000		Hanson, et al., 1965 Wilson, 1967 Wilson, 1963a Wilson, 1963b
2. Wheat *(Triticum vulgare)* leaves	RNA, with relative purine specificity; 2′3′ cyclic nucleotides also hydrolyzed	2′3′ cyclic nucleotides, 3′ nucleotides	5·2, but pH optimum changes in presence of salt		divalent cations in crude homogenates no inhibition with partially purified enzyme	Hadziyev et al., 1969 Matsushita, 1959 Sodek, 1970 Sodek, 1968
3. Oat *(Avena sativa)* leaves	rRNA, sRNA, with relative purine specificity-pA, pC, pU 2′3′ cyclic purine nucleotides also hydrolyzed	2′3′ cyclic nucleotides, 3′ nucleotides	5·5		divalent cations, p-chloromercuribenzoate; subject to product inhibition[c]	Udvardy et al., 1969 Wyen et al., 1969
4. Tobacco *(Nicotiana tabacum)* leaves and seedlings	RNA with relative purine specificity; 2′3′ cyclic purine nucleotides, pU hydrolyzed at a faster rate than pC	2′3′ cyclic nucleotides, 3′ nucleotides	5·1	approx. 32,000	some divalent cations	Bagi and Farkas, 1966 Reddi, 1958 Frisch-Niggemeyer and Reddi, 1957 Reddi, 1958

5. Pea *(Pisum sativum)* leaves	RNA, with relative purine specificity; 2′3′ cyclic purine nucleotides also hydrolyzed	2′3′ cyclic nucleotides, 3′ nucleotides	5·0			Holden and Pirie, 1955 Markham and Strominger, 1956
6. Mung bean *(Phaseus aureus)* sprouts	RNA, with relative purine specificity; 2′3′ cyclic purine nucleotides also hydrolyzed.	2′3′ cyclic nucleotides, 3′ nucleotides	5·5 activity at a given pH changes with NaCl concentration.		some divalent cations	Walters and Loring 1966 Stock and Vandendriessche, II, 1961 Stock and Vandendriessche, III, 1961

[a] Nomenclature proposed by Reddi (1966) and Wilson (1968) to include soluble ribonucleate nucleotido-2′ transferases (cyclizing) E. C. 2.7.17. All are probably endonucleases (Wilson, 1968).

[b] The ribonuclease activity of crude homogenates of other plant material shows similar characteristics at pH 5.

[c] Ribonuclease I isolated from intact leaves is identical to Ribonuclease I isolated from excised leaves incubated for 7·5 hours in the light.

Table 2

Characteristics of short-term changes in ribonuclease in intact tissues[a]

Plant material	Stress conditions which increase activity	Biochemical factors decreasing RNase activity *in vivo*	Chemical promotors of activity	References
1. Corn *(Zea mays)* seedlings	X-radiation of root tips			Cherry, 1962
2. Wheat *(Triticum vulgare)* coleoptile sections	detachment	pancreatic ribonuclease; indoleacetic acid, effect blocked by p-chloromercuribenzoate		Truelson, 1967
3. Oat *(Avena sativa)* leaves	detachment,[b] activity further increased by incubation in the light, sucrose substitutes for light	dichlorophenyldimethylurea, kinetin, cycloheximide		Udvardy et al., 1969 Udvardy et al., 1967
4. Pea *(Pisum sativum)* green stem internodes	detachment, incubation with sucrose	indoleacetic acid		Truelson, 1967
5. Tobacco *(Nicotiana tabacum)* leaves	rapid infiltration with water, mechanical damage, detachment	8-azaadenine, chloramphenicol, actinomycin D, kinetin, puromycin, p-fluorophenylalanine		Bagi and Farkas, 1968 Bagi and Farkas, 1967 Bagi and Farkas, 1966
6. *Rhoeo discolor* leaf sections	detachment	cyloheximide, alpha napthalene acetic acid[c], 5-fluorouracil 6-methylpurine, chromomycin	abscisic acid	DeLeo and Sacher, 1970

7. Tomato *(Lycopersicon esculentum)* leaflets	detachment, response prevented by excision of leaflet while immersed in isotonic solution	kinetin, indoleacetic acid, chloramphenicol, cytidylic acid, uridylic acid		Dove, 1971 Dove, 1967
8. Lentil *(Lens culinaris)* roots	excision	Indoleacetic acid[c]	abscisic acid	Pilet, 1970

[a] Enzyme assays use crude tissue homogenates unless otherwise noted.
[b] Partially-purified preparation.
[c] Antagonizes effect of abscisic acid.

Farkas (1966, 1967, 1968) produced elevated ribonuclease levels in tobacco leaf discs by infiltrating them with water. Wheat leaves synthesize abscisic acid when their water balance is disturbed by severe water loss (Wright, 1969; Wright and Hiron, 1969), and abscisic acid treatments increase the ribonuclease activity of leaves (DeLeo and Sacher, 1970), so that abscisic acid may be a chemical intermediate between the damage stimulus and the ribonuclease response.

Treatment of leaf tissue with growth regulators prevents the increase in ribonuclease activity while it stimulates synthesis of most proteins. Indoleacetic acid and kinetin are both effective in blocking the increase in ribonuclease activity which normally follows leaf detachment (Truelson, 1967; Udvardy et al., 1967; Dove, 1971). Inhibitors of RNA synthesis also prevent the increase in ribonuclease activity.

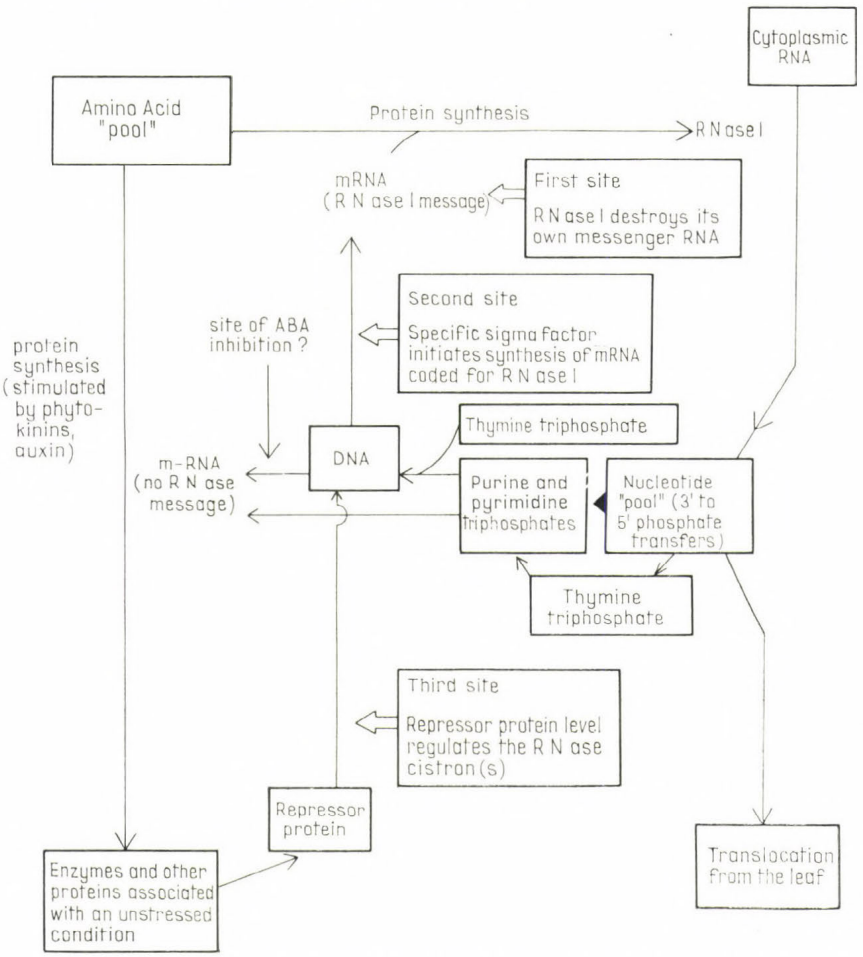

Fig. 1. Three possible sites for the metabolic regulation of leaf ribonuclease I. activity. Abbrevations: ABA-abscisic acid; RNase I. — ribonuclease I; mRNA — messenger RNA; rRNA — ribosomal RNA

Although much work remains to be done before precise models can be proposed to explain the metabolic regulation of ribonuclease I, some features of the control system are becoming clear: 1. Inhibitor studies indicate that ribonuclease I activity, which is stimulated by tissue damage, requires transcription of messenger RNA (mRNA) coded for ribonuclease synthesis. 2. The short duration of the ribonuclease "pulse" following detachment (two to four hours in tobacco leaves) and the rapid decline in ribonuclease I activity following the application of growth regulators or inhibitors of mRNA suggests that mRNA coded for ribonuclease I synthesis has a rapid turnover rate (Bagi and Farkas, 1966, 1967, 1968). 3. Growth regulators which stimulate synthesis of most types of RNA and proteins usually prevent synthesis of ribonuclease. 4. Stress conditions and abscisic acid treatments inhibit the synthesis of most types of RNA and protein while they stimulate synthesis of ribonuclease.

Most enzyme synthesis in unstressed leaves is probably greatest when phytokinin and auxin levels are optimal for growth. A smaller group of enzymes are synthesized primarily in damaged tissue. Synthesis of ribonuclease I, an enzyme of this latter group, might be regulated by any of three mechanisms (Fig. 1). First, ribonuclease I activity might be controlled by regulating the rate of destruction of the RNA which carries the message for ribonuclease I synthesis. A second possible regulatory site for ribonuclease I activity is the point on the DNA cistron where synthesis is initiated for the mRNA coded for ribonuclease I synthesis. As was mentioned earlier in the elegant paper by Dr. Biswas, a polypeptide called a sigma factor is required to initiate mRNA synthesis on a specific cistron of some bacteria and viruses (Watson, 1971). Similar initiation factors occur in higher plants, and it is possible that there may be different initiation factors for different stages of development. A specific initiation factor could initiate the synthesis of mRNA coded for ribonuclease I synthesis, along with other mRNAs coded for synthesis of other enzymes induced by stress. The other key factor in this positive control of enzyme synthesis is cyclic 3′5′adenosine monophosphate. In *E. coli* 3′5′cyclic AMP is required for the transcription of all mRNAs which are coded for the synthesis of enzymes inhibited by glucose catabolism. In higher plants, some specific nucleotide control factor could increase beyond threshold levels when the overall rate of RNA synthesis is decreased, and this increase in nucleotide level might promote synthesis of mRNA coded for ribonuclease I protein.

The third type of regulation would involve a repressor-derepressor system. A stress would inhibit synthesis of a repressor protein. Normal protein breakdown would deplete the previously-synthesized repressor protein, and the derepressed operator gene sites would synthesize mRNA coded for ribonuclease I protein. While the existence of such sites is highly speculative, it is hoped that the relationships discussed here will be useful in the future development of models based upon more complete experimental evidence.

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CYTOKININ-LIKE ACTIVITY OF A PYRIMIDINE DERIVATIVE

by

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PRELIMINARY EXPERIMENTS

As members of the Research Institute for Plant Protection in Budapest we were interested in the investigation of the inhibitory action of cytokinins on virus infections. It was shown that the suppression of TMV-infection by cytokinins is an indirect action, which is connected to the increased nucleic acid and protein synthesis by cytokinins in the host plant (Király et al., 1968).

We also investigated the mechanism of action of a series of pyrimidine antimetabolites in the inhibition of virus infections (Matolcsy et al., 1968). To our surprise, 6-methyluracil, β-ureidocrotonic acid (which is a possible precursor of methyluracil) and a third pyrimidine analogue exerted characteristic cytokinin activity in the chlorophyll retention test for cytokinins. This finding directed our attention to the pyrimidine derivative 6-methyluracil (Fig. 1) as a new candidate for cytokinins. It was known to us that all the available evidences suggested that an adenine nucleus was required for a typical cytokinin action assayed on the basis of growth-promotion (promotion of cell division), in other words by the tissue culture method. However, our finding with 6-methyluracil was supported only by an "undervaluated bioassay": the chlorophyll retention test, which, as used to say, suffers from lack of specificity.

Our pyrimidine derivative was active not only in this test, but also suppressed TMV-infection as did all the cytokinins. We concluded from a series of experiments that the bioassay based on the suppression of viral local lesions by cytokinins in tobacco leaves is a very sensitive one, although not very specific again.

It was also shown by several co-workers in the Research Institute for Plant Protection that 6-methyluracil stimulated the incorporation of precursors of nucleic acids and proteins (El-Hammady, 1968, Pozsár and Matolcsy, 1968, Matolcsy et al., 1968). As is known, the stimulation of protein and nucleic acid synthesis is also a characteristic feature of the cytokinin action. Again, this assay suffers from lack of specificity.

So, our pyrimidine derivative was active in 3 bioassays for cytokinins, but all of them suffered from lack of specificity. In the course of our own investigations we experienced that 6-methyluracil, like other cytokinins, is able to increase leaf growth of Pinto bean. Treating the halves

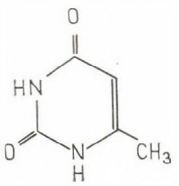

Fig. 1.
6-Methyluracil

of attached bean leaves with cytokinins or 6-methyluracil, we always were able to show an increase in leaf growth in the case of the treated half leaves. This assay involved a growth-promoting activity, supposedly a promotion of cell division. However, the quantitative estimation of leaf growth promotion is only a tentative assay.

TISSUE CULTURE BIOASSAY

After the preliminary experiments outlined above, it remained the last step, namely to apply the only "authorized" test: the tissue culture bioassay for the demonstration of the cytokinin action of 6-metyluracil.

The tissue culture test was carried out by using a local strain of callus culture from *Nicotiana tabacum* cv. *Samsun*. As is seen in Table 1, 6-methyluracil supported tissue growth even in 4 ppm concentration, which corresponds to 3×10^{-5} *M*. Actually, there was no difference between the action of 4, 20 or 100 ppm concentrations. Thus, on the basis of these investigations we claim that the methylated pyrimidine 6-methyluracil is a cytokinin. According to the definition of Mothes cytokinin is a substance which promotes cell division and exerts other growth regulatory functions in the same manner as kinetin. 6-Methyluracil fulfills these requirements because

(1) it is active in the retardation of senescence ("chlorophyll retention test"),

(2) it suppresses local lesions induced by TMV ("virus inhibition test"),

(3) it stimulates the incorporation of precursors into nucleic acid and protein ("test for protein and nucleic acid synthesis").

All thse properties are exerted by kinetin and other cytokinins, too. In addition, 6-methyluracil

(4) has a leaf growth-promoting activity ("leaf growth test"), and

(5) it stimulates cell division in tissue cultures ("tissue culture test").

These two last properties are specific for the cytokinin action.

SHOOT TIP CULTURE BIOASSAY

Finally, we would like to point out that the action of our methylated pyrimidine resembles to kinetin in shoot tip cultures too. Shoot tips of Samsun tobacco, consisting of the meristematic dome and leaf primordia,

Table 1

Effect of 6-methyluracil on the tobacco callus yield
Growth period: 25 days

	Fresh wt. mg/flask	Per cent
Control	1,900	100
6-Methyluracil		
0·8 ppm	2,090	110
4·0 ppm	2,660	140
20·0 ppm	2,774	146
100·0 ppm	2,755	145

were dissected from axillary buds and placed in a liquid medium according to Linsmaier and Skoog (1965). As is seen in Fig. 2, after a 4-month period of growth, both kinetin and 6-methyluracil were able to promote shoot and leaf formation and growth as a whole. This morphogenetic function

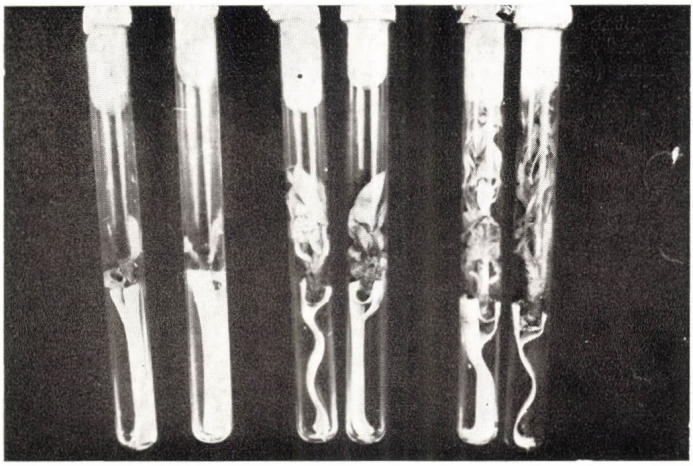

Fig. 2. Promotion of growth of tobacco in shoot tip cultures by kinetin (2·56 mg/liter) and 6-methyluracil (20 mg/liter). Control: two tubes on the left. 6-methyluracil: two middle tubes. Kinetin: two tubes on the right

of 6-methyluracil might be a sixth bioassay for the demonstration of the cytokinin character of our methylated pyrimidine.

Considering the fact that cytokinins increase the rate of methylation of *t*-RNAs (see the paper of Abeels et al. in this volume, pp. 69—74 as well as Abeels and Montasser Kouhsari, 1970) it is now easier to accept that 6-methyluracil, a methylated pyrimidine turned to be a cytokinin.

SUMMARY

A pyrimidine derivative, 6-methyluracil was active in six different bioassays for cytokinin, including tissue culture and shoot tip culture tests as the most specific ones. Accordingly, this compound is considered as a typical cytokinin.

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SOME PROBLEMS RELATED TO RNA METABOLISM IN ROOTS

by

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Using 5 mm long tips of 18 mm long roots *(Lens culinaris)*, it was observed that the cells of root caps contained less ribosomes than the cells of young tissues (quiescent center and meristem). The RNA content was much higher in young cells than in older ones. The RNA level was found to be related to the RNase activity and tha endogenous auxin content. Young cells contained more RNA and auxin than the old ones and their RNase activity was less.

NAA and IPC were both used to change the auxin cencentration; IPC was found to decrease the auxin level singnificantly. It was observed that neither NAA nor IPC had a direct action on the *in vitro* destruction of RNA, by a pure RNase.

