Symposia Biologica Hungarica 6

PROCEEDINGS OF THE SYMPOSIUM ON BACTERIAL TRANSFORMATION AND BACTERIOCINOGENY

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Akadémiai Kiadó, Budapest

PROCEEDINGS OF THE SYMPOSIUM ON BACTERIAL TRANSFORMATION AND BACTERIOCINOGENY

August, 13–16, 1963
Budapest

(Symposia Biologica Hungarica 6)

This work contains the lectures, debates and contributions to the Symposium organized by the Hungarian Academy of Sciences.

The volume contains also literary references and consists of three parts. Part 1 deals with the transformation of bacteria, Part 2 with the phenomena of bacteriocinogeny, and Part 3 with the genetic fundamentals of cell regulation.

AKADÉMIAI KIADÓ
BUDAPEST

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FOREWORD

A symposium with the title *Bacterial Transformation and Bacteriocinogeny* was held in Budapest from August 13 to 16, 1963, sponsored by the Hungarian Academy of Sciences. The purpose of this Symposium was to make an attempt to examine some recent developments in certain avenues of molecular genetics and it was so designed to bring scientists together to report recent findings. The three sessions of the Symposium were devoted to transformation, bacteriocinogeny, and DNA specificity and cell regulation and on each of the sessions authorities of these topics presented two invited lectures followed by contributory papers. The one-day excursion to Lake Balaton offered a further opportunity for informal exchanges of ideas and discussions. The papers given by the invited speakers and contributors and the discussions are presented in this volume. An attempt has been made to maintain the conversational tone of the extensive discussions, however, it is unfortunate, perhaps, that the published proceedings does not record them completely. We wish to express our appreciation to the many people whose contributions made the Symposium successful, and especially those who gave advice to us during the programming stage, the speakers and chairmen. Thanks are due to the co-operation of those who devoted efforts in arranging and running the programme. Particular thanks are due to Dr K. Szende who assisted in over-all editorial duties of the arrangements for publication of the proceedings. Gratitude is expressed to Mrs É. Szelei who provided secretarial services in the early phase of the Symposium and for helping the indexing and for checking the page proof. Acknowledgement is gratefully made to the Akadémiai Kiadó, Budapest, publishing this symposium volume.

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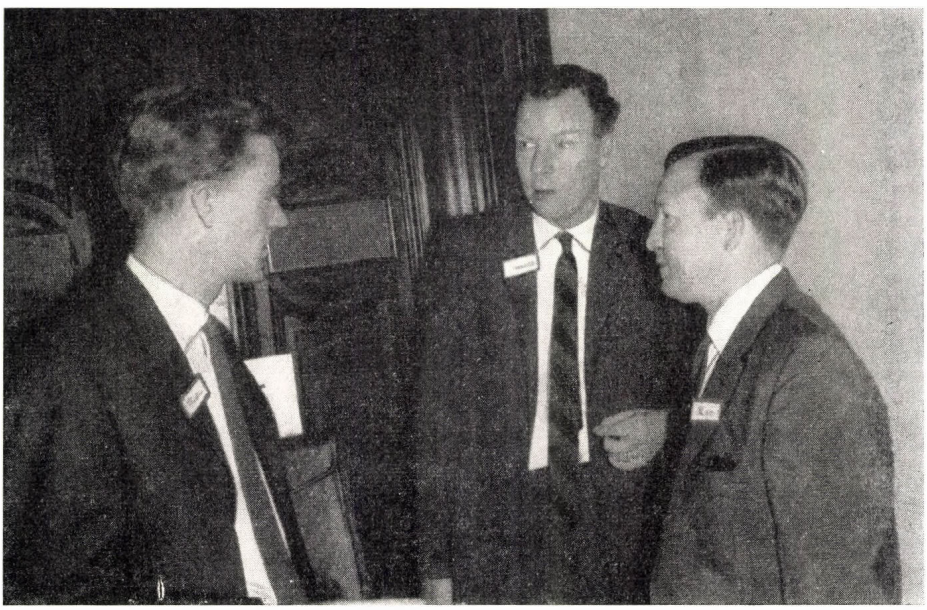

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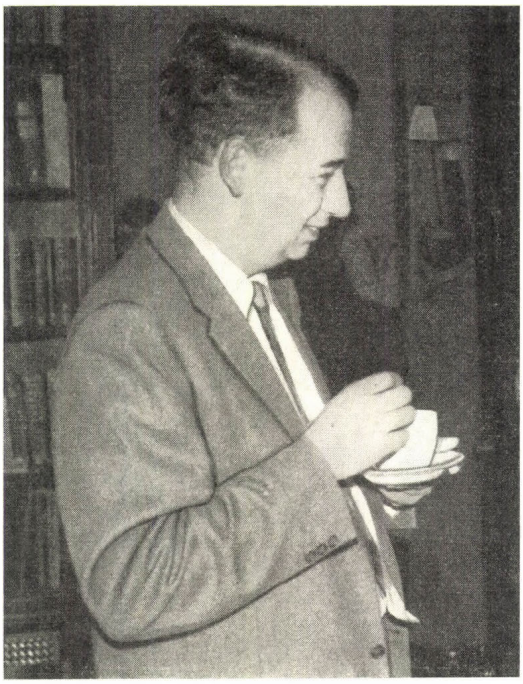

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OPENING ADDRESS

By

F. Bruno Straub

SECRETARY OF THE BIOLOGICAL SECTION,
HUNGARIAN ACADEMY OF SCIENCES, BUDAPEST, HUNGARY

On behalf of the Hungarian Academy of Sciences I am glad to welcome the participants of the *Symposium on Bacterial Transformation and Bacteriocinogeny*.

As a biochemist I am not in a position to make wise introductory comments on the subject of this Symposium, although it is clear to a biochemist that the analysis of transformation phenomena has influenced contemporary biochemistry quite profoundly. Our ideas on genetic information and on information transfer are based on this particular field. I am glad to see that well-known scientists from different countries have come to us to discuss some aspects of the present stand of research in this field. I think, one of the great problems of science still unsolved is the problem of information transfer between scientists. Maybe, the study of biological information and information transfer will help us perfect our methods for scientific information transfer and information retrieval.

Such a Symposium seems to me like an intellectual episomal effect, and I hope that some new thoughts introduced into these talks will manifest themselves in new media and produce new results. Our Academy of Sciences is giving special attention and help to the biological sciences nowadays, as we have realized that, in spite of scattered successes in some fields, we have several shortcomings in basic biological research, and, in particular, we did not do enough in the past to help basic research in genetics.

The convening of this Symposium is one of the measures to help geneticists and the field of modern microbiology. I sincerely hope that this Symposium will, apart from furthering international understanding, give much stimulating intellectual pleasure for participants.

Thank you !

Symp. Biol. Hung. 6, 17–9 (1965)

INTRODUCTORY REMARKS

By

BARNA GYŐRFFY

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It is my privilege and honour to heartily welcome you in the name of the Organizing Committee and of the Hungarian geneticists, on the occasion of the *Symposium on Bacterial Genetics* held for the first time in Hungary.