In root tips incubated for 10 h, RNase activity increased. This increase was reduced by NAA and stimulated by IPC. Apparently, two RNase systems are present in the root extracts; NAA and IPC appear to act on one of them only.

The RNA content decreased in the root time during the incubation. This decrease was lessened by NAA and enhanced by IPC. Interrelations between auxin level and metabolism are discussed.

INTRODUCTION

Many papers suggest that the action of auxin on the growth of plant cells is directly associated with the metabolism of RNA (Trewavas, 1968*a*). It has been confirmed recently that auxin stimulates the biosynthesis of RNA (Trewavas, 1968*b*) but very little is known as regards the effect of auxin on the *in vivo* RNA destruction (Phillips and Fletcher, 1969). It was found, however, (Truelsen and Galston, 1966) that RNase activity is related to the intensity of growth which, for *Triticum* coleoptiles, decreased after addition of RNase (Truelsen, 1967). A similar observation has been made with *Lens* root and an antagonistic interaction has been noted between auxin and RNase (Pilet, 1969). An inverse proportionality has recently been observed (Pilet and Braun, 1970) in *Lens* root tips, between RNA and RNase and it has been found that auxin treatment causes an increase of RNA and of auxin content, and a decrease in RNase activity.

In the work reported here, the relations between auxin level, RNA and RNase were analysed for the root tip of *Lens culinaris*. Also, β-naphtyl-acetic acid (NAA) and isopropyl-N-phenyl-carbamate (IPC) were used to test the effect of auxin on the RNase activity and RNA content. NAA was

chosen because it is an active auxin (Pilet, 1961), practically not destroyed *in vivo*, which is not the case for natural auxin, β-indolyl acetic acid (IAA). IPC was used because it reduces the endogenous auxin level (Mann et al., 1967).

MATERIAL AND METHODS

Biological test

Seeds of *Lens culinaris* (var. *Vilmorin*) were soaked (4 h) and placed (24 h) on filter paper in Petri dishes moistened with deionised water (dark; 25 °C). Seedlings were then selected (4 mm long roots) and cultivated under the same conditions. After 72 h, the 18 mm long roots were cut — when used for experimental purposes — at a distance of 5 mm from the tip. The apical segments were incubated (10 h) with shaking (dark; 25 °C) in 20 ml of 0·01 M Tris-(hydroxymethyl)-amino-methane-maleate (pH 6·5) containing 1·5% sucrose (Hamilton et al., 1965) and streptomycin (12 μg/ml). Only green light was used during the manipulations.

Auxin content

The method used for extracting and separating endogenous auxin by SiO_2 chromatography (Collet et al., 1964), and the technique for analysing the biological activity of the different regions of the chromatogram have already been described in detail (Pilet, 1963). Attention has so far been confined to the acid fraction of the ethyl–acetate extract, according to the technique of Thurman and Street (1960) and the chromatographic running solvent has been isopropanol: ammonia 28%: water (8 : 1 : 1). Bio-assays consisted of the *Lens* root section test (Pilet et al., 1960).

RNase activity

The technique has been discussed previously for similar material (Pilet and Braun, 1970). 500 mg of roots were ground in the presence of quartz sand in a mortar with 20 ml 0·1 M ammonium acetate buffer, pH 5·0. The homogenate was centrifuged 30 min at 12,000 g and the supernatant diluted 1 : 10 with the buffer. The assay was performed in a manner similar to that previously described (Truelsen, 1967). To 1 ml RNA solution (2 mg Fluka RNA per ml buffer), 0·5 ml diluted extract was added. The reaction was either stopped immediately (controls) or after 30 min of incubation at 37 °C. Undegraded RNA was precipitated by the addition of 0·25 ml perchloric acid (2·5 M) containing 0·75% uranyl–acetate. After allowing the tubes to stand at least 30 min at 4 °C, the samples were centrifuged

for 15 min at 5,000 g. The supernatants were diluted 1 to 10 with water and their optical density measured at 260 nm. The difference in absorbance of the controls and the incubated samples was taken as a measure of RNase activity, which was expressed in terms of ΔOD per 50 mg fresh weight.

RNA content

Extraction and analyses of RNA were performed in a manner similar to that previously described (Pilet and Braun, 1967).

Roots were ground in a mortar under liquid nitrogen. After sufficient homogenization and evaporation of the liquid nitrogen, 6 ml of TCA-acetone (5 g trichloracetic acid in a mixture of 47·5 ml water and 47·5 ml acetone) were added. After further homogenization the suspension was centrifuged for 15 min at 4 °C and 2000 rpm (= 1000 ×g). The supernatant (S_1) was discarded and the precipitate (P_1) was kept for extraction. The advantage of TCA-acetone (Daniel and Baldwin, 1964) as compared to the usual TCA or PCA (perchloric acid) extraction media consists in its ability to remove pigments and lipids, thus eliminating the necessity of extraction with alcohol and ether.

Precipitate P_1 was again washed with 6 ml of TCA-acetone and the suspension centrifuged as above. Supernatant S_2 was discarded and precipitate P_2 further processed. To P_2, 6 ml of 0·25 N PCA was added and the suspension was centrifuged as above. Supernatant S_3 was discarded and precipitate P_3 further processed. Washing with PCA was repeated resulting in precipitate P_3.

To precipitate P_4 3 ml 0·4 N NaOH was added and the mixture incubated 16 to 20 h at 37 °C. Then 1 ml 1·5 N PCA was added, thus precipitating DNA and proteins. To ensure complete precipitation, the suspension was kept for at least 1 h at 4 °C. The suspension was centrifuged for 15 min at 4 °C at 2000 rpm. Supernatant S_5 was stored in the cold, while 2 ml 0·25 N PCA was added to precipitate P_5. The resulting suspension was thoroughly mixed and centrifuged as above. Precipitate P_6 was stored in the refrigerator and supernatant S_5 and S_6 were combined for RNA assay, according to the method of Ceriotti (1955). One ml water (blank) or 1 ml suitable diluted ribose solution (standard) or 1 ml suitable diluted extract were added to 7·5 ml orcinol reagent. This reagent was prepared in the following way: Solution A: 68 mg $CuCl_2$ was dissolved in conc. HCl and brought to 100 ml with conc. HCl. Solution B: 1250 mg orcinol was slowly added to a mixture of 440 ml H_2O and 450 ml conc. HCl. Final reagent: 50 ml solution A and 950 ml solution B.

For hydrolysis, the tubes were placed in a boiling bath for 40 min. After hydrolysis, the tubes were cooled in cold tap water and the optical density was measured immediately in a spectrophotometer or colorimeter at 675 nm, without isoamyl-alcohol extraction. Assuming the hydrolysis of purine ribosides to be quantitative and the hydrolysis of pyrimidine ribosides to be zero (Ceriotti 1955), a conversion factor of 3·76 $\pm$ 0·13 was used to calculated mg RNA from the ribose content.

Presentation of the results

All data will be given (Pilet and Nougarède, 1970) both in units of N-protein determined by UV-absorption (280 nm) after elimination of the interferences of nucleic acids, (Warburg and Christian, 1941) and in units of cells according to a technique based on the use of the Navachine reagent and pectinase incubation (Humphries and Wheeler, 1960).

RESULTS AND DISCUSSION

Endogenous interrelations

The advantage of working with root tips is that they have both very young tissues (meristem and quiescent center) and older ones (root cap) (Pilet, 1969*b*).

With special guillotine, two series of sections were prepared from 18 mm long roots of etiolated *Lens* seedlings. The first sections (from 0 to 200 μ from tip) contain mainly old cells; in the second sections (from 200 to 500 μ), there are mainly young cells. Ultrastructural properties of the two regions were previously analysed (Pilet and Nougarède, 1965). The ribosome density significantly changes from the first region to the second one. The data obtained (Table 1) clearly show that the cells of the root cap contain less ribosomes

Table 1

Density of ribosomes in the root tips of Lens culinaris

	Old cells	*Young cells*	
	root cap	quiescent center	meristem
Distance from tip in μ	0 — 200	200 — 250	250 — 500
Number of ribosomes/5 μ^2			
mean values	592*	1,750	3,446
$\pm$	112	254	363

* The few cells of calyptrogen were not counted; 240 counts of an aera of 0·25 μ^2.

Table 2

Comparative RNA content of the root tips of Lens culinaris

RNA in μg	*Old cells*	*Young cells*
per 10 mg fresh weight	18·8	118·0
per 10^6 cells	22·3	45·6
per 0·1 mg N-protein	52·9	93·4

Each result is the average of 17 values.

than the cells of young tissues of the root tip. Such observation already indicate that the total RNA level will not be the same in the two regions under study.

In fact, the analysis of RNA concentration (Table 2) shows that there is far more RNA in the young cells than in the older ones, and this is also the case whatever parameter is used, for the expression of the results.

However, the level of total RNA in the root tip is directly related to the RNase activity on the one hand, and to the endogenous auxin concentration on the other. By expressing all the values in protein units (Table 3), it can be noted that if the young cells contain more RNA, they also possess more auxin, and lower RNase activity.

Table 3

RNA content, RNase activity and auxin level of the root tip of Lens culinaris

	Old cells	*Young cells*
RNA in μg per 1 mg N-protein	529	934
RNase in Δ OD_{260} per 0·5 mg N-protein	0·687	0·019
Auxin in 10^{-3} μg eq. IAA per 10 mg N-protein	1·78	706·07

Change in the auxin level

The auxin content of young and old cells differs greatly. The results presented in Table 3 show that the RNA level and RNase activity are also different in young and older cells. Does a causal relationship exist between the variations of auxin level and RNA metabolism?

First it was of interest to analyse, for the two regions considered, the effects caused by IPC on the endogenous auxin level. As can be seen (Table 4), IPC causes a significant decrease of auxin level as it has already been observed by Mann et al. (1967). One can note that the decrease in concentration of auxin extracted from root tips treated with IPC was significant only in the young cells.

RNase activity

In vitro experiments. In the presence of RNase, the increase of OD (at 260 nm) of a RNA solution, as resulting from the progressive loss of primary and secondary RNA structure (hyperchromicity analyses; Inman, 1964) can be considered as a reliable method to test the *in vitro* RNA destruction (Mahler and Cordes, 1966).

In the present assay, 30 μg/ml of RNA was incubated (37 °C) in 2·5 ml of 0·01 M Tris-HCl buffer (pH 7·4) in the presence or absence of NAA or IPC (20 μg/ml). At zero time, 0·05 ml of RNase (Pilet, 1969*a*), 2 μg/ml, was rapidly added. RNase was previously heated (10 min; 95 °C) to inactivae DNase.

Table 4

Auxin content of the root tip of Lens culinaris treated with isopropyl-N-phenyl carbamate (IPC)

Endogenous auxin 10^{-3} µg IAA equivalents per 10 mg N-protein

IPC in µg/ml	*Old cells*	*Young cells*
0	1·78	706·07
10	1·84	509·18
20	1·62	343·06

Each result is the average of 12 values.

Table 5

In vitro action of NAA and IPC on the hydrolysis of RNA by RNase

(Hyperchromicity analyses: see p. 317)
Values in Δ OD at 260 nm

Incubation time (min)	*Assays*		
	RNA + RNase	RNA + RNase + NAA	RNA + RNase + IPC
0	0·725	0·736	0·718
	±0·015	±0·017	±0·015
30	1·042	1·019	1·034
	±0·023	±0·021	±0·020
60	1·136	1·148	1·130
	±0·027	±0·032	±0·025

Each result is the average of 8 values.

Table 6

Effect of NAA on the RNase level of the apical segment (initial length: 5 mm) of 18 mm long roots of Lens culinaris. RNase activity in Δ OD_{260} per 50 mg of fresh weight. Incubation: 10 h

	Concentration of NAA (µg/ml)		
	0	10	20
0 h	0·135	—	—
10 h	0·161	0·147	0·139
0—10 h	0·026	0·012	0·004
% increase	19·2	8·8	2·9
±	4·1	2·6	0·8

Each result is the average of 15 values.

Table 7

Effect of IPC on the RNase level of the apical segment (initial length: 5 mm) of 18 mm long roots of Lens culinaris. RNase activity in Δ OD_{260} per 50 mg of fresh weight. Incubation: 10 h

	Concentration of IPC (μg/ml)		
	0	10	20
0 h	0·140	—	—
10 h	0·164	0·183	0·179
0—10 h	0·024	0·043	0·039
% increase	17·1	30·7	27·8
±	3·9	7·2	5·7

Each result is the average of 15 values.

Results are presented in Table 5. It can be seen that neither NAA nor IPC has a significant effect on the *in vitro* destruction of RNA by RNase.

NAA action. After 10 h of incubation, in the presence or absence of NAA (10 and 20 μg/ml), apical root segments were cut off and their RNase activity was tested. Results are given in Table 6. In the control, as previously described (Pilet and Braun, 1970), the RNase activity increased. NAA caused a significant, concentration dependent, inhibition of this increase

Previous observations have shown that the distribution of RNase activity is related to that of endogenous auxin: high RNase activity was found to be connected with low auxin content. Similar conclusions were drawn by Truelsen (1967) on the basis of experiments using *Pisum* stem and *Triticum* coleoptile treated by IAA.

IPC action. A similar assay was performed with IPC. As shown in Table 7, IPC caused a significant stimulation of the increase of RNase activity. No significant difference was found for the two concentrations tested, even though (Table 4) with an increasing concentration of IPC a decreasing level of endogenous auxin level was observed. The fall in auxin concentration, due to IPC (Mann et al., 1967), can be related to the stimulation

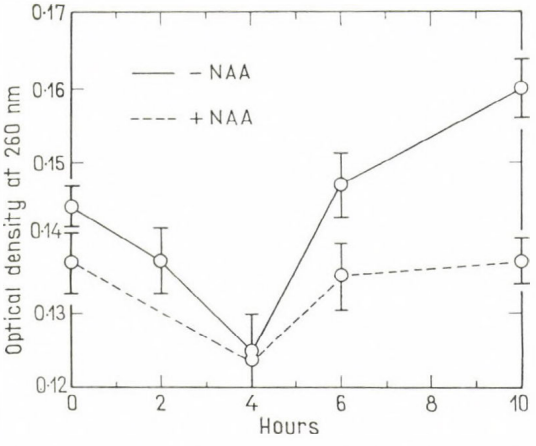

Fig. 1. Effect of NAA on RNase level in the apical segment (initial length: 5 mm) of 18 mm long roots of *Lens culinaris*. Change of RNase activity with the time (in h) of incubation. RNase activity is expressed in ΔOD at 260 nm per 50 mg of fresh weight

of RNase activity; this observation was, therefore, in agreement with earlier data.

Nature of RNase. The RNase activity was also analysed as related to the incubation time and treatment of the segments with NAA (Fig. 1) and IPC (Fig. 2). As can be seen, in both the control (—NAA and —IPC) and the treated (+NAA and +IPC) segments, the activity of RNase decreased during the first 4 h of incubation but increased rapidly afterwards.

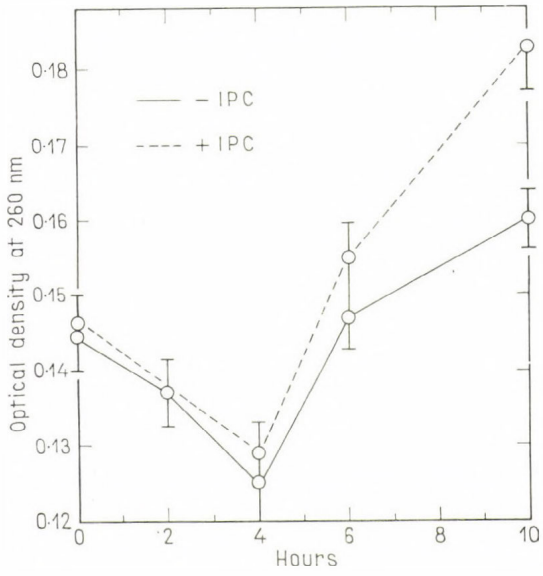

Fig. 2. Effect of IPC on RNase level in the apical segment (initial length: 5 mm) of 18 mm long roots of *Lens culinaris*. Change of RNase activity with the time (in h) of incubation. RNase activity is expressed in ΔOD at 260 nm per 50 mg of fresh weight

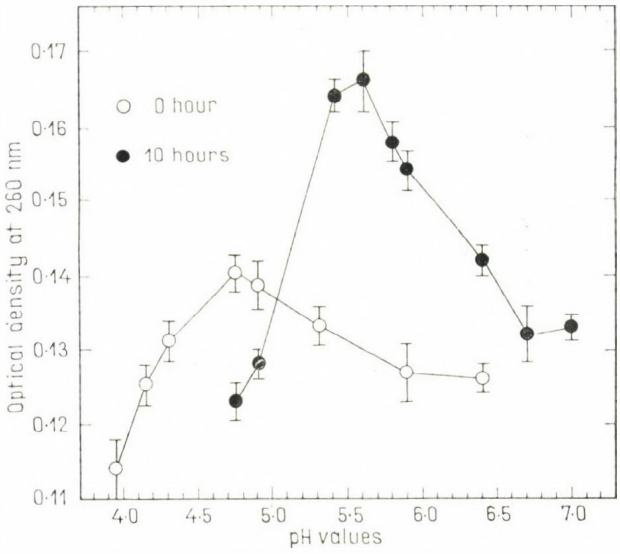

Fig. 3. Effect of pH on the RNase prepared from the apical segment (initial length: 5 mm) of 18 mm long roots of *Lens culinaris*. RNase activity is expressed in ΔOD at 260 nm per 50 mg of fresh weight

Such a curve might indicate the presence of at least two enzyme systems: the activity of the first one decreasing as incubation starts, and that of the second one, increasing. It is interesting to note that NAA and IPC (both used at a concentration of 20 μg/ml) have a small (NAA) or no (IPC) effect on the first part of the reaction, while the second part was clearly inhibited by NAA and significantly stimulated by IPC. It would, therefore, appear that the change in the RNase activity due to the effect of NAA or IPC, are confined to the second (hypothetical) enzyme system.

The existence of two different RNases is confirmed to some extent by studies on the optimum pH of the extracts. As seen in Fig. 3, the pH optimum at zero h incubation (just after extraction) is approximately 4·8 and that after 10 h incubation nearly 5·6. The presence of two RNase systems has already been observed in germinating *Triticum aestivum* seeds (Vold and Sypherd, 1968) and the optimum pH found for the two RNases is very close to those reported here.

RNA content

NAA action. After incubating the root with or without NAA, apical root segments were cut off and the total RNA concentration was analysed. Results presented in Table 8 show a decrease of RNA content in the control as previously noted (Pilet and Braun, 1967) and indicate that this decrease is less apparent with increasing NAA concentrations. The results confirm earlier observations made on similar material (Pilet and Braun, 1967, 1970). The decrease of endogenous RNA content could be largely counteracted by auxin treatment.

IPC action. Similar assays were performed with root segments treated with IPC. The effects are shown in Table 9. With IPC-treatment, the loss of RNA was greater than in the controls.

Table 8

Effect of NAA on the amount of RNA in the apical segment (initial length: 5 mm) of 18 mm long roots of Lens culinaris

RNA content in µg per 200 mg of fresh weight. Incubation: 10 h

	Concentration of NAA (µg/ml)		
	0	10	20
0 h	569	580	554
10 h	416	519	531
0 – 10 h	153	61	23
% decrease	26·9	10·5	4·1
±	5·0	2·2	0·9

Each result is the average of 12 values.

Table 9

Effect of IPC on the amount of RNA in the apical segment (initial length: 5 mm) of 18 mm long roots of Lens culinaris

RNA content in µg per 100 mg of fresh weight. Incubation: 10 h

	Concentration of IPC (µg/ml)		
	0	10	20
0 h	569	551	582
10 h	416	340	348
0—10 h	153	211	234
% decrease	26·9	38·2	40·2
±	5·0	6·2	7·3

Each result is the average of 12 values.

CONCLUSIONS

The present data clearly show that the change in RNA concentration is correlated with a change in RNase activity: a decrease in RNA level is in direct relation to the increase in RNase activity. Such a relationship has previously been analysed (Truelsen, 1967. Trewavas, 1968*a*; Grellet et al., 1968; Pilet and Braun, 1970). The inverse relationship between RNase activity and RNA content indicates that auxin may also control the RNA metabolism. When NAA was added, the RNase activity decreased, which may produce an increase in endogenous RNA. By adding IPC, the auxin level decreased, and a stimulation of RNase activity was observed. Consequently, a decrease in endogenous RNA was also found. It has, however, to be kept in mind that, for the moment, it is not possible to decide whether the RNase activity analysed here is the only RNase active *in vivo*. Close relationship can, however, be found between growth, controlled by auxin and RNA metabolism, which is also related to auxin action.

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RIBONUCLEIC ACID SYNTHESIS AND HORMONE ACTION IN LENTIL ROOTS

by

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The synthesis of specific RNA is needed for the indoleacetic acid induced formation of peroxidases associated with lentil root ribosomes. In lentil roots two different classes of rapidly labelled RNA can be fractionated on methylated albumin columns. One fraction (α) is uridine-rich, heterodisperse, with a mean life lower than 1 hr. The other fraction (β) is adenine-rich and contains polysomic mRNA. RNA synthesized in vitro, by isolated nuclei, have properties similar to these two RNA species. A close similarity exists between uridine-rich RNA described in animal cells and the nuclear rapidly labelled RNA of lentil roots contained in fraction α.

Short treatment of lentil roots with indoleacetic acid increases the amount of nuclear uridine-rich RNA. A prolonged treatment causes an increase in rapidly labelled RNA in fraction β. The possibility that D-RNA could play an important role in the regulation of gene expression is discussed.

INTRODUCTION

Previous studies from our laboratory have shown the presence of three isoperoxidases associated with the ribosomal fraction of lentil roots. During cellular fractionation, these haemoproteins are present on these particles and not in the other fractions. These peroxidases were isolated and obtained in a highly purified form by ion-exchange chromatography and molecular sieving. One is slightly acid whereas the others are slightly basic. The incorporation of ^{14}C-leucine into these haemoproteins has enabled us to show that the basic peroxidases are rapidly synthesized. Treatment of the roots with indoleacetic acid considerably stimulates the de novo biosynthesis of the two basic peroxidases but not that of the acid peroxidase (Penon et al., 1970).

The possibility that, in the hormone action, a regulational event could take place at the transcriptional level has been a concern of this paper. Our results show that induced peroxidase synthesis requires the synthesis of some RNA species. Two fractions of RNA obtained from methylated albumin columns, D-RNA and TB-RNA, were characterized and are apparently involved in the tissue response to hormone treatment. Among the RNA synthesized in vitro by isolated nuclei exist RNA species with chromatographic and sedimentation properties similar to the two RNA species previously described. Thus, knowledge obtained from the in vivo experiments may be applicable to the investigations on isolated nuclei.

Particular attention has been given in this paper to D-RNA which for its properties (metabolic instability, base composition, sedimentation velocity) can be considered homologous to animal HN-RNA. The significance of this RNA, considering its possible role in regulation, will be discussed.

METHODS

The methods utilized in these studies have been published elsewhere (Penon et al., 1970; Miassod et al., 1970). Details pertinent to various experiments are described in the legends of figures and table.

RESULTS

Figure 1, curve 1, shows the effect of hormone on peroxidase synthesis in lentil roots. Auxin, after an initial lag period of about 5 h, causes an increase of peroxidase level in the ribosomal pellet. This increase results from the de novo biosynthesis of the two basic peroxidases. A similar lag period was observed by Gayler and Glasziou (1968) during the induction of peroxidase activity in sugarcane slices. The addition of RNA synthesis inhibitors, such as 6-methyl purine, at the same time as the hormone (Fig. 1, curve 2), completely inhibits peroxidase formation. No change in peroxidase activity can be observed for at least 15 h in this experiment. When 6-methyl purine is added to lentil roots, without preliminary hormonal treatment (Fig. 1, curve 3), the peroxidase level remains stable for the duration of the experiment which has been extended up to 15 h. If added after 6 h of indoleacetic acid treatment (Fig. 1, curve 4), 6-methyl purine causes a rapidly diminishing rate of increase in peroxidase activity. There is no subsequent loss of enzyme activity. These data show that initially messenger RNA synthesis is limiting for peroxidase formation.

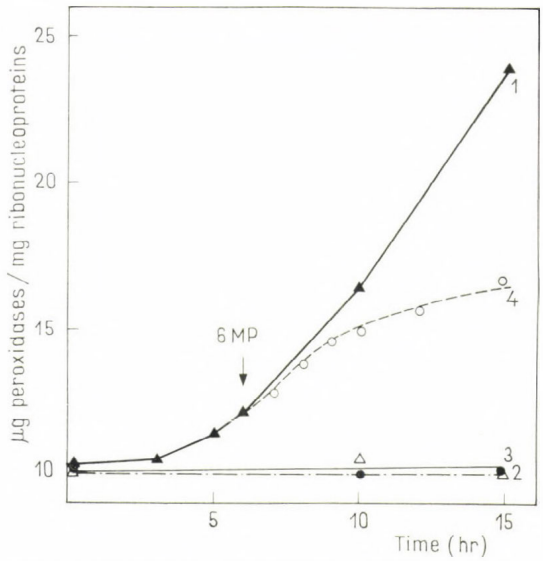

Fig. 1. Effect of 6-methyl purine on IAA induced peroxidase synthesis in lentil roots. Tissue samples were incubated in a medium containing IAA 0·15 mM, for the periods shown (curve 1). Other samples were incubated in a medium containing either IAA 0·15 mM and 6-methyl purine 0·1 mM (curve 2), or 6-methyl purine only (curve 3). After 6 h, some IAA treated samples were transferred to 6-methyl purine 0·1 mM (curve 4). Tissue samples were ground at the intervals shown, ribosomes were extracted and assayed for peroxidase activity

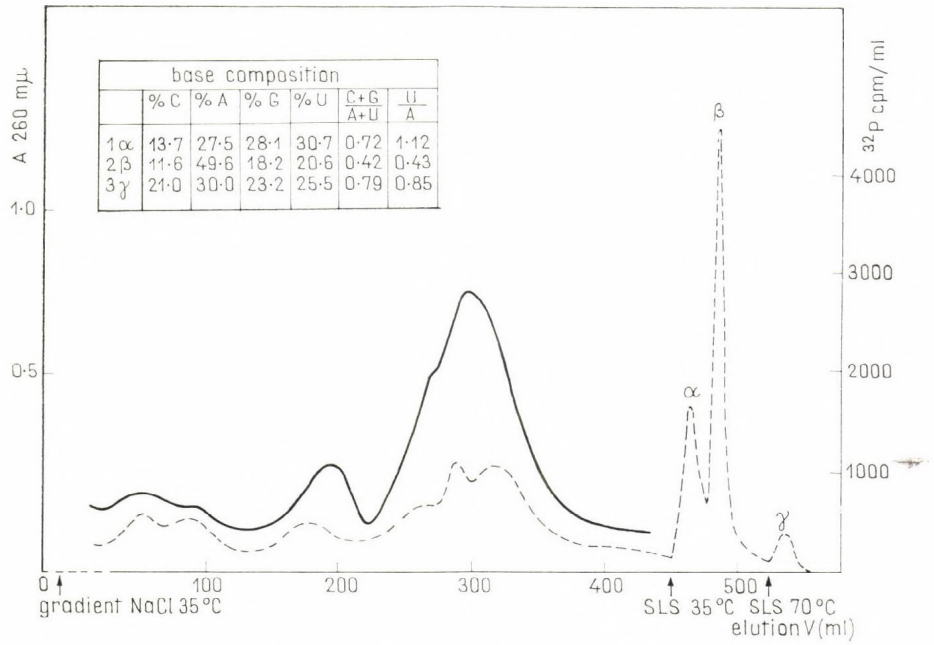

Fig. 2. Fractionation of (^{32}P) labelled RNA from lentil roots on MAK column. Tissues were labelled, for 15 min, with (^{32}P) and RNA was extracted as previously described. The RNA (4·6 mg) was applied to a methylated-albumin kieselguhr column and eluted by a linear NaCl gradient (0·2 M to 1·3 M). TB-RNA was eluted by a medium containing SDS 2% and EDTA 1 mM, first at 35 °C then at 70 °C. Absorbancy at 260 nm was measured and radioactivity was determined as described by Miassod et al. (1970). Aliquots of fractions α, β, γ were hydrolysed and base composition was determined

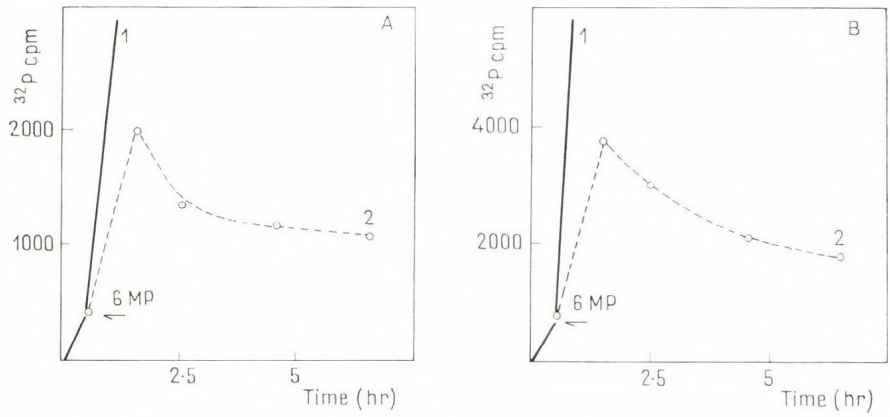

Fig. 3. 6-methyl purine chase of (^{32}P) labelled RNA from α and β fractions. The roots are labelled with (^{32}P) (curves 1). Some samples (curves 2), after 30 min of labelling, are transferred in a chase medium containing NaH_2PO_4 0·1 M and 6-methyl purine 0·1 mM, for the intervals shown. RNA are extracted and analyzed by MAK chromatography. Figures A and B show the kinetics of incorporation and chase of RNA from peaks α and β

An interpretation of these observations is that indoleacetic acid is involved in the regulation of synthesis of some specific RNA. It was of interest to investigate RNA synthesis in lentil roots. In an attempt to characterize the rapidly synthesized RNA in these tissues, we have fractionated (^{32}P) labelled RNA from roots on MAK columns, and determined the base

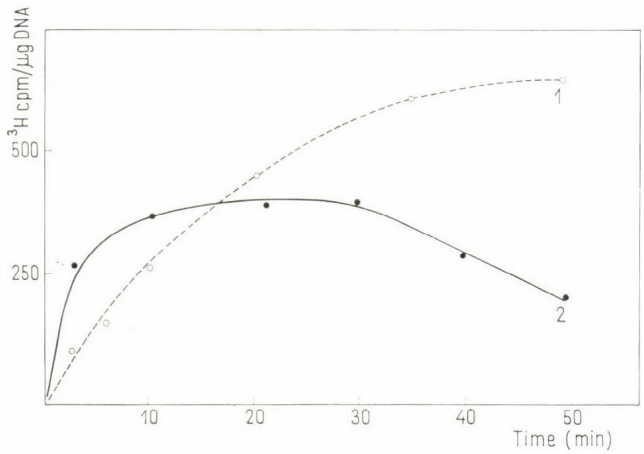

Fig. 4. Kinetics of ^{3}H-UTP incorporation by isolated lentil root nuclei. Reactions were carried out in 400 µl, at 15 °C (curve 1) and 37 °C (curve 2). The reaction mixture contained ATP, CTP, GTP, 0·125 mM each, ^{3}H-UTP 0·0125 mM (1 µCi/0·005 µmole), phosphoenol pyruvic acid 0·5 mM, pyruvate kinase 8 µg, Cleland reagent 3·5 mM, spermine tetrahydrochloride 0·2 mM, $MgCl_2$ 6·5 mM, ammonium sulphate 2·5 mM, Tris HCl pH 7·8, 125 mM

composition of various fractions. The RNAs (^{32}P) labelled for 15 min were eluted with a linear gradient of sodium chloride from 0·2 M to 1·3 M. The major part of TB-RNA was eluted by 2% sodium dodecyl sulphate, containing 1mM EDTA, at 35 °C. The rest could be recovered by continuing the SDS elution and elevating the temperature to 70 °C. This procedure prevents degradation of TB-RNA (Ellem, 1966).

The elution pattern of TB-RNA can be seen in Fig. 2. Three peaks α, β, γ can be observed. Peak α has a base composition characterized by a high content of uridine and shows some similarity in relative guanine + cytosine content to lentil root DNA (the molar G + C content of lentil root DNA is about 42% as estimated from its buoyant density $\varrho = 1{\cdot}693$). The composition of peak β differs from that of the former RNA and is characterized by a higher adenosine content. Previous experiments have shown that RNA of fraction α is polydisperse with high sedimentation velocity. The RNA of peak β has a lower sedimentation coefficient (12 S) than RNA from fraction α. We could detect no significant change in base composition of fraction β at any labelling time (Miassod et al., 1970).

The decay of these RNAs was measured by incubation of the roots in (^{32}P) for 30 min, followed by a chase in a (^{31}P) medium containing

a RNA synthesis inhibitor. Since penetration of large molecules, such as actinomycine D, took at least 2 or 3 h and introduced errors into measurement of decay time for short lived RNA, 6-methyl purine 0·1 mM was used. The specific activity of RNA increased during the first hour of the chase and then decreased. After 2 h of chase (Fig. 3A, curve 2), a substantial fraction (about 40%) of the label in peak α disappeared. There is no subsequent loss of radioactivity. Indeed, we have observed (Miassod et al., 1970) that on prolonged labelling, rRNA precursors and mature rRNA are present in peak α. Such experiments indicate a half life lower than 1 h for the rapidly labelled unstable RNA. Under similar conditions of chase, the RNA of fraction β is broken down with a half life of around 2 h (Fig. 3B, curve 2).