The task of the opening this Symposium is not only a great honour to me, but a source of deep personal gratification.

Genetics has come to occupy a key position in modern biology and, recently, to serve as a focal point also for biochemistry, physical chemistry, virology and so on. The extensive studies made during the last decade resulted in a shift from the classical approaches of genetics to the molecular view of the mechanism of heredity, which results in reiteration of the danger in the unity of genetics.

Before beginning our sessions, we should do well to take a little time off for considering the topic of this Symposium and its perspectives in the general scheme of genetics.

It was already in the last quarter of the 19th century when Wilson stated: "The transmission of a specific substance or idioplasm, which we have seen reason to identify with chromatin, the general role of the nucleus in metabolism is of vital importance to the theory of inheritance. The cytoplasm is, in a measure, the substratum of inheritance, but it is so only by virtue of its relation to the nucleus, which is — so to speak — the ultimate court of appeal. The nucleus cannot operate without a cytoplasmic field in which its peculiar powers may come into play, but this field is created and molded by itself. Both are necessary to development, although the nucleus alone suffices for the inheritance of specific possibilities of development."

Since that time remarkable progress has been made in our understanding of the basic mechanism of heredity, many major and exciting problems were solved along the avenues of the classical genetics, and during the last decade genetics has been developing spectacularly fruitfully, with ever increasing speed, and brilliant advances have been achieved, particularly in the field of molecular genetics. While this revolutionary and triumphant genetics (as Sonneborn paraphrased) makes an unexpected and illuminating substantial progress from one week to the other, there seems to be also a certain amount of anxiety that there is a fundamental difference between the classical genetics and the molecular genetics that may cause an unbalance in the future development of genetics. I think, it is wrong to make such a characterization, as the difference is far from being fundamental. There is only a difference in the aspects of methodology.

I do not agree with the somewhat exaggerated views that every problem of genetics will sooner or later be solved in molecular terms and that the formal genetics of the future will consist in matching in test tubes DNAs and messenger RNAs from vegetative or somatic cells, or that the molecular genetics is not genetics at all but much more biochemistry or biophysics. I am convinced that the molecular genetics attacking first the basic problems by simplifications at the molecular level, will proceed further to the next, more complicated sets of problems at the cellular, individual and even at the population level, like it has been done by the classical genetics. The future balanced integration of both genetical aspects is obvious.

It seems very often that the problems of molecular genetics predominate and are much more in the focus of interest than any others. I firmly believe, however, that the *XIth International Congress of Genetics* in some weeks at The Hague will witness to the balanced progress in many fields of genetics.

Considering the past and present progress as well as the prospects of genetics, the prediction seems not to be improbable that there are three main avenues along which the genetics will progress in the foreseeable future: first, the molecular and cellular level analysing the further details of classical chromosomal heredity as well as the extrachromosomal inheritance, second, the individual level attacking the still faintly explored genetical problems of differentiation and development, and lastly, the population level revealing the fundamental problems of evolution.

From the general geneticist's point of view, I think, many of the problems of molecular genetics and molecular biology to be discussed in the coming sessions are in interrelations with many other classical fields of genetics and a correlation can only contribute to the balanced unity of genetics.

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Now I wish to recall a few phases of the story of this Symposium. It wa already two years ago when it was proposed to the Hungarian Academy of Sciences that a Symposium on bacterial genetics should be held in 1962 in Budapest. Owing to the severely limited possibility to sponsor such a special Symposium, the subject to be considered was limited to bacterial transformation. However, the organization of the Symposium was postponed until this year, which afforded the opportunity for correlating it in some way with the *International Congress of Genetics* as well as with the *Third Erwin–Baur-Gedächtnisvorlesungen* to be held before the Congress at The Hague, in Gatersleben. When the arrangements of the Symposium really started at the end of the last year, it was thought more profitable if the subject of the Symposium would be extended to bacteriocinogeny, in which field results have been achieved also in Hungary.

It was a courageous step, indeed, that we took over when we started this Symposium and we sent our first informal letters to many of the research workers who had made significant advances in these areas. However, the response was far beyond our expectations and even the interest aroused much greater than we could have imagined. Thus, taking into account that, owing to other commitments, several of the leading workers in transformation had declined our invitation and as a result of the replies from those able to come to Budapest and participate, we decided to extend the topics for discussion also to problems of regulation.

Our idea was that this Symposium should give an opportunity to examine the current states of research work in certain avenues, to hear the presentations of personal contributions to the progress, to discuss problems of mutual interest, and last but not least, to strengthen the links between scientists of different nations.

Our desire, to have a Symposium on bacterial genetics here in Budapest, is now realized and we are proud of such an élite gathering since many of our guests who accepted our invitation and have undertaken the effort to come here contributed remarkably to our understanding of the basic problems of genetics. On the other hand, we regret very much indeed that Professor Bresler, Dr Goldfarb, Professor Maaløe and Professor Shugar cannot be here among us.

In spite of all our efforts, we know, there are also faults in our arrangements, therefore we earnestly request you to bear in mind that we are really rank amateurs in this kind of work and overlook our errors. We will do our best to ensure that these shortcomings are outweighed by our efforts and good-will, and we hope that, with your sincere co-operation and understanding, the Symposium will be successful.

In conclusion, I would like to express appreciation to the Hungarian Academy of Sciences for accepting our proposal and sponsoring this Symposium. I should like to thank also all those who so graciously collaborated in the arrangements and are now assisting in the running of the whole of the Symposium.

So now, on behalf of the Organizing Committee, it is my pleasant duty to declare the *Symposium on Bacterial Transformation and Bacteriocinogeny* open.

Thank you!

SECTION I

BACTERIAL TRANSFORMATION

Symp. Biol. Hung. 6, 23–32 (1965)

MECHANISM OF RECOMBINATION IN DNA-MEDIATED TRANSFORMATIONS

By

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The transformation of receptive bacterial cells by isolated deoxyribonucleate (DNA) from related strains provides one of the simplest genetic systems. Since the classic work of Avery et al. (1944) it has become increasingly clear that the active DNA is a simple form of the genetic material that, organized into chromosomes, is present in almost all living organisms. The demonstrations that a variety of bacterial properties, such as drug resistances and various enzymatic traits, are similarly transferred by DNA justifies the expectation that this determinant material represents the functional matter of many genes, as well as many kinds of cells.

The advantages of this genetic system over most other ones is that the genetic material can be manipulated chemically and physically to a far greater extent when it is in the form of isolated DNA than when it is present in cells, nuclei or even viruses. For in these other cases, the biological activity can no longer be estimated if the manipulation has seriously altered the complex container in which the DNA is carried. Therefore, with transformation systems one has the opportunity to investigate the chemical and physical properties of genetic material, its sensitivity to chemical or physical change, the creation of mutations, the structural requirements for its activity. In what follows, I shall discuss some recent work which, utilizing this approach, begins to show complex behavior previously not known to be associated with single particles of DNA.