Table 1

Requirements for ^{3}H-UTP incorporation into RNA by isolated lentil root nuclei

System	Incorporation cpm/μg DNA	% control	
Complete	480	100	(4)
— CTP, ATP, GTP	38	7	(1)
— Mg^{2+}	6	1	(3)
Actinomycin D 75 μg/ml	53	11	(3)
Rifampicin 75 μg/ml	496	103	(3)

Reaction was carried out at 15 °C, for 30 min, in 0·4 ml as described in the legend of Fig. 4. Incorporation is stopped by addition of 2 ml of 5% TCA. After centrifugation, the pellet is washed three times, by 5% TCA, then hydrolyzed in 5% TCA at 90°C, for 30 min. Hydrolysate is counted in a dioxane scintillation solution containing 5 gm PPO, 0·35 gm POPOP and 100 gm naphthalene per lit. The non specific incorporation is 7 cpm/μg DNA. The results are mean values and the numbers of experiments are given in parentheses

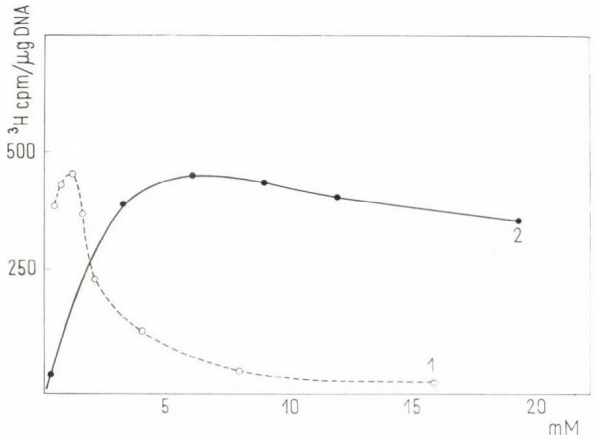

Fig. 5. Effect of divalent cation concentration on polymerase activity of isolated lentil root nuclei Incubation conditions were as described in Fig. 4. Reaction was carried out at 15 °C, for 30 min. Mg^{2+} concentration was varied as indicated (curve 2). Mn^{2+} effect (curve 1) was studied with a modified medium containing Mg^{2+} 3 mM and ammonium sulphate 0·2 M

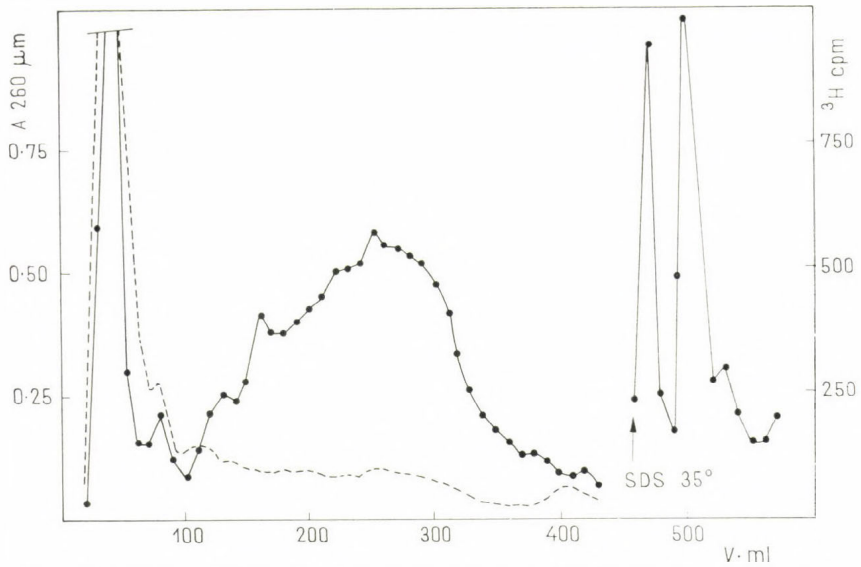

Fig. 6. Fractionation of ^{3}H-RNA synthesized in vitro on MAK column. Incubation was done, in the presence of Mn^{2+} 2 mM and ammonium sulphate 0·2 M, in 10 ml, at 15 °C, for 30 min, as described in Figs 4 and 5. Elution conditions were as described Fig. 2. Elution pattern of TB-RNA eluted with SDS 2% at 70 °C, is not represented

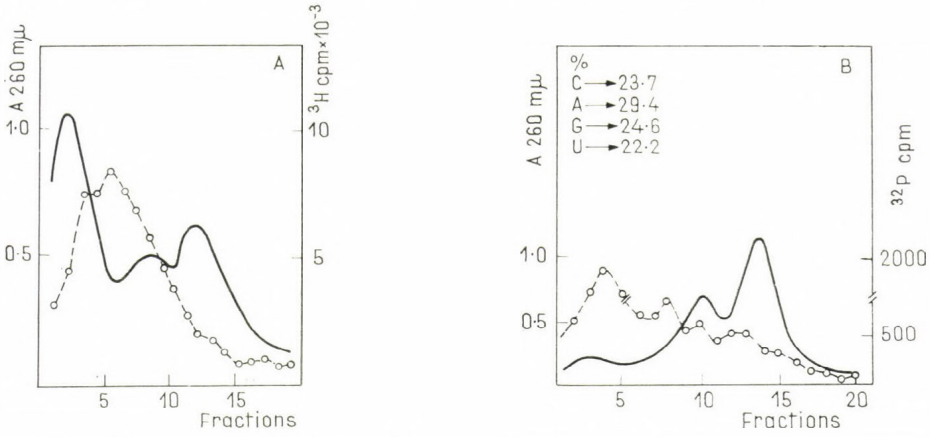

Fig. 7. Sedimentation profiles in SDS-sucrose density gradient of nuclear RNA synthesized in vitro and polysomic RNA extracted from lentil root polyribosomes:

A. ○--○--○ Nuclear RNA (120 µg) synthesized by isolated nuclei, and ribosomal RNA (200 µg) added as marker was centrifuged in a SDS-sucrose gradient from 5 to 20% at 20 °C, for 2 h 30 min, at 37,500 rpm in a SW 39 rotor. Incubation was done in 10 ml, at 15 °C, for 30 min as described Fig. 6

B. ○--○--○ Polysomic RNA (300 µg) was extracted by a phenol-SDS mixture, at 4 °C from roots labelled for 30 min with (^{32}P). Centrifugation was carried out at 50,000 rpm, in a SW 50 L rotor, at 20 °C, for 2 h, in a SDS-sucrose gradient from 10 to 30%. Fractions from the 5 to 12 S region were pooled, hydrolyzed in 0·3 N KOH, and base composition determined (Miassod et al., 1970)

The localization of these RNAs within the cells was examined (Miassod et al., 1970). RNA of peak α is apparently restricted to the nucleus. RNA of peak β is found in nuclei and polyribosomes. Furthermore, RNAs with chromatographic and sedimentation properties of these RNAs have been observed among the RNAs transcribed in vitro by isolated nuclei. These organelles are extracted from lentil roots according to Mertelsmann (1969). The isolated nuclei incorporate ^{3}H-UTP into acid-insoluble material, up

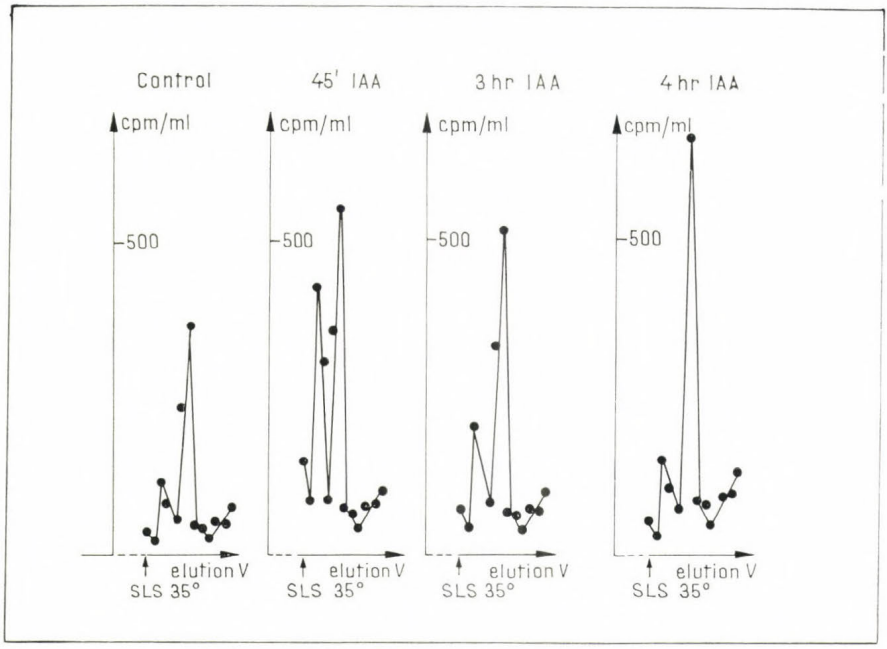

Fig. 8. MAK column chromatography of RNA synthesized by control and indoleacetic acid treated lentil roots. Control roots and roots incubated, for the periods shown, with indoleacetic acid 0·15 mM, were (^{32}P) labelled for 15 min. RNA was extracted as previously described (Miassod et al., 1970). The preparations were applied to a MAK column and eluted as described in Fig. 2

to 30 min, at 15 °C (Fig. 4, curve 1). At 37 °C (Fig. 4, curve 2), the reaction is complete in ten minutes but total incorporation is lower than in the first experiment. On prolonged incubation, ribonuclease activity causes a decrease in the amount of acid-insoluble material. The incorporation is dependent on the presence of magnesium, the four nucleotides are needed. The reaction is inhibited by actinomycin D and not by rifampicin (Table 1). The effect of divalent cations is shown in Fig. 5. RNA polymerase activity of isolated nuclei is stimulated by magnesium at low ionic strength and manganese at high ionic strength. A similar result was obtained in experiments on animal RNA polymerase activities (Tata and Widnell, 1966). The products of the reaction, in the presence of manganese, were charac-

terized by MAK chromatography and sucrose gradient centrifugation. The RNA, synthesized in vitro, contains molecular species with chromatographic properties of RNA from α and β fractions previously studied (Fig. 6). However, TB-RNA obtained from in vitro experiments contains a higher percentage of RNA, eluted as the fraction α, than total, in vivo labelled, RNA. The sedimentation pattern of these RNAs is similar to that of RNAs from either peak β or polysomic RNA (Fig. 7), but a slight amount of rapidly labelled RNA with high sedimentation velocity can be detected in RNA extracted from isolated nuclei.

The effect of hormonal treatment on in vivo synthesis of RNA from both α and β fractions can be seen in Fig. 8. Treated samples were incubated with indoleacetic acid for the time intervals shown. Then, control and treated samples of roots were labelled with (^{32}P), for 15 min. After short hormonal treatment, the major increase in RNA synthesis occurs in the nuclear, uridine-rich RNA fraction (peak α). A prolonged treatment brings about an enhancement of the synthesis of RNA from fraction β.

DISCUSSION

The lentil root RNAs, tenaciously bound to MAK columns, are separated in two major fractions α and β, by elution with sodium dodecyl sulphate at 35 °C. At 70 °C, a minor fraction, γ, is obtained. In the case of a short labelling period, RNA of fraction α is uridine-rich, and may contain high-molecular weight species. The mean life of this RNA is lower than 1 h. On the other hand, RNA of fraction β is adenine-rich, its sedimentation coefficient is about 12 S and its half life is higher than 2 h. The RNA from peak α is apparently restricted to the nucleus and polysomic messengers are present in fraction β. Molecular species, present in RNA synthesized by isolated nuclei, resemble to RNA from fractions α and β in sedimentation and chromatographic properties.

These results are in agreement with previous observations. Two species of unstable RNA have been postulated in the TB-RNA fraction of soybean (Key, 1969), one being similar in composition to soybean DNA (D-RNA) with the other containing much more AMP and less UMP than the DNA (TB-RNA). RNA of peak β is similar to TB-RNA described by Johri and Varner (1970). RNA of peak α has properties of "mRNA" found in dwarf peas by these workers. It is also similar to Q_2RNA isolated in animal cells (Ellem, 1966; Yoshikawa et al., 1965). This RNA interact at a smaller extent than TB-RNA with methylated-albumin. The discrepancy in the elution pattern between peak α and "mRNA" or Q_2RNA can be explained by the salt gradient used in our experiments (0·2 M to 1·3 M). Furthermore, freshly preparated methylated-albumin was used in our investigations. Indeed, Koch and Kubinsky (1964) have found that by aging, the retention properties of methylated-albumin are decreased and much more of the rapidly labelled RNAs were eluted by the salt gradient. Some papers report that the large size of plant D-RNA is due to aggregate formation on MAK column (Johri and Varner, 1970). However, molecular species with high S values can be observed in plant nuclear RNA, before MAK chromatography. Furthermore, high molecular weight RNA with base composition, stability

and localization within the cell, which closely resemble to plant D-RNA, exists in animal cells (Scherrer et al., 1966; Attardi et al., 1966). Thus, these results favour the idea that the transcriptional activity in plant cell nuclei has many features in common with that in animal cells. A part of this activity results in the formation of large heterogeneous molecules confined to the nucleus (peak α) which are different from mRNA found in polyribosomes (peak β).

One hypothesis postulates that D-RNA is a precursor to polysomic mRNA (Ryskov and Georgiev, 1970). In an effort to compare the sequence of D-RNA and mRNA, we began to perform RNA-DNA hybridization experiments with both RNA species. Unfortunately, these experiments do not allow a firm conclusion that sequences in mRNA are derived from D-RNA because the RNA which hybridizes easily binds to reiterated regions of DNA (Britten and Kohne, 1968). Fractionation of lentil root DNA is being undertaken to obtain non reiterated DNA. However, recent results (Penman et al., 1970), showing a differential inhibition of synthesis of nuclear heterogeneous RNA by cordycepin, suggest that mRNA and D-RNA are transcribed separately by distinct polymerases with different sensitivities to the drug. This hypothesis is supported by other observations. In animal cells at least three distincts polymerase activities have been isolated by chromatography and show, apparently, different transcriptive roles (Roeder and Rutter, 1969). Thus, the function of D-RNA remains to be defined. Its function could be other than those usually attributed to RNA.

We have shown that an early effect of auxin occurs at the transcriptional level. D-RNA appears directly involved in the tissue response to hormone treatment. This result is in agreement with previous observations in plants (Key, 1969; Tester and Dure, 1967). Moreover, RNA synthesized in various tissues after a short hormone treatment is uridine-rich (Cooper, 1968), has a large size (Dingman et al., 1969) and presents high nucleotide sequence complementarity to DNA (Hamilton et al., 1968). Consequently, D-RNA could play an important role in the regulation of gene expression. For instance it could be involved in a control mechanism analogous to that proposed by Britten and Davidson (1969). A study of RNA synthesis by distinct polymerase activities isolated from plant nuclei, including experiments on the effect of hormone, associated with its receptor, on these systems, is needed to test this hypothesis.

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SECTION V

NUCLEIC ACIDS AND PROTEINS IN PLANT DEVELOPMENT

Symp. Biol. Hung. **13**, *pp. 337—343 (1972)*

NUCLEIC ACIDS AND THEIR DERIVATIVES IN THE CONTROL OF DEVELOPMENT

by

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Modern concepts of the biochemical control of growth and development tend to be primarily, if not entirely, based on the Jacob-Monod model of the regulation of enzyme synthesis. According to these concepts, development is the net result of a DNA-programmed sequence of events in which the synthesis of individual key enzymes is switched on or off to a predetermined pattern. There are, however, several reasons for believing that this system is, at best, a crude control. For example, there is the question of the persistence of an individual mRNA after its formation had ceased; even more importantly, the persistence of the activity of an individual enzyme after its synthesis had been repressed. Next, there is the question of apoenzyme versus holoenzyme, i.e. the synthesis of enzyme protein is to no avail if the requisite coenzyme or prosthetic group is not available to convert it into the active form. Then there is the question of what happens in a cellular emergency, as for example occurs following damage. In such circumstances, there is usually a rapid wound response often followed by a regenerative phase. Even if this is taken care of by a 'programme', it must represent an unplanned departure from the original programme.

The conclusion which these and several other points indicate is that a 'fine control' of growth and development exists and functions in cooperation with the Jacob-Monod type of control. It is becoming more and more apparent that this fine control system operates through the free-nucleotides by virtue of their roles as allosteric effectors and coenzymes.

It appears that part, perhaps the major part, of the RNA of many cells exists in a form which is readily depolymerized (Grunberg-Manago et al., 1955; Ochoa and Heppel, 1957) to its constituent nucleotides. An important function of this RNA may be its role as a reserve of 5'-nucleotides which can be released at a time of metabolic need just as glycogen acts as a store of glucose. The controlled release of nucleotides in this way could be an important means by which the cell regulates its activity (Kaplan et al., 1954; Morton, 1958). Soodak (1966) has calculated that there is something like a fiftyfold excess of DNA in human cells, i.e. excess over that which can be explained as template for enzyme protein synthesis. He also points out that the weight of DNA in a rat liver cell is equal to the entire weight of an *E. coli* cell. Soodak suggests that in the higher forms of life where a more complex order of programming is involved in embryogenesis etc., this excess DNA serves to order the assembly of yet another type of RNA, whose ultimate function is to serve as a source of 'Leloir coenzymes'.

Strominger (1960) has made similar suggestions linking the free nucleotide pool with DNA via specialized RNA fractions.

At this point in considering possible links between nucleic acids and the free-nucleotide pools, a relevant question is the biochemical significance of the fact that so many coenzymes and prosthetic groups have nucleotide moieties. Further, does the distribution of metabolic function amongst the various nucleotides have some chemical or enzymic significance? For example, uridine nucleotides appear to be solely associated with the activation and transformation of sugars (aldehydes and ketones). Cytidine nucleotides have similarly unique roles in the activation of alcohols, and adenosine nucleotides serve predominantly as oxidation-reduction intermediates and as carriers of various types of acids. Does some feature of the chemistry of the base components of these nucleotides make each particularly suited for its special function?

In summary, there are three relationships to be considered. First, there is the relationship of the free nucleotide pattern of a tissue to the metabolic activity of that tissue. Second, the relationship of the free nucleotide pattern to nucleic acid metabolism and third, the relationship of the base type of a nucleotide coenzyme to its metabolic function. These relationships are considered in more detail below.

NUCLEOTIDE PATTERN AND METABOLIC STATE

It has been known for some time that the free nucleotide pattern of a particular tissue reflects the type and extent of metabolic activity within that tissue. Primarily on the basis of work with animal tissues, Schmitz (1961) described two extreme types of pattern, (a) the *energietyp* (e.g. chromatogram of heart muscle tissue), in which those nucleotides most concerned in energy metabolism, i.e. adenine and nicotinamide nucleotides, constitute about 90% of the total nucleotide content, and then mostly as the energy-rich di- or tri-phosphates, and (b) the *stoffwechseltyp* (e.g. chromatograms of regenerating liver) which is characterized by its lower content of adenine and pyridine nucleotides (less than 50%) and its increased content of nucleotides more especially concerned in metabolic transformations and biosynthesis, such as those of uridine and cytidine. We have found similar trends in working with plant tissues. For example, during the imbibition phase, germinating seeds show a pattern comparable to the energietyp but as germination proceeds this changes towards the stoffwechseltyp (Brown, 1962; 1965). In our experience, the root tissue of seedlings is usually characterized by relatively high concentrations of UDP-glucose. Leaf tissue, on the other hand, has a relatively high concentration of adenine and pyridine nucleotides. The nucleotide pattern of free-cell cultures of sycamore (*Acer pseudoplatanus* L.) is of interest in this respect, initially the predominant nucleotide is ATP but by the end of the lag phase of growth, its place has been taken by UDP-glucose. During the lag phase, there is a 46fold increase in UDP-glucose concentration and a sevenfold increase in that of UTP. By the time the period of rapid cell division has started, the individual concentrations of ATP, guanosine

nucleotides, UTP and UDP-glucose have started to fall. In contrastat this time, NAD shows a marked increase (Brown and Short, 1969).

In brief, the nucleotide pattern of a tissue is characteristic of that tissue and when the metabolic state alters during growth and differentiation, significant changes occur concomitantly in the nucleotide pattern. The important question is, however, whether the changes in nucleotide pattern cause the changing metabolic emphasis or are merely a result of it. In an attempt to answer this question, we have induced changes in the nucleotide pattern of plant tissue and have followed associated metabolic changes.

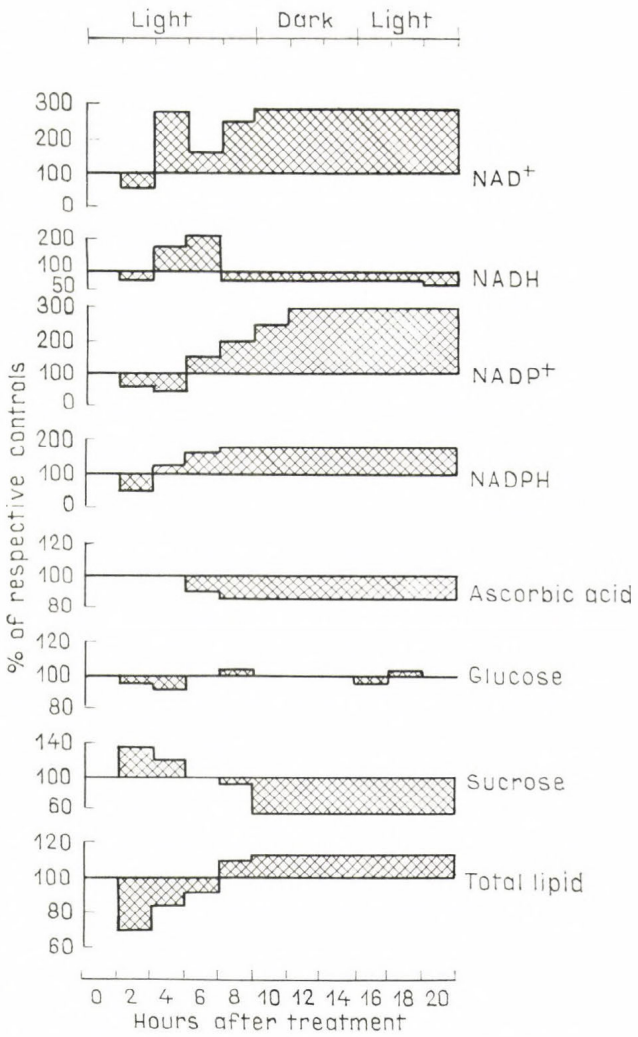

Fig. 1. Sequence of changes in kinetin-treated tissues relative to those occuring in untreated tissues over the same period. Control values (i.e. concentrations of specified metabolites in untreated tissue) are shown as 100% at each time interval. Treatment of seedlings was at zero time

The obvious way of inducing a selective change in nucleotide pattern would be to introduce a relatively large amount of one of the nucleotides into that tissue, however, with the tissues with which we have worked, this does not appear to be practicable because of the non-specific phosphatases which rapidly dephosphorylate nucleotides as they are introduced. A variety of purine and pyrimidine derivatives other than nucleotides were screened for their ability to induce selective changes in the nucleotide pattern and kinetin (6-furfuryladenine) was finally chosen as being suitable. This substance caused a number of changes, most noticeable of which were those in the concentrations of pyridine nucleotides (Fig. 1).

Concomitant changes in metabolic activity were reflected by a significant increase in glyceride content and a fall in sucrose and ascorbate concentrations (Fig. 1). Sampling at intervals of 2 h was, however, insufficient to determine if the changes in pyridine nucleotide concentration preceded those in sucrose and glyceride concentrations. An observation which does support this sequence of events following kinetin treatment, is the significant increase both in lipid concentration and in the activity of NADP-dependent isocitrate dehydrogenase (E.C. 1.1.1.42), for, Yamamoto (1969) has demonstrated similar effects by treating plant tissues directly with $NADP^+$.

Very little work has been reported concerning attempts to manipulate nucleotide patterns with a view to altering metabolic state but one very recent report is of interest, albeit from animal work. Decker and Keppler and their co-workers (1971), working with rats, have shown that D-galactosamine induces a liver damage essentially similar to that of viral hepatitis and that the biochemical characteristics of this is an apparent depletion of the nucleotides UMP, UDP, UTP. UDP-glucose and UDP-galactose. In the liver of galactosamine-treated animals this is due to the rapid formation of UDP-galactosamine and its metabolites which are only slowly metabolized further, i.e. the uridine nucleotides are trapped and the nucleotide pool becomes depleted in this respect.

NUCLEOTIDE PATTERN AND NUCLEIC ACIDS

As it would appear that selective changes in nucleotide pool accompany, and in some cases direct, metabolic changes during growth and development, the relationship of nucleotide pool to the central control, i.e. DNA, must be considered. As was discussed above, cells of higher organisms contain DNA and RNA in excess over that required for enzyme protein synthesis, and it has been suggested that the RNA in question is produced as a storage form of 5'-nucleotides. The storage may be of information as well as of material. Sequential release of nucleotides from, say an adenine-rich RNA would substantially boost the adenine nucleotide component of a nucleotide pattern and steer metabolism towards energy production (respiration).

Table

Fluctuations in the concentrations of free and RNA-bound nucleotides

Period (day)	Concentration (mμmole									
	Uracil nucleotides					Guanine nucleotides				
	Bound in RNA fraction:*					Bound in RNA fraction:				
	Free	I	II	IV	V	Free	I	II	IV	V
0–1	+125	+120	−50	+10	+1000	+20	−90	−60	+20	+1590
1–3	+280	−100	0	+70	+290	+30	+80	−20	+20	+210
3–6	+200	+30	+40	0	+100	−35	+60	+60	+90	+170

* RNA fractions I, II, IV and V are sRNA, low mol. wt. ribosomal RNA, light ribosomal

We have attempted to examine this hypothesis by inducing severe but non-lethal changes in the metabolism of a plant and following the subsequent changes both in free-nucleotide pattern and in the nucleotide composition of the various species of RNA. The plant material chosen for this work was petiolar tissue from primary leaf cuttings of *Phaseolus vulgaris* L. These cuttings root readily in water without additional treatment and provide an experimental system in which petiolar tissue undergoes the necessary metabolic changes to produce roots. The results of this work (Brown and Mangat, 1970) are summarized in Table 1. It should be noted that during the period studied, no significant changes occurred in either the free-nucleotide pool or nucleic acid profile of the associated laminae, i.e. the petiolar tissue was a self-contained system with respect to nucleotides. Although many significant changes occurred in the free-nucleotide

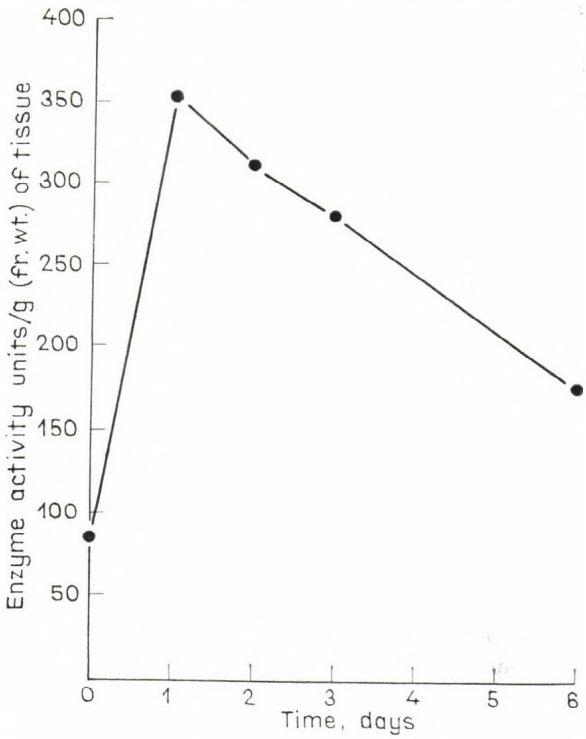

Fig. 2. Phosphodiesterase activity towards RNA in extracts of bean petioles during root formation. Leaves were detached at day 0 and showed root formation by day 6. Assay incubations were at 37 °C for 1 h, at pH 5·1: one unit of enzyme activity corresponds to an increase in $E_{260\ nm}$ of 0·01 under these conditions

1

during rooting of bean petioles

10 g fr. wt. of tissue)

Cytosine nucleotides					Adenine nucleotides				
	Bound in RNA fraction:					Bound in RNA fraction:			
Free	I	II	IV	V	Free	I	II	IV	V
+30	−70	−80	+10	+1210	+235	−50	−80	+20	+1000
+30	+50	+40	−10	+60	+310	+40	+30	0	+220
−30	+70	+20	+40	+70	−70	+50	+40	+50	+10

RNA and heavy ribosomal RNA, respectively.

pool, note for example the increasing UDP-glucose concentration as roots develop, these changes could not be linked quantitatively with the accompanying changes in the base composition of the petiolar RNA fractions. It is, however, pertinent to point out that the phosphodiesterase activity towards RNA within the petiole, rose to a relatively high level immediately after the cuttings were taken, (Fig. 2): this activity gradually fell again as roots developed.

Although none of the RNA fractions examined appeared to contribute significantly to the free-nucleotide pool, a number of reports over the last few years have described RNA fractions that are not extracted by the hitherto standard procedures used in the work described. Suffice it to mention the AMP-rich RNA isolated from rat liver and which is not extracted with the aqueous phase during the phenol extraction procedure (Hadjivassiliou and Braverman, 1966) and the AMP-rich RNA which remains firmly bound to MAK columns after elution of the fractions examined above (Ewing and Cherry, 1967). Clearly, depolymerization of such fractions, rich in AMP, could have important implications especially in the light of Atkinson's 'adenylate charge' concept as an important regulatory factor in metabolism (Atkinson, 1968).

RELATIONSHIP OF THE BASE-TYPE OF A NUCLEOTIDE COENZYME TO ITS METABOLIC FUNCTION

Consideration of this topic, i.e. why uridine nucleotides are primarily involved in sugar metabolism whereas cytidine nucleotides are uniquely associated with the activation of alcohols, and adenine nucleotides are especially associated with acids, again brings to mind the idea of a link between nucleotide coenzymes and nucleic acids, with all that implies for the regulation of growth and development. Nevertheless, at present, no answers can be given to these questions; this remains a problem for the future.

CONCLUSION

Whereas much information is now accumulating on the role of nucleotides as coenzymes and allosteric effectors in the control of metabolism, relatively little is known of the relationship of this localized control to that of the central control system, i.e. DNA—RNA—protein synthesis. Even less is known of the relationship between the structure of the 'Leloir coenzymes' and their individual roles. Yet, answers to these questions must be forthcoming for a full understanding of the biochemistry of growth and development.

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NUCLEIC ACID SYNTHESIS IN THE PEA SHOOT APEX

by

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The RNA of pea shoot apical meristems was labelled by placing a drop of [^{3}H]-uridine on each shoot tip for 2·5 or 6 h. The relative amounts of incorporation of label into different regions of the shoot apex were measured on autoradiographs of sections and were taken as a measure of the relative rates of RNA synthesis. [^{3}H]-uridine incorporation occurred throughout the whole apex but was most rapid and the specific activity of the RNA was highest in the slowly dividing cells (the central zone) at the summit of the apex. The lengths of the DNA synthesis (S) phase of the mitotic cycle in different regions of the apex were calculated from the percentages of cells which became labelled 2 h after a drop of [^{3}H]-thymidine had been placed on the apex. S was longest in the slowly dividing cells of the central zone. These cells at the summit of the apex seem to show a more rapid rate of RNA synthesis and breakdown, but a slower rate of DNA synthesis, than the faster dividing cells elsewhere in the apical meristem.

INTRODUCTION

There is considerable evidence which suggests that the cells at the summit of the apical meristem of the shoot divide less frequently than the cells on the flanks of the apical dome where leaves are initiated (Gifford and Corson, 1971). In the pea shoot apex direct measurements have shown that the rate of cell division at the summit of the apex is about half, or less, of the rate of division in the more basal parts of the apical dome (Lyndon, 1970*a*). Histochemical measurements showed that cells in all parts of the apex, irrespective of their different rates of division, contain on average the same amounts of DNA, RNA and protein (Lyndon, 1970*b*). Since all cells in the pea apex appear to be dividing (although at different rates), and divisions are asynchronous (Lyndon, 1970*a*), these are average values for the composition of cells in which the amounts of DNA, RNA and protein are increasing as each cell grows and synthesises new material during interphase of the mitotic cycle. If, in the pea apex, RNA and protein are synthesized throughout interphase, as they appear to be in other cells (Woodard et al., 1961; Van't Hof, 1963, 1967; Lyndon, 1967; Mitchell, 1969), then it follows that the rates of net increase in the amounts of RNA and protein are proportional to the rates of growth and division of the cells, i.e. slowest at the summit and faster down the flanks of the apex where leaves are initiated. If the rates of net increase of RNA and protein are a function of their rates of synthesis, then the rates of synthesis of RNA and protein should also be proportional to the rates of cell division and one would expect to find a region at the summit of the apex, corresponding to the region where cell division and growth is slower, where the rates of

synthesis of RNA and protein are also slower to the extent of being half or less of the rates of synthesis elsewhere in the apex.

When labelled precursors of RNA were supplied to shoot apices of *Brachychiton* and *Sinapis* an unexpectedly uniform distribution of incorporation of label into RNA was found (West and Gunckel, 1968; Bernier Bronchart, 1970). However, this might simply have reflected a uniform distribution of the rates of cell division and growth (which were not measured) in these apices. For the pea the rates of cell division and growth throughout the apex are known (Lyndon, 1970*a*, *b*) and it should therefore be possible to decide whether or not the known differences in the rates of net increase of RNA throughout the pea apex are a function of differing rates of RNA synthesis.

DNA is synthesized and increases in amount only during the S period of interphase. If the rate of DNA synthesis during S differs in different parts of the apex this will be apparent as differences in the length of S.

By supplying the pea shoot apex with radioactive precursors which label RNA or DNA it should be possible to compare the relative rates of synthesis of nucleic acid as indicated by the relative rates of incorporation of label, in the different regions of the apex known to show different rates of cell division and growth.

METHODS

Peas (*Pisum sativum* cv. *Lincoln*) were grown in sand at 23 °C in a controlled environment room with a 12 h light/12 h dark cycle as already described (Lyndon, 1968).

Labelling the shoot apices

Various methods of supplying radioactive substances to the shoot apex have been reviewed by Bernier and Bronchart (1963). The method they found most useful, in which the plant is partly defoliated, is the one used here. When the peas were 9 or 10 days old, having 9 or 10 leaves and primordia (plus the 2 epicotylary leaves), enough of the younger leaves and stipules were cut off to expose the shoot apical dome. A drop of water was placed on the apex to prevent its drying out. When all the plants had been so prepared, the drops of water were carefully removed with filter paper and on each apex was placed a drop of radioactive solution containing approximately 20 μCi of either [^{3}H-5] uridine (specific activity 30 Ci per mmole) or [^{3}H-methyl] thymidine (specific activity 27·8 Ci per mmole), which are specific labels for RNA and DNA respectively. The plants were then left in the controlled environment (in the light) for 2 or 6 h. The shoot tips were then excised and immediately fixed in ethanol: acetic acid (3 : 1) for several hours before transfer to 70 per cent ethanol. They were then passed through an ethanol series to absolute ethanol and eventually xylene and were embedded in paraffin wax. Longitudinal sections were cut 6 μm thick and autoradiographs were prepared with Ilford K2 emulsion. After

4 to 14 days exposure at 1 °C the autoradiographs were developed in Ilford ID 19 developer, fixed in Hypam and washed. The sections were then stained in methyl green/pyronin (Casselman, 1959) and mounted in Canada balsam.

Sections labelled with [^3H]-uridine

Comparison of the silver grain density in different parts of a section by counting grains was almost impossible except in apices where the grain density was very low and label was evenly spread over the cells. In cells which had accummulated appreciable label the labelling was non-uniform, for grain density in nuclei and nucleoli was usually greater than in the cytoplasm and the relative amount of label in the nucleus and nucleolus differed from cell to cell. In order to compare grain densities in different parts of the apex it was necessary to use a method by which the grain density over a relatively large area, including several cells, could be measured quickly. It proved possible to use a Barr and Stroud integrating microdensitometer for this purpose. The absorption spectrum of sections stained in methyl green/pyronin showed a region of minimum absorption from 400 to 450 nm. All measurements were therefore made at 420 nm. With the ×100 objective it was possible to measure a sufficiently small area of section so that the number of silver grains in it could be counted. With the instrument set at Extinction = 1·0, the recorded absorption was a direct function of grain density except at extremely high grain densities where half or more of the field was obscured (Fig. 1). Having established this, it was then possible to use a lower power objective (×45) and a larger field to obtain a measure of relative grain densities in different parts of the section. A standard area of 420 μm^2 was measured. Most measurements gave the same absorption with Extinction = 0·75 and = 1·0, showing that there was no underestimation of the values for regions of high grain density. In the few cases where the measurement at Extinction = 1·0 was higher

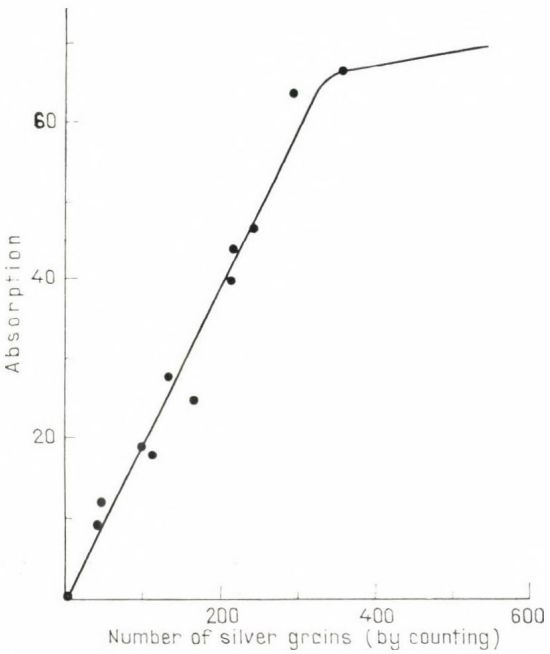

Fig. 1. Proportionality of absorption (arbitrary units) and number of silver grains per unit area of section

than at Extinction = 0·75, the former value was used. Measurements were made at the positions shown in Fig. 2. Each value is the mean from 3 sections i.e. 6 sections from each of 2 apices at each sampling time.