Let us first recall that when the results of a transformation could be measured quantitatively (Hotchkiss 1951), it was found that most pairs of traits carried by DNA were separately introduced into different cells. Thus, for a pair of markers X and Y, independent transformants T were found:

$$\text{cells(wild)} + \text{DNA}(XY) \text{ gives } T_X \text{ and } T_Y$$

The double transformant T_{XY}, if recovered, appears only in the low frequencies resulting from successive single transformations. On the other hand, if marker Y is linked to Z (Marmur and Hotchkiss 1960):

$$\text{cells(wild)} + \text{DNA}(XYZ) \text{ gives } T_X + T_{YZ} + T_Y + T_Z$$

In such cases, the linked pair YZ seems to be carried by a single DNA particle, which undergoes separation by recombination whenever T_Y or T_Z are produced. Most pairs of markers behave independently like X and Y.

One set of linked markers of *Diplococcus pneumoniae*, designated *Sadb*, was occasionally transformed together, *Sadb* or *adb*, but was generally sepa-

rated into the smaller units, *Sa*, *ad* or *d* or *S*, etc. (In these, *S* represents streptomycin resistance, *a*, *d* and *b* are separably identifiable sulfonamide resistance traits manifested in a folic acid synthetase.) The hypothesis was, therefore, proposed that there was a size limitation on the amount of genetic material that could be effectively transferred by transformation (Hotchkiss 1958, Balassa and Prévost 1962). Recombination was also being investigated at this time in the population of DNA particles resulting from infection of single cells with mixed bacteriophages (Chase and Doermann 1958, Steinberg and Edgar 1962, Luria 1962). We proposed to explain the quantitative anomalies (non-additivity and high coincidence of multiple transfers) in this and other finely analyzed systems as due simply to one-directional transfer or copying of short regions of the DNA strands. In this view, the usual recombination event in DNA strands is a double one (switch-over and nearby switch-back) so that those exchanges observed in three-factor crosses, in which one is obliged to formulate as double events, would still be ordinary ones. Consequently, their frequent occurrence is expected and there is little or no actual 'high negative interference'. By adhering to the single event (one switch-over) as the unit recombinational process, phage geneticists have had to postulate not only double, but triple and quadruple events, all clustered in, and occurring often in actively recombining regions of DNA (Luria 1962).

Since that time, it has seemed more doubtful whether there is any sharp basis for distinction between the small molecular strands of DNA and the often far larger strands of chromosomes. If there is, it will probably be most important to learn in what way individual DNA molecules are joined to each other—perhaps by cations, organic bases or other substances—in making up the large chromosomes. If there turns out to be no important discontinuity between the DNA molecule and the chromosome, then it will become all the more significant to understand how DNA molecules behave in the recombination that occurs in bacterial transformation.

By a study of the effect of DNA concentration and of time of exposure to DNA on transformation yield, it has been possible to determine how many particles of DNA are required to effect transformation of one cell. This represents some advantage over the phage recombinations which occur between ill-defined populations of phage genomes increasing by synthesis and decreasing by maturation but numbering perhaps several hundred particles within each host cell randomly infected with a few phages of each kind.

In an early work with the non-quantitative antigenic markers, there was a rough proportionality between DNA concentration and the numbers of *sub-populations* of bacteria in which transformant cells were detected (McCarthy et al. 1946, Ravin 1954, Stocker et al. 1953). When individual transformed cells could be counted by the use of the quantitative selective traits, it was shown that *transformation yield* was similarly related to DNA concentration (Hotchkiss 1951, 1957). In the useful cases of streptomycin resistance transformation of both *Pneumococcus* (Hotchkiss 1957) and *Hemophilus influenzae* (Alexander et al. 1954), it was shown that the number of transformed cells was proportional to DNA concentration over a 1000fold range. It was concluded from such work that a single particle of DNA reacting with a cell is sufficient to bring about a single transformation for the

markers which it contains. The fact that unlinked markers were co-transformed at a frequency which is approximately the product of the individual marker frequencies (Hotchkiss 1951, Marmur and Hotchkiss 1960) indicates that practically all the cells are equally transformable. This relationship was shown by Goodgal (1961) to apply over a wide concentration range. Co-transformation of linked markers, on the other hand, remains a linear function of concentration in constant proportion to single transformations (Marmur and Hotchkiss 1960, Balassa and Prévost 1962, Goodgal 1961). Thus, one may approximately characterize a linkage relation by the ratio of the frequencies of the linked group to one of its constituent markers. We may now rewrite the expression given above as

$$\begin{array}{lll} \text{cells(wild)} + \text{DNA}_X + \text{DNA}_Y & \text{gives} & T_X \text{ and } T_Y \\ T_X + \text{DNA}_Y & & T_{XY} \\ T_Y + \text{DNA}_X & & T_{XY} \end{array}$$

while in the case of the linked pair

$$\text{cells(wild)} + \text{DNA}_{YZ} \text{ gives } T_{YZ} + T_Y + T_Z$$

with corresponding two-step transformations when DNAse is also present.

In a recent work with Dr Joan Kent, we have been able to measure transformation yields at a constant DNA concentration as a function of duration of exposure time of cells to DNA. This approach takes the advantage of the uniform time-course that can be observed for the reaction between cells and DNA at 30° (Fox and Hotchkiss 1957), a temperature below that at which the cells are normally grown. The exposure is controlled by adding the DNA at a known time and terminating contact by adding at chosen times pancreatic deoxyribonuclease to break down the unused DNA.

Linear rates of entry are found for individual markers, and these rates are characteristic of the marker (Kent and Hotchkiss 1964). At a given concentration of DNA bearing several markers, the relative effectiveness and concentration of different markers can in this way be very accurately determined. Figure 1 illustrates that linear rates of entry are obtained also for linked markers, although the lowest rates shown are perhaps 3 per cent of the rates for single markers. The relative slopes for the linked and single markers in a DNA solution give a measure of the co-transformation efficiency or linkage of a marker pair, far more reliable and characteristic of the linkage group than when a single time point is used.

In this way, it was possible to show that from a linkage group of three markers, all three, or any pair, including the two outside markers can be taken up as a result of contact with single DNA particles. An example of this last case is the introduction of markers *S* and *d* from group *Sad* (Kent et al. 1963). In all the cases in which this 1–3 incorporation occurred, it proceeded linearly with time, although at a very low rate. Therefore, it seems to be demonstrated that a single DNA particle entering a recipient cell may donate to the latter two separate portions of itself in a recombination event in which four breakage–fusion events have occurred. A very interesting finding is that this more complex kind of recombination virtually disappears when the transformation is carried out at the 'normal' growth temperature of 37° (Kent et al. 1963).