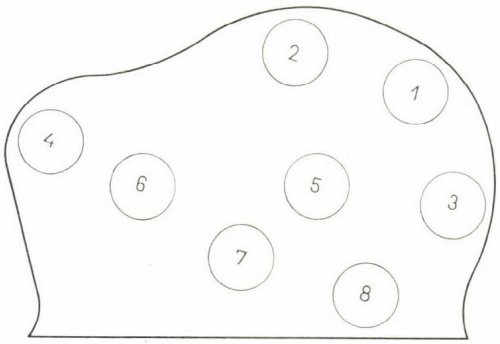

Fig. 2. Areas of the longitudinal section of the pea apex in which silver grain densities were measured microdensitometrically

1 = summit of apex (central zone) in which the rate of cell division is slow
2 = site of next but one leaf primordium (I_2)
3 = site of next leaf primordium (I_1)
4 = developing leaf primordium
5 = region of fastest dividing cells
6, 7, 8 = incipient pith and axial tissues

Sections labelled with [^3H]-thymidine

The positions of labelled nuclei in alternate serial sections right through each of 6 apices were recorded on tracings with the aid of a camera lucida. The use of alternate sections reduced the possibility of recording the same (cut) nucleus in adjacent sections. Cell counts were made on the same sections, the tracings of the sections being divided for this purpose into the regions of central zone, I_1, I_2, primordium and axis as described by Lyndon (1968).

RESULTS

RNA synthesis

In all plants the incorporation of [^{3}H]-uridine into RNA, after a 2·5 h application, was surprisingly uniform throughout the whole apex (Fig. 3), and microdensitometric measurements of relative silver grain densities in different regions of the apex confirmed this (Table 1). Contrary to expectation, there was as much incorporation of [^{3}H]-uridine at the summit of the apex, where the cells are known to be dividing slowest, and increasing in RNA content slowest, as in the faster dividing cells further down the apical dome. Since the concentration of RNA is lowest at the summit of the apex, the specific activity of the RNA after supplying [^{3}H]-uridine is in fact highest in these slowly dividing summit cells (Table 1). After 6 h of labelling the total amount of [^{3}H]-uridine incorporation into

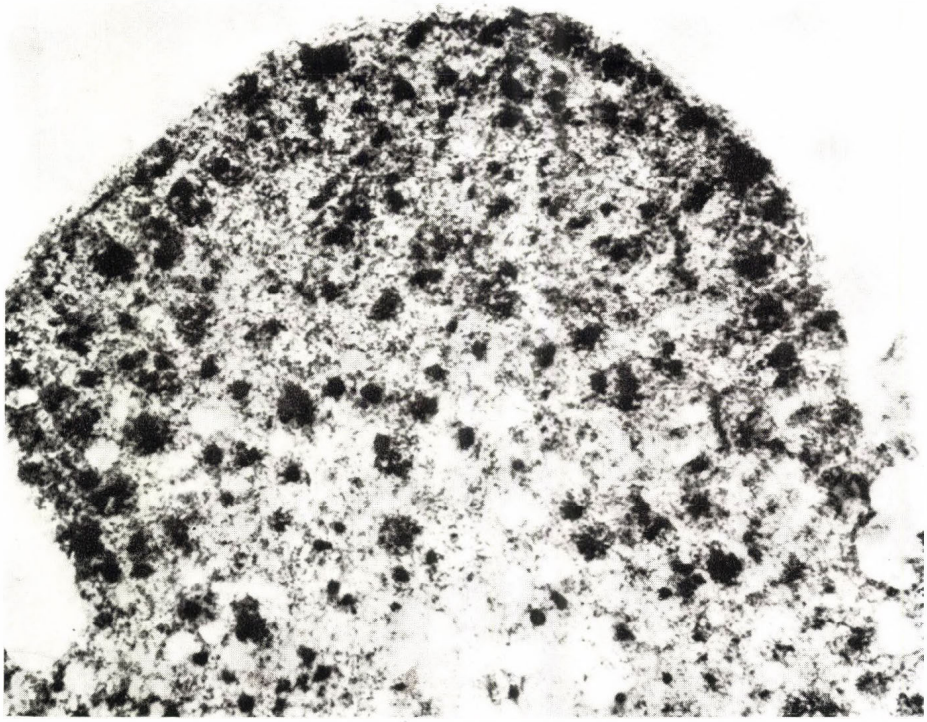

Fig. 3. Autoradiograph of L. S. of a pea shoot apex after labelling with [^{3}H]-uridine for 2·5 h

RNA had increased (as shown by the doubling of the grain count per unit area of section even though the exposure time of the autoradiographs was cut by two thirds) but the pattern of labelling was almost identical with that after 2·5 h (Table 1).

The distribution of label within the cells, with the nucleoli being the most intensely labelled, did not appear to differ appreciably after labelling for 2·5 or 6 h.

DNA synthesis

The percentages of cells with labelled nuclei, after 2 h labelling with [^{3}H]-thymidine (Fig. 4), in the different regions of the apex are shown in Table 2, and these values represent the percentages of the mitotic cycle which the cells spend in the S phase. The lengths of the mitotic cycles are also given and hence the length of S in hours in the different regions of the apex can be calculated (final column of Table 2). The length of S is about the same (6 to 8 h) in all parts of the apex except for the slowly dividing cells at the summit where it is longer (11 h). Since nuclei with only the 2C and 4C amounts of DNA have been found in the pea shoot

Table 1

Relative amounts of [^3H]-uridine incorporated into the different areas of the pea shoot apex

Area of apex*	Relative amount of [^{3}H]-uridine incorporation**		Relative concentrations of RNA+	Relative specific activity of RNA	
	2·5 h	6 h		2·5 h	6 h
1	100	100	100	1·00	1·00
2	99	101	114	0·87	0·89
3	95	109	131	0·73	0·83
4	96	103	138	0·70	0·75
5	78	93	—	—	—
6	78	94	114	0·68	0·82
7	70	89	—	—	—
8	80	99	117	0·68	0·85

* As shown in Fig. 2.
** Measured by microdensitometry.
+ Data from Lyndon (1970*b*).

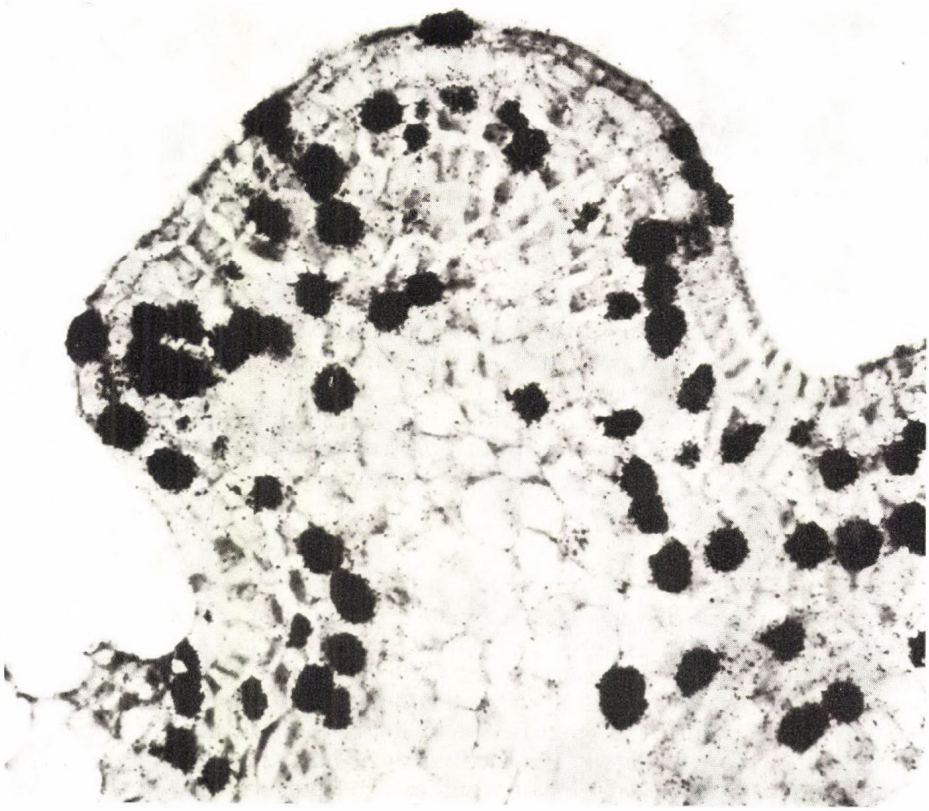

Fig. 4. Autoradiograph of L. S. of a pea shoot apex after labelling with [^{3}H]-thymidine for 2 h

Table 2

Incorporation of [^3H]-thymidine into nuclei in the pea shoot apex

Area of apex*	Percentage of labelled cells	Mitotic cycle (h)**	DNA S-period (h)
1	16	69	11·0
2	25	30	7·5
3	28	28	7·8
4	26	29	7·5
6, 7 and 8	23	26	6·0

* As shown in Fig. 2. The areas examined included the cells adjacent to the numbered areas and corresponded with the regions of central zone (1), I_2 (2), I_1 (3), primordium (4), and axis (6, 7, 8), for which measurements of the lengths of the mitotic cycle were made (Lyndon, 1970*a*).

** Data from Lyndon (1970*a*).

apex (by microdensitometry after Feulgen staining) (Lyndon, unpublished), it follows that during the S period of the mitotic cycle all cells synthesize the same amount of DNA, the 2C amount which is approximately 9·5 pg (Lyndon, 1967). The rate of DNA synthesis is therefore slower in the slowly dividing cells at the summit of the apex than in the cells elsewhere in the apex.

DISCUSSION

The fairly even distribution of label shows that the rate of incorporation of [^3H]-uridine into RNA in these experiments is not a function of the rates of cell division and growth which, in the central zone at the summit of the apex, are half or less of the rates of division and growth elsewhere in the apex (Lyndon, 1970*a*, *b*). On the contrary, incorporation of the RNA precursor was as rapid in the summit cells as in cells elsewhere in the apical dome and in the developing primordia. This is the same as has been found for the vegetative shoot apices of *Lolium* (Knox and Evans, 1968), *Brachychiton* (West and Gunckel, 1968), and *Sinapis* (Bernier and Bronchart, 1970).

In the pea the rate of net increase of RNA is a function of the rate of cell division and so will be slower in the summit cells than in the other cells. Since the rate of synthesis is the same or greater in the summit than in the other parts of the apex, but the rate of net increase (or accumulation) in the summit is only half of that elsewhere, it follows that the rate of RNA breakdown is greater in the summit than elsewhere in the apex.

On this interpretation the slowly dividing cells of the summit of the pea shoot apex show a more rapid turnover of RNA than the more rapidly dividing cells of the apex. Another characteristic of these summit (or central zone) cells is that the volume of their nuclei is 30 to 40 per cent greater than that of nuclei with the same DNA content elsewhere in the

apex (Lyndon, unpublished). In having a rapid rate of RNA turnover and having enlarged nuclei these cells at the summit of the apex resemble the slowly dividing or non-dividing cells in the more mature regions of the root which have large nuclei (Lyndon, 1967) and a rapid rate of RNA turnover (Jensen, 1961). Enlargement of the nucleus is also associated with an increased rate of RNA synthesis when nuclei are reactivated (Gurdon and Brown, 1965), and Harris (1967) has shown that in reactivated erythrocyte nuclei there is a direct relation between the volume of the nucleus and the rate of RNA synthesis. This raises the interesting possibility that the disappearance of the central zone in the pea apex when a new leaf is initiated (Lyndon, 1968) is a reflection of changes in nuclear size and synthetic activity.

There is no evidence for increased labelling of either the sites of initiation of new leaf primordia (I_1 and I_2 — areas 3 and 2 in Fig. 2) or the young primordium itself (Table 1 and Fig. 3). This is consistent with the view that leaf initiation results from changes in the direction of growth rather than changes in the rates of growth in the apex (Lyndon, 1970 *a*, *c*).

The interpretation of the data which has been given depends on the rate of incorporation of [^{3}H]-uridine being a measure of the rate of synthesis of RNA. However, the possibility cannot be ruled out that this is not so. One cannot be certain until one knows that the synthesis of RNA itself is the rate-limiting step in the incorporation of label in all parts of the apex and that the specific activity of the uridine at the site of incorporation into RNA is the same throughout the apex. It could also be argued that the cells at the summit of the apex have been stimulated into activity, including RNA synthesis, by mechanical damage during the defoliation and application of the radioactive solution. This is unlikely, because the apices of non-defoliated plants and of excised shoot tips immersed in radioactive solution, although only lightly labelled, showed the same pattern of relatively uniform distribution of label. Also, some apices which could be seen to be damaged showed much less incorporation of label in the damaged region.

The rate of DNA synthesis in the summit cells, unlike RNA synthesis, is slower than elsewhere in the apex and takes about 50 per cent longer (Table 2). This is almost the same as Jacqmard (1970) found for *Rudbeckia*, the only other shoot apex in which the length of S has been measured. Jacqmard's data suggest that the lengths of G1 and G2 are also extended in the slowly dividing cells at the summit of the apex. Taken together with the observation that the proportion of cells with the 2C and 4C DNA content is similar at the summit and elsewhere in the apex (Steeves et al. 1969; Lyndon, 1970*b*), this points to the whole of interphase being extended in the slowly dividing cells of the shoot meristem.

The slowly dividing cells in the root meristem have, however, different characteristics. It is only the G1 phase of the mitotic cycle which is extended. The length of S, and hence the rate of DNA synthesis, is the same as elsewhere in the root (Clowes, 1965; Thompson and Clowes, 1968). Also the rate of RNA synthesis in these cells is low, and RNA turnover will be correspondingly low (Clowes, 1956; Jensen, 1961; Barlow, 1970). Whatever interpretation is placed on the labelling data it is clear that the slowly dividing cells of the shoot apex are readily labelled with nucleic acid precursors

whereas those cells in the root are not. These differences allow us to speculate that the mechanism of controlling the rate of cell division may be different in the root and shoot meristems.

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CHANGES IN ISOZYMES OF HOST AND PATHOGEN FOLLOWING SOME FUNGAL INFECTIONS

by

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For several years we, in Madison, have been engaged in a study of the effects of infection by plant pathogens upon host proteins, particularly isozymes. We are interested in the biochemistry of disease and of disease resistance. Since resistance is often determined by genes of the host, we have studied the proteins of resistant and susceptible plants before and after inoculation. Soluble proteins were separated by gel electrophoresis and the isozyme bands were made visible by enzymatic reactions that produce colored products. This allowed us to see the isozyme patterns and the effects of infection on them.

Southern bean mosaic

Before discussing fungal infections, I would like to review our studies with two Hungarian scientists upon a viral and bacterial infection. The first was carried out with our conference chairman, Dr. G. L. Farkas (Farkas and Stahmann, 1966). We wondered if the increase in enzyme activity in diseased plants was due to activation or synthesis.

There was a marked change in the peroxidase isozymes of bean leaves following inoculation with Southern Bean Mosaic Virus. Young, healthy bean leaves gave two peroxidase bands. Two additional bands appeared when virus lesions developed. One of these bands was also seen in old leaves or leaves floated on water. These four bands were further purified by gel filtration and ion exchange chromatography and shown to be different proteins. The formation of the new bands was blocked by inhibitors of protein synthesis. We concluded that some of the increase in peroxidase in virus infection was due to synthesis.

Wildfire

Another Hungarian scientist, L. Lovrekovich, had shown that resistance in tobacco leaves to a bacterial disease was induced by injection of heat-killed bacteria or virus infection. Two peroxidase isozymes were detected in healthy tobacco leaves; an additional two bands formed within two days after 10^9/ml heat-killed cells of *Pseudomonas tabaci* were injected into the leaves. The development of disease symptoms was suppressed when half leaves were injected with killed bacteria or 50 μg/ml of commercial peroxidase two days before both halves were inoculated with 10^7 living *P. tabaci* cells. Severe disease symptoms developed within four days on

the half leaves that were injected with buffer, but no symptoms appeared on the half that received killed bacteria or peroxidase (Lovrekovich et al., 1968*a*).

These results, which resemble vaccination, suggest that the disease was suppressed by the injected peroxidase or peroxidase induced by killed bacteria. Since peroxidase did not inhibit bacterial growth and there was no protection when peroxidase was injected with living bacteria, we believe that the disease was suppressed by products formed by peroxidase or by other enzymes whose synthesis was induced by peroxidase. Tobacco mosaic virus infection also increased peroxidase and increased resistance to Wildfire (Lovrekovich et al., 1968*b*).

Black rot

Inoculation of sweet potato tissue with pathogenic and some non-pathogenic isolates of *Ceratocystis fimbriata* produced an increase in the number or intensity of peroxidase isozyme bands just below the inoculated surface. We thought that this increased peroxidase activity may be a defense reaction and therefore did experiments to see if the non-pathogens would induce a protection against the pathogen. Pieces of sweet potatoes from a susceptible and resistant variety, were left uninoculated or inoculated with a non-pathogen two days before being challenged by a second inoculation with the pathogenic isolate (Weber and Stahmann, 1966). Only the susceptible tissue not inoculated with the non-pathogen developed severe symptoms. In the resistant tissue and in the susceptible tissue first inoculated with the non-pathogen, growth of the fungus was confined to the surface layers.