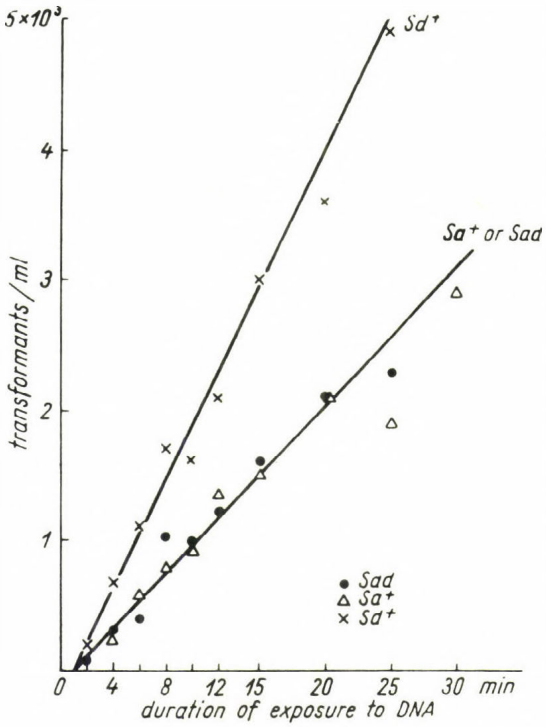

FIG. 1.—Rate of accumulation of specified transformants as a function of exposure of *d* cells to *Sa* DNA. The triple transformant *Sa*+ (that is *Sad*$^+$ or *SaD*) is produced as frequently as the double *Sad*. The class *Sd*$^+$ (or *SD*) includes two types; by subtracting the values for the directly measured classes, *Sd*$^+$ minus *Sa*$^+$, one obtains the 'doubleswitch recombinants', *Sa*$^+$*d*$^+$ (or *SAD*). (*S* = streptomycin resistance, *a* and *d* = different distinguishable sulfonamide resistance factors)

Thus, we may conclude that a single DNA strand *is able* to transfer non-adjacent markers in a transformation event; this is one of the ways DNA may behave. There may, however, be restricting influences, favored perhaps by evolution, which reduce the extent of such complex events unless the DNA pairing contact is maintained abnormally long by unphysiological conditions. As mentioned, this type of event seems to occur in populations of growing phages, but it is difficult in that case to determine the number of particles involved and whether the true number of switch events is the same as that inferred. When two markers coming from separate donors (mixed DNA) are incorporated, the transfer rate is always quadratic, showing that exactly two DNA particles are needed to produce the paired transformation. This is also true for unlinked markers coming from the same donor (Kent and Hotchkiss 1964) (see Fig. 2) and for previously linked markers after subcritical heat inactivation (localized depurination) (Kent et al. 1963).

In summary, these new kinetic criteria for transformation rates give strong indications that the number of DNA molecules involved in particular transformations of individual recipient cells are as follows.

Transformations effected by single DNA molecules: (1) single markers, (2) linked markers from single donors, (3) pairs of markers from linkage group even when intermediate markers are not incorporated.

Transformations requiring two DNA molecules: (1) unlinked markers from single donors, (2) markers of a linkage group when derived from different donor strains (in mixed DNA of the two strains, (3) a small proportion of linked markers (proportional to single transformation frequency) may enter by successive transformations by two molecules from the same strain, (negligible, except when concentration of DNA is high or time of expo-

sure is prolonged), (4) linked markers from the same donor, when localized depurination or other damage has inactivated many of the individual markers.

One corollary of this finding is that, while pairing of DNA molecules gives increased opportunity for linked markers to be incorporated, it does not substantially decrease the probability for a second DNA particle to donate markers to the same or different parts of the same cell genome.

It has been pointed out that markers appearing to be unlinked might really be part of the same structure but merely so far apart that the large structure was unable to be incorporated at once (Hotchkiss 1958). Balassa and Prévost have made more detailed calculations of such probabilities by assuming a size-influenced incorporation (Balassa and Prévost 1962) instead of a maximum size-limited one as was done earlier (Hotchkiss 1958). It now seems that the kinetic criteria described above are able to show that most, if not all, of the 'unlinked' double transformations we have observed do indeed involve markers of separate molecules.

This type of analysis is proving to be of value in the study of another new feature of transformation, involving apparently heterozygotic regions of DNA. This work is largely carried out in collaboration with Dr Mihoko Abe and is now in preparation for detailed publication. A sulfonamide resistance marker, *c*, discovered some time ago in work with Miss Evans (Hotchkiss and Evans 1958), had been observed to be 'unstable' when strains containing it were repeatedly passed for many generations. When reexamined by Dr Abe, it was found that approximately one progeny cell from each five or six divisions (1 in 32 to 64 cells) lost its sulfonamide resistance. Since the sensitive cells produced were stable and indistinguishable genotypically or phenotypically, from wild type (+) cells, the process appears to be as shown schematically in Fig. 3. The unstable *c* strains arising either by mutation or by transformation give a mixed population. Taking advantage of specific sulfonamide-related drugs, the population may be selected for its phenotypic traits into two kinds. Selected in

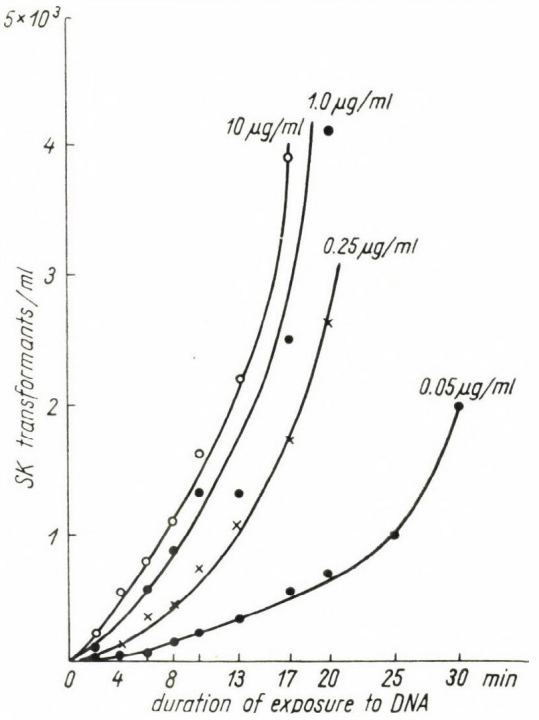

FIG. 2.—Rate of accumulation of unlinked markers *S* and *K* (streptomycin and micrococcin resistance) at different DNA concentrations. All the curves are essentially quadratic; that is, (transformants) 1/2 is a linear function of duration of exposure

sulfanilamide (SA) the population becomes 'pure' *c* cells, which, however, soon lead to production of wild (+) cells again. Selected in para-nitrobenzoic acid (NOB), selection is against *c* cells, and a wild type population results which shows no ability to turn back into, or recombine to give, resistant cells again.