This immunity induced by non-pathogens resembled the natural immunity and that produced by vaccination. However, the protection was confined to a few cell layers around the infection site. Associated with it, was an increase in peroxidase, polyphenol oxidase, and alkaline phosphatase (Weber et al., 1967).

An unexpected observation that uninoculated tissue incubated in closed chambers with infected pieces also showed an increased peroxidase activity, led us to suspect a volatile factor which was identified as ethylene. Peroxidase and polyphenol oxidase was increased 10 fold or more when treated with ethylene (Stahmann et al., 1966). Subsequently, other workers have confirmed an increase in peroxidase synthesis after exposure to ethylene and reported that ethylene induced the formation of cellulase and phenylalanine ammonialyase also. Resistance in very susceptible sweet potato tissue was also increased by exposure to low concentrations of ethylene prior to inoculation. Severe symptoms developed in the piece without ethylene; those receiving ethylene gave the resistant reaction.

Late blight

The isozyme pattern of peroxidase from white potato tuber tissue was changed by inoculation with an incompatible race of *Phytophthora infestans*. There was no difference in the patterns from the control uncut tuber;

a cut and uninfected tuber; and pieces that were cut and inoculated with the compatible race 1 to which the tissue was susceptible. However, the pieces that were cut and inoculated with the incompatible race 0 to which the tissue was resistant developed two new positively charged peroxidase bands. These new peroxidase bands were seen in the resistant combination only (Tomiyama and Stahmann, 1964).

Pea wilt

Isozymes of 16 enzymes from healthy pea plants, from infected plants 15 days after inoculation with *Fusarium oxysporum* f. sp. *pisi* race 1, and from the culture grown fungus were compared (Reddy and Stahmann, 1971). The intensity of the single band of catalase, alkaline phosphatase and glutamate dehydrogenase was increased by infection. The intensity of some bands of peroxidase, acid phosphatase, esterase and aldolase increased while others decreased after infection. Thus, three bands of acid phosphatase increased in intensity while three others decreased after Fusarium wilt infection.

Figure 1 illustrates this change with acid phosphatase. In this figure, gel1 is the acid phosphatase pattern of healthy pea stems. Gel 2 is that of infected stems. At the top of gel 2, bands are missing that were seen in the gel from healthy plants and from the fungus. There is a marked increase in the intensity of other bands. Thus, some isozyme bands increase, others decrease after Fusarium infection. Soluble isozymes of fumarase, succinate dehydrogenase and isocitrate dehydrogenase were not detected in extracts of healthy plants but were seen only in infected plants.

New isozyme bands of eight enzymes were seen from extracts of stems of Fusarium infected plants. These new isozymes which did not correspond to those of either the healthy plant or the fungus were not detected in either host or pathogen before infection. Such new isozyme bands were seen with peroxidase, aldolase, glucokinase, NADP-glutamate dehydrogenase, 6-phosphogluconate dehydrogenase, NAD- and NADP-malate dehydrogenase after infection.

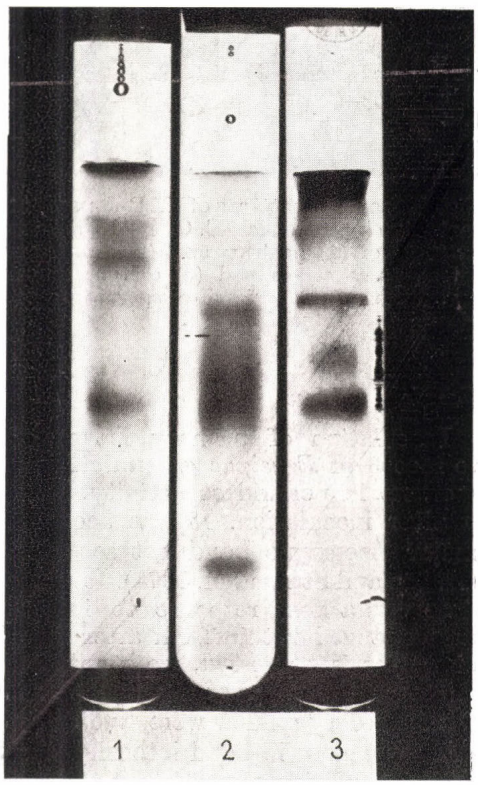

Fig. 1. Isozyme patterns of acid phosphatase (E. C. 3.1.3.2) from extracts of (1) healthy and (2) *Fusarium* infected pea stems and (3) culture grown mycelium of *Fusarium oxysporum* f. sp. *pisi* Race 1

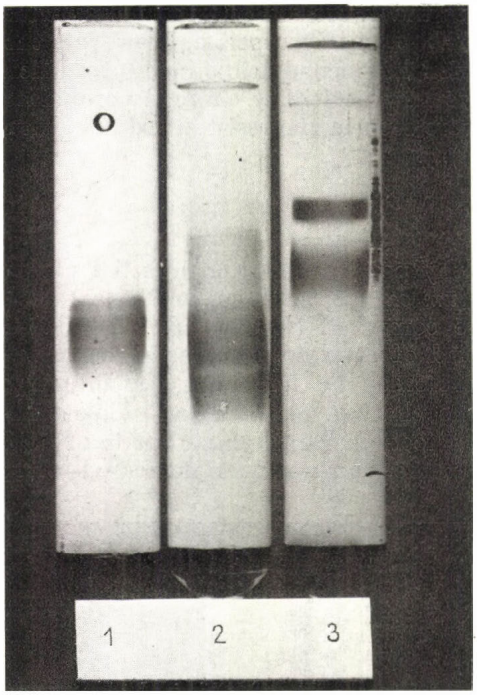

Fig. 2. Isozyme patterns of NAD-dependent malate dehydrogenase (E. C. 1.1.1.37) from extracts of (1) healthy and (2) *Fusarium* infected pea stems and (3) culture grown mycelium of *Fusarium oxysporum* f. sp. *pisi* Race 1

Figure 2 illustrates these new isozymes of NAD-malate dehydrogenase. Gel 1 was from healthy tissue. Gel 3 was from the fungus. Gel 2 is malate dehydrogenase from infected stems. It contains a fat moving band not seen in healthy tissue or the fungus.

Table 1 compares some properties of NAD-malate dehydrogenase from healthy or infected pea stems and *F. oxysporum* f. sp. *pisi*. Note that the K_M which represents the binding affinity of the enzyme was lowered. The lower K_M from infected plants means that the enzyme around infected sites has a higher affinity for its substrate than that in healthy parts. Such an increased affinity may be the biochemical basis for the accumulation of metabolites into the "metabolic sink" around infection sites. This new dehydrogenase band was much more stable to heat; and it had different physical and enzymatic properties than bands from either host or pathogen.

Powdery mildew

Inoculation of primary leaves of barley of differing compatibilities to race 3 of *Erysiphe graminis* f. sp. *hordei* resulted in a three to five fold increase in peroxidase activity. This increase was evident as soon as 24 h after inoculation. It was accompanied by the appearance of one new major isoenzyme band not seen in conidia or in healthy barley leaves (Hislop and Stahmann, 1971). It was concluded that the increase in peroxidase was a host response to infection. Since it was the only enzyme that changed within 24 h after inoculation, the period when the resistant character is expressed, we suspect that it may play a role in resistance.

Isozyme patterns of 14 enzymes from near-isogenic barley lines inoculated with powdery mildew were studied (Sako and Stahmann, 1971). A summary of the changes in band intensity and the appearance of new bands 7 days after inoculation of a resistant and susceptible barley line is given in Table 2. These data suggest that following inoculation of the resistant variety, only peroxidase showed a new band. However, the intensity of bands from enzymes involved in the pentose pathway, the hydrolysis of fatty acid esters, or protein and amino acid metabolism seem to increase.

Table 1

Properties of NAD-malate dehydrogenase from healthy and infected pea stems and from F. oxysporum f. sp. *pisi*

Property	Healthy	Infected	Fungus
K_M[a]	$3{\cdot}0\times10^{-5}$ M	$1{\cdot}5\times10^{-5}$ M	$1{\cdot}9\times10^{-5}$ M
Heat stability[b]	35	100	4
Specific activity[c]	6	17	—

[a] K_M = Michaelis constant measured with oxaloacetate as substrate .
[b] Minutes required for 50% loss of activity when heated at 45°.
[c] Units of activity in crude extracts per mg protein.

Table 2

Changes in isozyme bands following inoculation of resistant and susceptible barley lines with Erysiphe graminis f. sp. *hordei*

The number of bands showing an increase (+) or decrease (−) in intensity 7 days after inoculation and the number of new bands in extracts from inoculated tissue not present in extracts of uninoculated leaves are listed

Enzyme	Resistant (ml-g)		Susceptible (ml-g)	
	Intensity change	New band	Intensity change	New band
Oxidases				
Peroxidase*	+2	1	+2	1
Pentose Pathway				
Glucose-6-phosphate dehydrogenase	+1	0	+1, −5	0
Phosphogluconate dehydrogenase	+2	0	+1, −1	0
Hydrolases				
Acid Phosphatase	0	0	+3	1
Acetylesterase	+2	0	+2	2
Leucineaminopeptidase	+1	0	+1	0
Amino Acid Metabolism				
NADP-Malate dehydrogenase	+3	0	+3	1
Glutamate dehydrogenase	+1	0	+1	0
Glutamate dehydrogenase (NADP)	+1	0	+1	0
Glycolytic Pathway				
Glucokinase	0	0	+1	0
Glucosephosphate isomerase	0	0	+2, −1	0
Alcohol dehydrogenase	0	0	0	0
Krebs Cycle				
Succinic dehydrogenase	0	0	−3	1
Malate dehydrogenase	0	0	−1	1

* The increase in intensity and the new band were visible 24 h after inoculation.

In the susceptible line these changes also occurred but in addition there was a decrease in the intensity of many bands involved in the pentose pathway and the Krebs cycle and an increase in those involved in the glycolytic pathway.

Eleven enzymes from inoculated susceptible lines showed an increased intensity of one or more bands; four enzymes showed a decrease. A hypersensitive line (M1-k) showed similar changes. With the resistant line (M1-g) there was an intensity increase in one or more bands of seven enzymes. Such changes in isozymes are biochemical symptomes of disease and may be a basis for the increased metabolism associated with infection. It suggests that there are profound interactions between parasite and host at the enzyme level. There is an increase in oxidative and hydrolytic enzymes and enzymes involved in RNA synthesis following inoculation of both resistant and susceptible lines. However, only in the susceptible was there an increase in the energy generating pathways. Therefore we suggest that oxidases, hydrolyases and enzymes of the pentose pathway may be involved in resistance and that an enhanced glycolysis may favour susceptibility.

Alteration of histones and proteins by peroxidase

Since we saw an increase in the number or intensity of peroxidase isozymes with every fungal, bacterial or viral disease, we wondered if peroxidase were involved in disease resistance and could act on proteins or nucleic acids. Because peroxidase forms reactive free radicals and quinones, we suspected that peroxidase might alter enzyme activity or protein synthesis. Preliminary experiments in which polylysine, proteins and histones were treated with a peroxidase system revealed peroxidase induced changes in mobility and ultraviolet absorption. Peroxidase caused the formation of a substance, probably lysyl aldehyde, which could be oxidized to α-amino adipic acid.

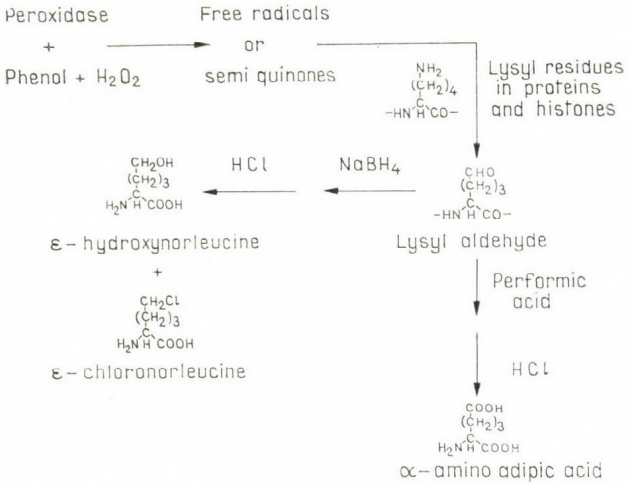

Fig. 3. Proposed reactions of a peroxidase system (peroxidase, phenol and hydrogen peroxide) with the amino groups of proteins and histones

Figure 3 shows the proposed reactions of a peroxidase system (peroxidase, a phenol and hydrogen peroxide) with proteins and histones. We suggest that free radicals or quinones react with lysyl residues to cause deamination with the formation of an aldehyde. We believe an aldehyde intermediate is involved because oxidation or reduction of the reaction mixture before acid hydrolysis gave rise to α-amino adipic acid or γ-hydroxynorleucine, respectively and the reaction mixture gave positive tests for aldehydes.

Table 3

The binding of the peroxidase oxidation product(s) of indole-3-acetic acid-2-^{14}C (IAA) to crude calf-thymus histone separated on Sephadex G – 25

Peroxidase (10 μg/ml) and IAA – 2 – ^{14}C ($2{\cdot}0 \times 10^{-4}$ *M*, $4{\cdot}1 \times 10^{6}$ CPM/ml) were incubated at room temperature for 60 min at pH 5·0 before adding an equal volume of crude calf-thymus histone (8·0 mg/ml) to make 1 ml total volume. After 30 min at room temperature, the mixtures were flushed with N_2, stored at – 20 °C and chromatographed on a 30 ml Sephadex G – 25 column.

Incubation mixture		CPM incorporated into high M. W. fraction[1]	per cent
Peroxidase + IAA + histone	(pH5)	133,000[2]	100
Peroxidase + IAA + buffer	(pH5)	3,500	3
IAA + histone	(pH5)	12,400	9
Peroxidase + IAA + histone	(pH8)[3]	84,600	64
IAA + histone	(pH8)[3]	6,090	5
Peroxidase + IAA + histone + glutathione	(pH5)[4]	90,300	68
Peroxidase + IAA + histone (heated)	(pH5)[5]	337,000	254

[1] The high molecular weight (M. W.) fraction (> 5,000 M. W.) eluted at 13 – 23 ml and the IAA eluted at 33 – 43 ml at pH 8·0 and 37 – 46 ml at pH 5·0.

[2] The 133,000 CPM incorporated is equal to about $6{\cdot}0 \times 10^{-9}$ moles of IAA.

[3] The histone was incubated at pH 8·0 and chromatographed at pH 8·0.

[4] Reduced glutathione ($2{\cdot}0 \times 10^{-4}$ *M*) was included with the histone.

[5] The peroxidase and IAA mixture was heated after incubation at 100 °C for 10 min and cooled to room temperature before adding histone.

Experiments are being conducted to test the hypothesis that through such reactions peroxidase may alter histones and that the altered histone is a less effective repressor of DNA-dependent RNA synthesis (Demorest and Stahmann, unpublished). We have found two peroxidase systems which can alter calf thymus histone. As shown in Table 3, the peroxidase oxidation products of indole-3-acetic acid bind to calf thymus histone. The data shows that the binding of IAA-2-C^{14} was increased 10 fold by treatment with peroxidase. Binding was further increased 2·5 fold by heating the reaction mixture which is known to increase the formation of 3-methyleneoxindole and decreased by the addition of glutathione or raising the pH.

Figure 4 shows the proposed oxidation of IAA to form an indole aldehyde and 3-methyleneoxindole which react with the amino group of lysyl residues or the sulfhydryl group of cysteinyl residues of histones. We suggest

that such reactions may alter their repressor properties and cause increased synthesis of RNA and proteins.

Chemical evidence for the alteration of calf-thymus histone by peroxidase and catechol is shown in Table 4. This table shows that when very lysine-

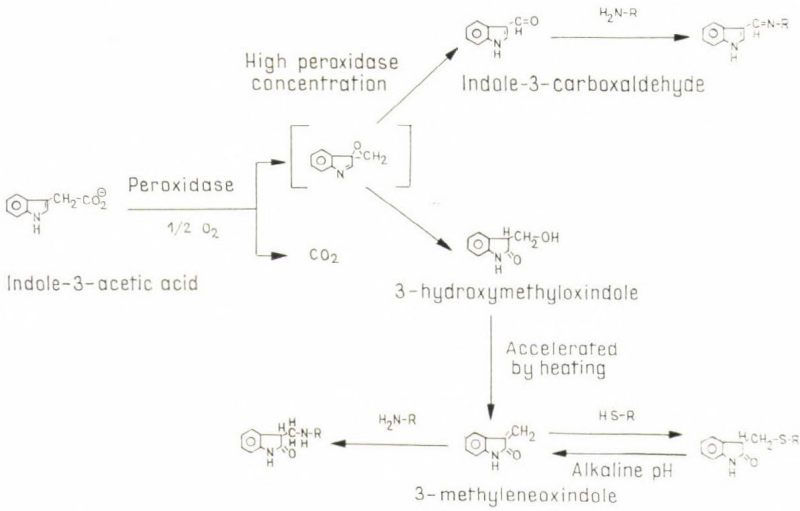

Fig. 4. Proposed oxidation of indole-3-acetic acid by peroxidase and reactions of the oxidation products with amino and sulphydryl groups of histones or proteins

rich histone is incubated with peroxidase, catechol and hydrogen peroxide, three times more α-amino adipic acid is formed and twice as much catechol is bound to the histone than when peroxidase is omitted. There may be some air oxidation of catechol in the absence of peroxidase and a binding of oxidation products. When the crude histone was treated with peroxidase and subjected to gel electrophoresis, fast moving histones were converted to a heterogeneous, slower moving group. Such data show that peroxidase can alter the physical and chemical properties of histones. Some preliminary experiments suggested that the template activity of isolated pea internode chromatin was increased by treatment with IAA and peroxidase. These results give support to the idea that some of the hormonal effects of IAA may be due to the binding of its peroxidase oxidation products to histones and that peroxidase may play a role in the derepression of the genome during injury or disease.