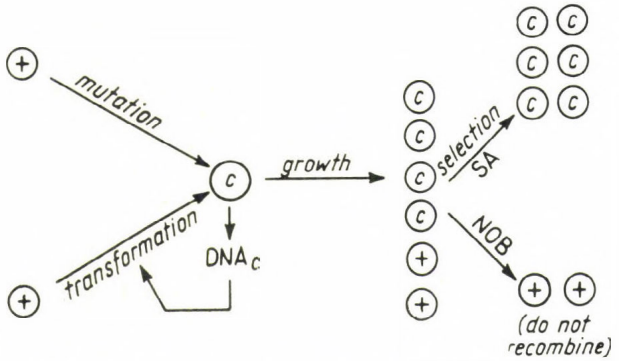

FIG. 3.—Scheme of growth, segregation and selection of *c* sulfonamide resistant cells

This could be viewed as a mutation with a high reversion rate, but it appeared like a segregation of a *c* marker away from some heterozygotic form which contains both the *c* and its wild-type allele *C*. This heterozygotic form could be an overlapping structure of some sort, or some episome-

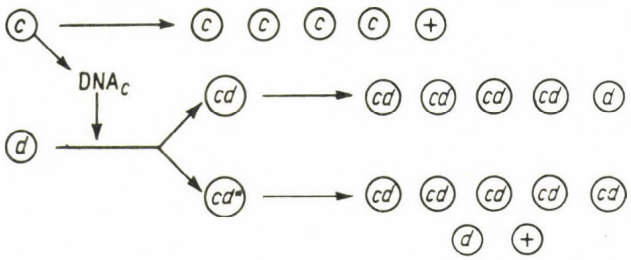

FIG. 4.—Scheme showing segregation of *c* and two kinds of *cd* cells

bearing form as suggested by the very interesting recent work of Dr Watanabe, reported at this Symposium and elsewhere (Watanabe 1963). The combination of marker *c* with resistant strains *a* and *b* threw no light upon the matter, for *ac* (or *bc*) strains segregated or reverted to *a* (or *b*) strains in the same way as when *a* and *b* were not present.

However, when we transferred marker *c* to a *d* strain, a closely linked pair, *cd*, was produced. Now, if *c* merely mutates back into *C*, then *cd* strains should change back only into *d* strains (*Cd*). But, if a heterozygotic structure *c*(*C*)

were the basis of the *c* instability, one might expect that the DNA carrying the *c* marker would sometimes introduce its *D* neighbor into the *cd* strain.

This latter is what apparently happens. When *cd* strains are examined by selection in the drug para-nitrobenzoic acid, two types are found. One type can produce stable *d* strains, while the other gives both stable *d* and (+) types. The recovery of (+) cells (*CD*) means that marker *D* has also been introduced presumably from the *c* donor (*cD*). Furthermore, when the *c* marker leaves the strain, it appears sometimes to take with it the *d*, sometimes the *D*, marker.

The phenotypes found are summarized in Fig. 4. These results are explainable if the *c* strain carries marker *C* unexpressed, and the *cd* strains carry

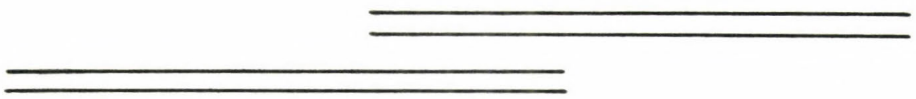

FIG. 5.—Overlap model

in the one case *C* and in the other *CD*, in an unexpressed state. So we examined the DNA of these strains for latent markers, not expressed in the strain itself, but transferable to recipients of the suitable genotype. In Table I are shown the results. It is apparent that the suspected latent markers are all present. By using the sophisticated kinetic test for the linkage mentioned above, it is found that the pairs *cd* and *CD* are linked to each other, while *c* is not linked to *D*. By an occasional recombination, the *Cd* type is formed and segregates out as an apparently stable product. If *D* is not present, this is the only detected rearrangement; if *D* is present, the *CD* type (wild type) segregates out even more frequently.

TABLE I

Transforming agents recovered from cd strains

Phenotype	Inferred genotype	Transforming agents recognized*
c	$\frac{C}{c}$	*c*
cd	$\frac{C}{c\ d}$	*cd*, *c*, *d*
*cd**	$\frac{CD}{c\ d}$	*cd*, *CD*, *c*, *d*, *D*
*Scd**	$\frac{CD}{S\quad c\ d}$	*Scd*, *cd*, *Sc*, *CD* (and *S*, *c*, *d*, *D*)

* Note: marker *C* cannot be recognized alone in transformation

These findings indicate that marker *c* becomes more intimately associated with preexisting bacterial markers (*d*) than it would be if it were borne on a free episomal particle. Since virtually all of it behaves this way all of the

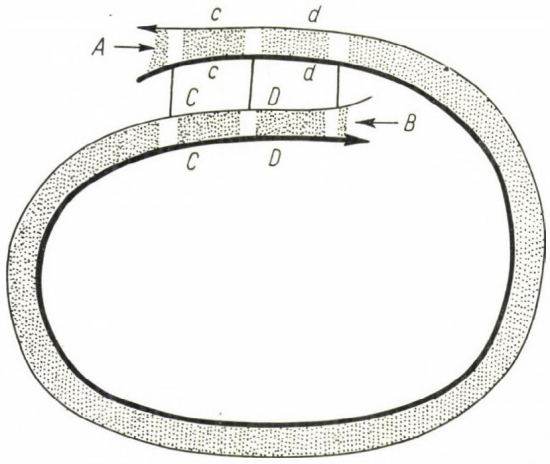

FIG. 6.—Model of pneumococcal chromosome that would account for stability and behavior of *cd* (*CD*) strains. *AB* = possible sites of initiation of replication and of formation of messenger, *CD*, *cd* = markers in heterozygotic region

time, an episome seems to be excluded. Furthermore, the *CD* or *C* markers so carried are not functional. This was learned from the absence of a folic acid synthetase having the known drug sensitivity corresponding to these markers (until recombination occurs). The DNA bearing these markers does not seem to be in any way physically abnormal. We will discuss these and other studies on these strains in more detail in later publications.

An adequate structural model for the heterozygote *cd* (*CD*) should, therefore, show the following properties: (1) two regions having duplicated genes carried by (2) double-stranded DNA in both regions, with (3) possibility for recombination between the two regions, but (4) with normal DNA replication usually reproducing both regions and heterozygosity, however (5) these regions to be asymmetric in their relations to the rest of the genome so that (6) creation of gene product is not equal for both, therefore, presumably, sites of initiation of RNA transcription (initiator, operator, punctuation) being absent or not functional in one of the two regions.