A proposed ribosome cycle catalyzed by peroxidase

Ten years ago Uritani and Stahmann (1961) observed an increase in peroxidase and in microsomes in diseased sweet potato tissue. In Fig. 5 we propose that a ribosomal peroxidase may catalyze the synthesis of new ribosomes by derepression of genes involved in ribosome synthesis. This

Table 4

Content of α-amino adipic acid and catechol oxidation products in peroxidase treated calf-thymus very lysine-rich histone

The mixtures (histone, 1·0 mg/ml; peroxidase, 0·1 mg/ml; H_2O_2, 2·5 mM; catechol, 0·25 mM) were incubated for 2 h at room temperature and then passed through a Sephadex G—25 column. The high molecular weight fraction was well separated from the catechol and H_2O_2. The high molecular weight fraction was then dialyzed at 0 °C for 48 h. The α-amino adipic acid content in the dialyzed preparation was determined by standard amino acid analysis after performic acid oxidation.

Incubation mixture	μmoles α-amino adipic acid/μmole histone[1]	% catechol[2]
Histone	0·0	0
Histone + peroxidase + H_2O_2	0·0	0
Histone + peroxidase + catechol	0·0	19
Histone + H_2O_2 + catechol	0·3	28
Histone + peroxidase + H_2O_2 + catechol	1·0	67
Peroxidase + H_2O_2 + catechol	0·0	0

[1] The μmoles of histone was determined from the alanine and glycine content and the known molecular weight of very lysine-rich histone.

[2] The % catechol or catechol oxidation products bound to histone in the high molecular weight fraction (> 5,000 M. W.) was calculated as the % of material absorbing at 280 mμ, not due to histone, peroxidase, or unbound catechol oxidation products, which was recovered from the original mixture.

admittedly speculative hypothesis suggests that derepression occurs during injury or infection to induce an increase in the number of ribosomes. We suggest that this derepression results from the peroxidase catalyzed alteration of histones causing a reduced binding to DNA. The oxidation products

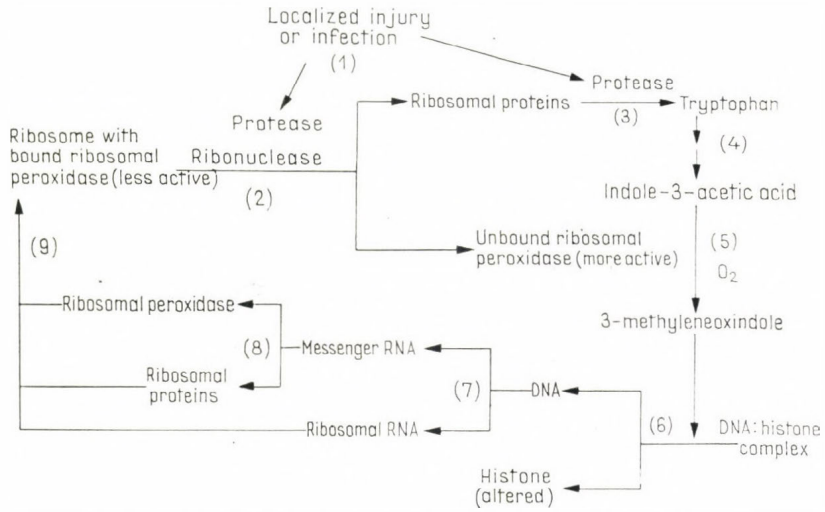

Fig. 5. Proposed ribosome cycle catalyzed by peroxidase

of IAA may react with amino, sulfhydryl, or guanidino groups; oxidation products of phenols may also be involved. These reactions expose DNA from the repressed DNA-histone or protein complex which leads to the synthesis of ribosomal and messenger RNA's. This triggers synthesis of ribosomal proteins and ribosomal peroxidase which assemble into new ribosomes.

In this manner the hydrolytic enzymes released as a result of cellular damage (1), disrupt ribosomes (2), to release proteins and peroxidase which through reactions (3), (4), and (5) form 3-methyleneoxindole that binds to histone. This binding derepresses the DNA (6), and leads to the formation of messenger RNA and ribosomal RNA (7). These code for ribosomal proteins including ribosomal peroxidase (8) which assemble into new ribosomes (9).

We realize that this is a highly speculative hypothesis and propose it for your consideration because we want to emphasize that peroxidase may play a role in the regulation of metabolism, particularly in injury or disease. A stimulation of the germination and differentiation of wheat rust spores by added peroxidase observed by Macko, Woodbury and Stahmann (1968) may support this hypothesis.

In summary we have shown:

(1) That in plants inoculated with selected virus, bacteria or fungi there was a rapid increase in peroxidase and often the appearance of new peroxidase isozymes followed by changes in the activity and isozyme patterns of other enzymes. Some isozymes disappeared and new isozymes not seen in healthy plants or the pathogen appeared after infection.

(2) That biological or chemical agents that induced an increase in peroxidase also induced disease resistance and caused changes in isozyme patterns.

(3) We have suggested from chemical and physical evidence that the peroxidase oxidation products of indole-3-acetic acid or phenols may activate genomes to increase the synthesis of proteins and ribosomes.

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CHANGES IN ENZYMES OF CARBOHYDRATE OXIDATION DURING DIFFERENTIATION OF THE ROOT OF *PISUM SATIVUM*

by

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Differentiation may be viewed as the controlled, sequential, expression of different proportions of the genotype of the cell. Thus, changes in the maximum catalytic activities of enzymes that control metabolic pathways are an essential part of the mechanism of differentiation. As part of a study of the biochemistry of differentiation of the root of *Pisum sativum*, we have measured the activities of enzymes of carbohydrate oxidation in extracts of different regions of the root (Fowler and ap Rees, 1970; Wong and ap Rees, 1971). From this work we have selected, for this paper, data that bear on the following questions. First, do the maximum catalytic activities of phosphofructokinase (EC 2.7.1.11) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) change during the differentiation of the root? Second, do any such changes contribute to alterations in the relative activities of glycolysis and the pentose phosphate pathway during differentiation? We have chosen these enzymes because they catalyse the first irreversible steps unique to their respective pathways. Thus, unlike subsequent enzymes in the pathways, they could control the entry of glucose-6-phosphate into glycolysis and the pentose phosphate pathway, respectively.

We worked with the tip 26 mm of roots of peas that had been germinated in the dark at 25° for 5 days. The methods that we used have been described (Fowler and ap Rees, 1970; Wong and ap Rees, 1971). We emphasize that we have presented evidence that our measurements of enzyme activities reflect the catalytic activities of the tissues examined.

Regions of the root at different stages of differentiation were obtained by transverse sectioning of the root tip. The activities of the enzymes in extracts of these segments are related to the protein in the extract in Table 1. All segments contained appreciable activity of both enzymes. The value for phosphofructokinase did not vary significantly between 5 and 20 mm from the tip but was appreciably higher in the tip 5 mm than in the rest of the segments. There were no significant changes in the activity of glucose-6-phosphate dehydrogenase. These results indicate that all four segments had appreciable capacities for glycolysis and the pentose phosphate pathway and that the relative capacities of the two pathways changed in favour of the latter as the apical cells of the root matured.

Evidence that the above changes are significant in relation to metabolism *in vivo* is provided by the distribution of ^{14}C from 1-^{14}C and 6-^{14}C glucose supplied to root segments (Table 2). The distribution in the region 1·6—3·0 mm from the tip is representative of the first 6 mm of the root, whilst that in the region 6—16 mm from the tip is representative of the root

Table 1

Activities of phosphofructokinase and glucose-6-phosphate dehydrogenase in extracts of segments of pea root tips

Root segment (mm from tip)	Activity* (nmoles substrate consumed/min/mg protein)	
	Phosphofructokinase	Glucose-6-phosphate dehydrogenase
I 0–5	95±3 (7)	55±7 (8)
II 5–10	55±5 (7)	44±5 (7)
III 10–15	55±7 (7)	39±1 (8)
IV 15–20	59±6 (7)	47±2 (7)
	Fisher's P values	
I vs II	<0·001	N.S.
I vs III	<0·01	N.S.
I vs IV	<0·01	N.S.
II vs III	N.S.	N.S.
II vs IV	N.S.	N.S.
III vs IV	N.S.	N.S.

* Values are means ± S. E. The number of extracts assayed is given in parentheses. sisher's *P* values are given for comparison of activities. Values of 0·05 or less are considered Fgnificant. Values greater than 0·05 are given as N. S. (not significant).

Table 2

Distribution of ^{14}C recovered from segments of pea root tips supplied with 1–^{14}C and 6–^{14}C glucose

Cell fraction	Percentage of absorbed ^{14}C per cell fraction			
	Segment 1·6–3·0 mm from tip		Segment 6–16 mm from tip	
	1–^{14}C	6–^{14}C	1–^{14}C	6–^{14}C
CO_2	15	13	23	9
Organic acids	22	24	22	28
Amino acids	10	12	9	12
Nucleotides	2	2	1	1
Sugars	23	21	17	19
Material insoluble in 80% ethanol	24	25	23	20

6–26 mm from the apex. The pattern in the region 6–26 mm is that expected of a tissue in which both glycolysis and the pentose phosphate pathway made significant contributions to carbohydrate oxidation (ap Rees and Beevers, 1960). The patterns in the tip 6 mm differ from those in the more differentiated cells. The differences are those that would be

Table 3

Activities of phosphofructokinase and glucose-6-phosphate dehydrogenase in extracts of stele and cortex of pea roots

Enzyme	Activity* (nmoles substrate consumed/min/mg protein)		Fisher's P values, stele vs cortex
	Stele	Cortex	
Phosphofructokinase	46±2 (10)	52±2 (10)	N.S.
Glucose-6-phosphate dehydrogenase	98±4 (8)	63±4 (8)	<0·001

* Values are given as in Table 1.

Table 4

Release of $^{14}CO_2$ from 1—^{14}C glucose and 6—^{14}C glucose supplied to isolated steles and cortices

Interval (min after addition of ^{14}C-glucose)	$^{14}CO_2$ released per interval as percentage of uptake at 120 min			
	Stele		Cortex	
	C-1	C-6	C-1	C-6
0—20	0·79	0·15	0·33	0·11
20—40	1·00	0·53	0·79	0·44
40—60	1·11	0·81	1·18	0·75
60—90	2·21	1·90	2·08	1·63
90—120	3·44	2·82	2·23	1·63

Table 5

Relative activities of phosphofructokinase and glucose-6-phosphate dehydrogenase during differentiation of the pea root

Region of root	Activity* (nmoles substrate consumed/min/mg protein)		Fisher's P values, PFK vs G-6-P DH
	Phosphofructokinase	Glucose-6-phosphate dehydrogenase	
Whole segment 0—5 mm from tip	95±3 (7)	55±7 (8)	<0·01
Whole segment 5—10 mm from tip	55±5 (7)	44±5 (7)	N.S.
Cortex 6—26 mm from tip	52±2 (10)	63±4 (8)	<0·05
Stele 6—26 mm from tip	46±2 (10)	98±4 (8)	<0·001

* Values are given as in Table 1.

expected if the contribution of glycolysis, relative to that of the pentose phosphate pathway, were greater in the tip than in the region 6—26 mm from the tip. Thus we have evidence that the change in the relative activities of phosphofructokinase and glucose-6-phosphate dehydrogenase in favour of the latter coincided with an increase in the activity of the pentose phosphate pathway relative to that of glycolysis.

The above changes occurred roughly at the point in the root where development of the stele becomes appreciable (Popham, 1955). Consequently the changes could have been a characteristic of the stele. We tested this hypothesis in experiments with stele and cortex isolated from the root 6—26 mm from the apex (Wong and ap Rees, 1971). The reliability of our separation of stele from cortex is demonstrated in Fig. 1.

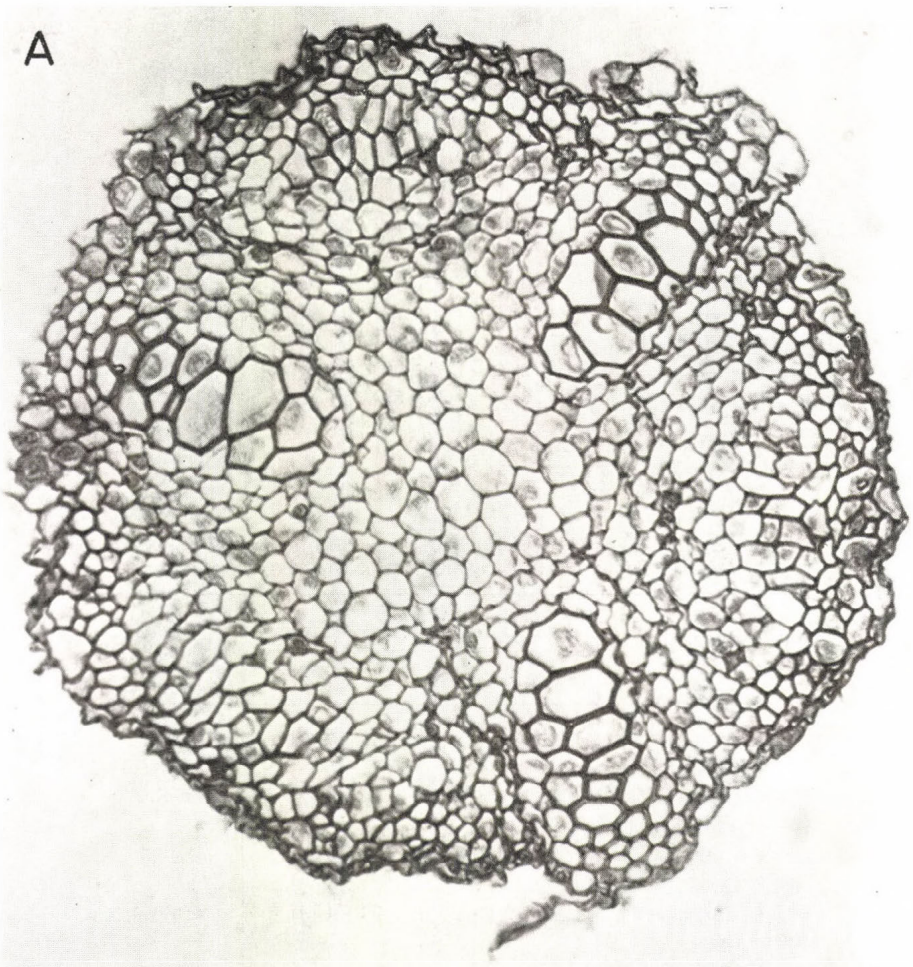
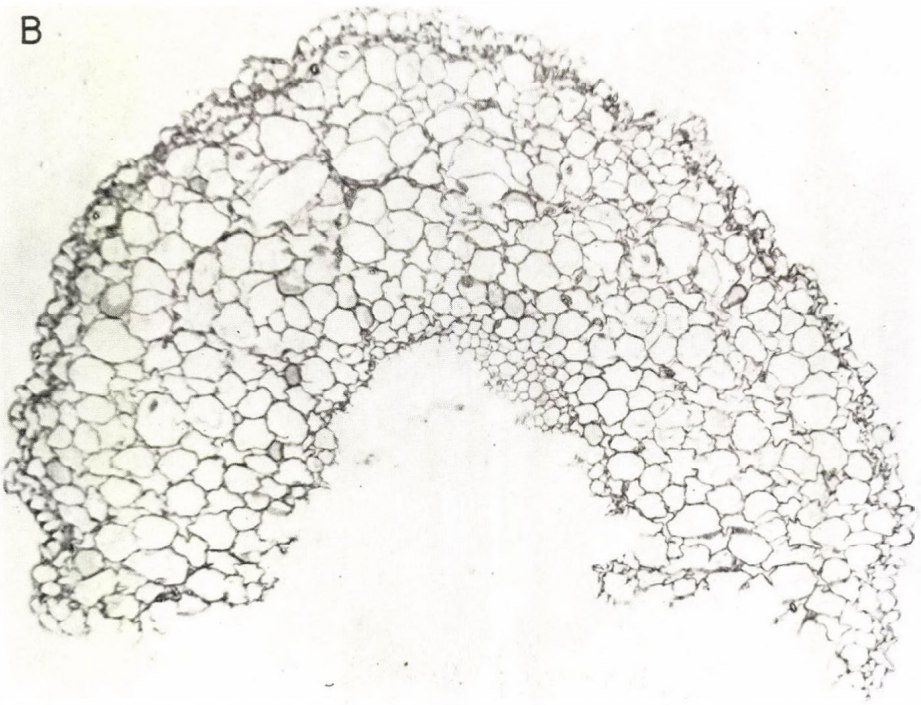


Fig. 1. Transverse sections of, A, stele (×440) and, B, cortex (×210) isolated from pea

Considerable activities of both enzymes were found in both stele and cortex (Table 3). The activity of phosphofructokinase was similar in extracts of stele and cortex but that of glucose-6-phosphate dehydrogenase was greater in extracts of stele than in those of the cortex. These results indicate that both tissues have considerable capacities for glycolysis and the pentose phosphate pathway and that the capacity of the latter pathway is higher in the stele than in the cortex. The patterns of $^{14}CO_2$ production from 1-^{14}C and 6-^{14}C glucose are those expected of tissues in which both pathways make substantial contributions to glucose oxidation (Table 4). The technique is not sensitive enough to show whether the pentose phosphate pathway was even more active in the stele than in the corresponding cortex.

Changes in the relative activities of the two enzymes during differentiation of the root are emphasized in Table 5. We conclude that all parts of the root examined possess marked capacities to catalyse glycolysis and the pentose phosphate pathway and that during differentiation the relative capacities of the two pathways change in favour of the pentose phosphate pathway. This change is more marked in the stele than in the cortex. We think that the changes in enzyme activities are meaningful in terms of carbohydrate oxidation *in vivo* because in general they are accompanied by the expected changes in the pattern of glucose oxidation. The mechanism of the changes and their role in root metabolism have not yet been discovered. The simplest working hypothesis that occurs to us is that the



roots 16—21 mm from the apex. Both sections were stained with safranin and fast green

changes are due to variation in the amounts of enzymes and that this variation determines the cells' capacity to provide $NADPH_2$ required for biosynthesis during differentiation of the root.

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