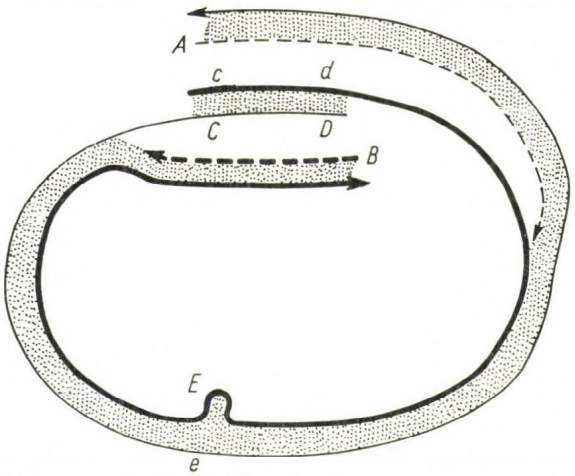

FIG. 7.—Model of Fig. 6 after commencement of polarized single strand (Kornberg) replication. Note that at *cC* the resting ends stabilize each other. Note also that heterozygosity at *E* would segregate at this next division, while heterozygosity at *cd* is replicated

The first three of these properties hold for a simple overlap model (Fig. 5). The last three properties seem more satisfactorily accomodated by a model which we have been considering for some time, the one presented in Fig. 6. Doubtless, other models also would serve in varying degrees, but this one has the additional advantage of giving a chromosome that would appear

to be circular in linkage tests (and in electron micrographs). It would allow stability of the loose ends also during a 'Kornberg replication' of single strands (Kornberg 1961), starting from opposite ends, as suggested in Fig. 7. If replication of both strands actually proceeded synchronously from one end, as suggested by recent autoradiographs of dividing *Escherichia coli* cells (Cairns 1963), then the ends could still hold each other.

More important, the model gives asymmetric positions to the repeated regions *cd* and *CD*. We may think of *cd* as being associated with a site of initiation of transcription ('initiator' or 'operator'), perhaps to the right of *d* in Fig. 7, which is absent entirely at the *CD* end of the strand (at *B*). This would not affect the repeated complete replication of the DNA including the overlap, but would prevent formation of any gene product ('messenger') from the (non-functioning) *CD* part.

Although we were unaware of it, this model with terminal redundancy had been suggested for phage heterozygotes some time before by Streisinger and discussed informally at various times, being finally summarized in an excellent presentation by Dr Stahl at this Symposium. We have not begun to make as many experimental tests of the model as he has outlined, but we may perhaps be considered to have offered another not too rigorous application of the model and arguments consistent with it. In addition, it is of interest that we had a somewhat different reason for considering the model: the balance of DNA replication, and the imbalance of DNA function, in the two parts.

In summary, we can now state with some confidence that recombination in transformation occurs between the bacterial genome and one or more parts of a single incoming DNA molecule. A second DNA molecule is not excluded from acting on the same recipient cell subsequently, but this process can be distinguished from the other by kinetic criteria. At least one transformation introduces a duplicate marker or linked pair of markers to produce a heterozygotic structure. This structure is so constituted that only one part of it is phenotypically expressed in cell function. It is replicated intact, however, except that an occasional recombination occurs, with the production of a stable segregant having a different assortment of the markers in the functional state.

REFERENCES

Alexander, H. E., Leidy, G. and Hahn, E. (1954) *J. exp. Med. 97*, 797

Avery, O. T., MacLeod, C. T. and McCarthy, M. (1944) *J. exp. Med. 79*, 137

Balassa, G. and Prévost, G. (1962) *J. theor. Biol. 3*, 315

Cairns, J. (1963) *J. molec. Biol. 6*, 208

Chase, M. and Doermann, A. H. (1958) *Genetics 43*, 332

Fox, M. S. and Hotchkiss, R. D. (1957) *Nature (Lond.) 179*, 61

Goodgal, S. H. (1961) *J. gen. Physiol. 45*, 205

Hotchkiss, R. D. (1951) *Cold Spr. Harb. Symp. quant. Biol. 16*, 457

Hotchkiss, R. D. (1957) in *Chemical Basis of Heredity*, ed. W. McElroy and B. Glass, Johns Hopkins Univ. Press, Baltimore

Hotchkiss, R. D. (1958) The biological replication of macromolecules, in *Symp. Soc. Exp. Biol. XII*, 49

Hotchkiss, R. D. and Evans, A. H. (1958) *Cold Spr. Harb. Symp. quant. Biol. 23*, 58
Kent, J. L. and Hotchkiss, R. D. (1964) in preparation for publication
Kent, J. L., Roger, M. and Hotchkiss, R. D. (1963) *Proc. nat. Acad. Sci. (Wash.) 50*, 717
Kornberg, A. (1961) Enzymatic Synthesis of DNA, *Ciba Lectures in Microbial Biochemistry*, John Wiley and Sons, New York
Luria, S. E. (1962) *Ann. Rev. Microbiol. 16*, 205
Marmur, J. and Hotchkiss, R. D. (1960) *Proc. nat. Acad. Sci. (Wash.) 40*, 55
McCarthy, M., Taylor, H. E. and Avery, O. T. (1946) *Cold Spr. Harb. Symp. quant. Biol. 11*, 117
Ravin, A. W. (1954) *Exp. Cell Res. 7*, 58
Steinberg, C. M. and Edgar, R. S. (1962) *Genetics 47*, 187
Stocker, B. A. D., Krauss, M. R. and MacLeod, C. T. (1953) *J. Path. Bact. 66*, 330
Watanabe, T. (1963) *Bact. Rev. 27*, 87

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KINETICS OF PROVOKED COMPETENCE IN STREPTOCOCCAL CULTURES AND ITS SPECIFICITY

By

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Transformability of bacteria is so far limited to a small number of species and within these species only to some strains. The ability to undergo transformation is defined as competence but the nature of this phenomenon is not well understood.

After a careful study of competence in streptococcal cultures, we found that the appearance of competence resembles in many respects the kinetics of accumulation of enzymes and toxins, such as streptodornase, streptokinase, hialuronidase and streptolysin 0 in cultures of these bacteria. We assumed, therefore, that streptococci do not develop competence until a substance produced by the cells themselves, which is capable of converting non-competent cells into competent ones, is accumulated. This was found to be true and a factor provoking competence was discovered in sterile supernatants of transformable and competent streptococcal cultures.

Before the characterization of this factor, it has to be mentioned that, under natural conditions, competence of streptococci develops only in media supplemented with either serum or albumin. In the absence of these substances transformation does not occur or, if present, is extremely low. The onset of competence, the period of its duration and the time of its disappearance are strictly dependent on the size of the initial inoculum in the culture. The smaller the inoculum the later the appearance of competence. Thus, the conversion to competence of the non-competent bacteria with the use of the provoking factor can be demonstrated in two different conditions: (1) in cultures grown in the absence of serum or albumin, (2) in cultures grown in media containing serum or albumin. As for the second condition, the culture is started with a small amount of initial inoculum and the cells are exposed to the action of the provoking factor for 2 h before natural competence can be expected.

In all experiments, except one, the first method of competence provocation was applied. The marker used in the transformation experiments was streptomycin-resistance. The recipients and donors were group H haemolytic streptococci and strains of *Streptococcus sanguis*, Types I, II and I/II regarded to be closely related to group H streptococci. Strains of *S. sanguis*, Type I/II share the C-polysaccharide characteristics of group H streptococci.

With the factor provoking competence available, the following procedure of transformation was developed. Bacteria grown in the absence of serum or albumin for 2 h were exposed to the action of the provoking factor. After 30 min, the cells were spun down, the supernatant containing the provoking factor discarded, the cells suspended in fresh medium, exposed to the action

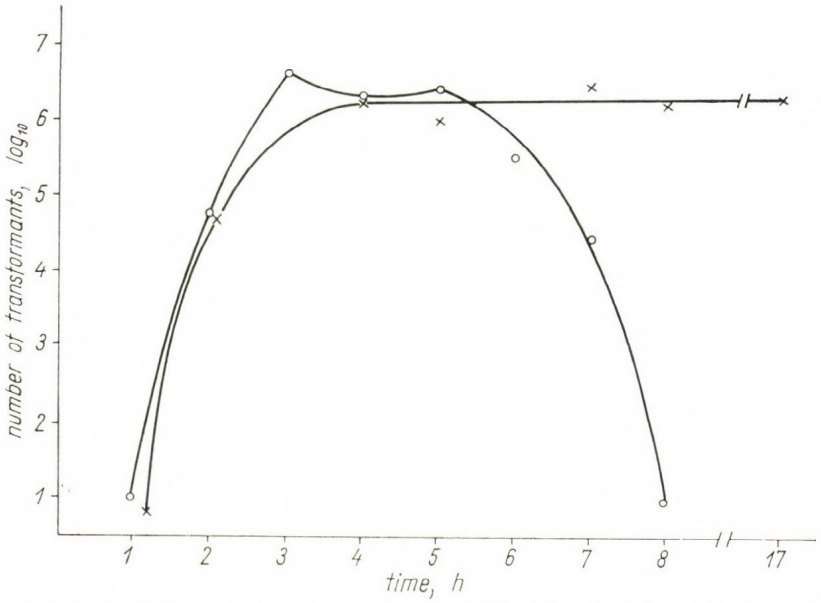

FIG. 1.—Development of competence in a culture of strain Challis and the competence-provoking activity of supernatant fluids prepared at times indicated. The competence-provoking activity was tested on homologous organisms in the absence of serum or albumin. ○ = competence, × = competence-provoking activity of the supernatant fluid

of DNA for 10 min and then allowed to grow in the absence of the antibiotic for 110 min for phenotypic expression. Transformants were scored after 40 h at 37° on blood agar plates containing 250 μg/ml of streptomycin.

Figure 1 illustrates time as a function of development of competence and of the production of the provoking factor in a culture of strain Challis, a group H streptococcus. As can be seen, competence disappeared at the end of logarithmic phase of growth but the competence-provoking activities of the sterile supernatant fluids prepared even after 17 h of growth of the culture did not differ markedly from those prepared at the time of maximum competency.

The provoking factor can be precipitated from the supernatant by ammonium sulphate (80–90% saturation) or by cold ethanol (8 volumes). As shown in Fig. 2, the first method of precipitation is more efficient.

The provoking factor is heat sensitive. Samples of ammonium sulphate concentrates of provoking factor dissolved in nutrient medium, in 0.01 M phosphate buffer or in saline, all adjusted to p_H 7.2, were heated for 10 min at temperatures indicated in Fig. 3, and then their competence-provoking activity was tested. Obviously, non-saturating amounts of provoking factor were used in order to observe any decline of activity caused by heating. As can be seen in Fig. 3, the provoking factor seems to be protected by some compounds present in the nutrient medium.

The conversion to competence of non-competent cultures with the use of the provoking factor is temperature dependent. The provoking factor is not

active at 0°, 10° and 20° (Fig. 4). Maximum activity was observed at 37°. Temperatures higher than 40° could not be tested, because they affect growth and viability.

Provocation of competence is also time dependent. The efficiency of transformation was found optimal after 25–35 min of action of provoking factor.

The provoking factor seems to be rather non-dialysable. Visking dialysis tubings known to retain particles of molecular weight greater than 10^4, were filled with crude concentrates of provoking factor and poured into glass vessels which contained 20 volumes of 0.01 M phosphate buffer. Some loss of activity was observed but it was not higher than 15–20% of initial activity. It seems, therefore, that no cofactor is necessary for the action of the provoking factor.

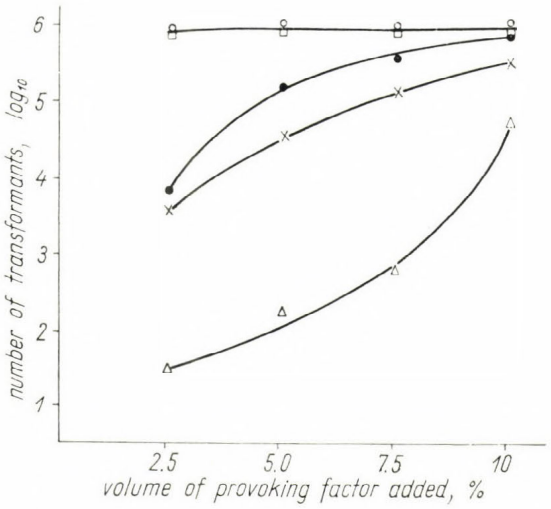

FIG. 2.—Competence-provoking activity of a supernatant fluid and of crude concentrates of provoking factor. ● = supernatant fluid, ○ = ammonium sulphate precipitate dissolved in 1/10 of the initial volume, □ = ethanol precipitate dissolved in 1/10 of the initial volume, × = ammonium sulphate precipitate dissolved and diluted to the initial volume of supernatant fluid used for precipitation, Δ = ethanol precipitate dissolved and diluted to the initial volume of supernatant fluid used for precipitation

The efficiency of provoked transformation is dependent on the medium in which bacteria are suspended for the time of action of the provoking factor. This is demonstrated in Table I. The highest yield was observed when the provoking factor acted on bacteria in nutrient medium. In saline, which contained various amounts of nutrient medium, the efficiency of conversion to competence was higher when more nutrient medium was added to the saline. Chloramphenicol in a concentration of 7.5–10 mg/ml completely blocked conversion to competence.

A given portion of provoking factor, concentrated or not, can be used several times to provoke competence. Obviously, in this kind of experiment, bacteria were treated repeatedly with small amounts of provoking factor, which were much below the saturating concentration of even a highly active preparation. Under such conditions, therefore, any irreversible fixation of the provoking factor by the first portion of cells should result in the diminution of the effect of action on the second portion of cells. This is, however, not so as can be seen in Table II. On the contrary, the effect of the second application of the factor is superior to the effect of its first use and the results of the third application are, on most instances, as good as those of the first one. We have no explanation for the high activity of the

TABLE I

Conversion to competence in non-growth media

Experiments	Media					
	ET3	saline + 25% of medium ET3	saline + 15% of medium ET3	saline + 5% of medium ET3	saline	ET3+7.5 mg/ml of chloramphenicol
1.....	$200 \cdot 10^4$ transformants/ml 100%	61.0%	32.2%	16.1%	7.1%	0
2.....	$334 \cdot 10^3$ transformants/ml 100%	55.1%	34.7%	17.4%	6.0%	0
3.....	$256 \cdot 10^3$ transformants/ml 100%	49.8%	23.2%	11.9%	5.7%	0
Mean value	100%	55.3%	30.0%	15.1%	6.3%	0
Increase of viable count during 30 min action of provoking factor	1.8 times	1.6 times	1.6 times	1.3 times	1.3 times	0.97

provoking factor at its second use. It can be assumed, however, that the first portion of cells absorbs a substance from the crude preparation of the provoking factor so inhibiting provocation of competence.

Competence is a transient state. Streptococcal cells, which have become competent upon the action of the provoking factor, lose the ability to be

TABLE II

Effects of the repeated use of the provoking factor on conversion to competence

Experiments carried out with concentrates of the provoking factor	Effects		
	first use	second use	third use
1	$460 \cdot 10^2$	$330 \cdot 10^3$	$490 \cdot 10^2$
2	$780 \cdot 10^3$	$347 \cdot 10^4$	$355 \cdot 10^3$
3	$308 \cdot 10^2$	$240 \cdot 10^3$	$312 \cdot 10^2$
4	$326 \cdot 10^3$	non-tested	$322 \cdot 10^3$
Experiment carried out with the supernatant of a competent culture as a source of provoking factor			
5	$206 \cdot 10^2$	$680 \cdot 10^2$	$616 \cdot 10^2$

transformed if DNA is not added at the proper time. The rate of decline of competence is a function of time and temperature as shown in Fig. 5. In this experiment bacteria grown for 2 h in nutrient medium were exposed to the action of the provoking factor for 30 min, centrifuged, resuspended in fresh nutrient medium and maintained either at 22° or at 37°. At time intervals, indicated in Fig. 5, aliquots were removed, supplemented with DNA and permitted to grow for phenotypic expression. The transformants were scored in the routine way. The number of viable units forming colonies was also determined. As shown in Fig. 5, competence disappeared almost completely at 37° after 60 min, but at 22° it was little affected within 3 hours.

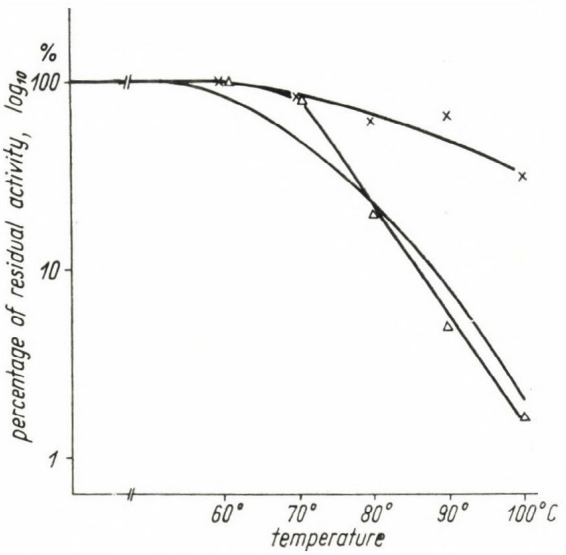

FIG. 3.—Heat sensitivity of competence-provoking factor. The activity of the unheated factor is indicated as 100%. × = concentrate of provoking factor diluted in nutrient medium before heating, Δ = diluted in buffer, unmarked curve = concentrate diluted in saline

In order to determine whether the rapid loss of transformability at 37° is a result of cell division, aliquots of competent cells were suspended in nutrient medium, which contained 7.5 mg/ml of chloramphenicol, or in buffered saline. The bacteria were kept in a 37° water bath and their capacity to undergo transformation tested at times indicated in Fig. 6. Chloramphenicol in a concentration of 7.5 mg/ml does not decrease the viable count but it does inhibit cell division. The rates of drop of competence in buffered saline and in nutrient medium with chloramphenicol are nearly the same as those in nutrient medium without

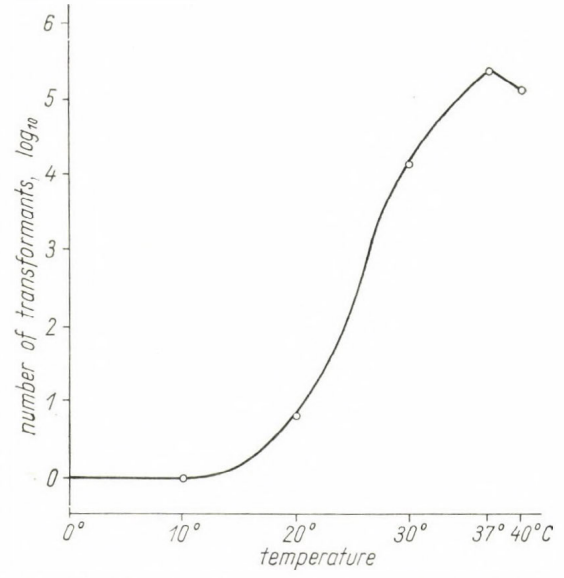

FIG. 4.—Dependence of activity of the provoking factor on temperature

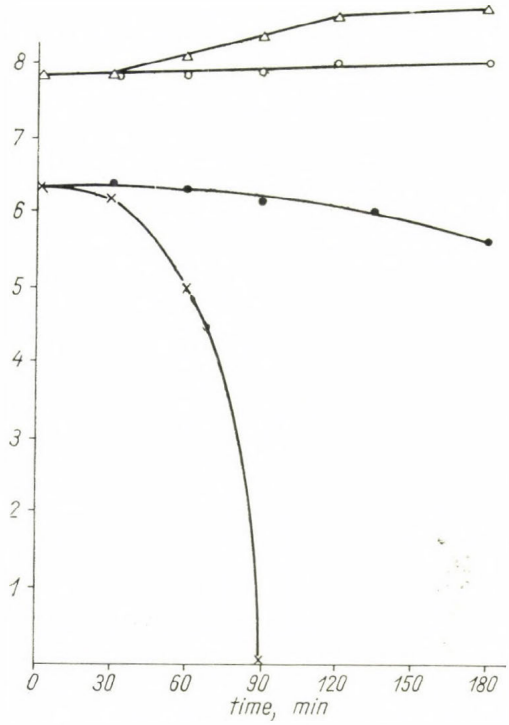

FIG. 5. — Loss of competence in nutrient medium at 37° and 22°. × = loss of competence at 37°, ● = loss of competence at 22°, △ = increase of the number of viable units at 37°, ○ = increase of the number of viable units at 22°

chloramphenicol, at 37°. In the presence of chloramphenicol, however, there was no increase in the number of viable units and in saline it was very insignificant. We conclude, therefore, that it is not cell division, but some metabolic processes taking place at 37° but not at 22°, that result in a decline of competence.

The specificity of the action of the provoking factor is of considerable interest. The kinetics of development of competence was studied in cultures of three transformable streptococcal strains: in those of two strains of group H streptococci, Challis and 3437/48, and of one strain of *Streptococcus sanguis*, Type I/II. All these strains share the C polysaccharide common to group H streptococci. The *S. sanguis* strains, however, are alfa haemolytic and also differ from the beta haemolytic group H streptococci with regard to some fermentation reactions. In the presence of serum or albumin all the three strains produce exocellular factors capable of provoking competence in conditions in which it never occurs naturally, e.g. in the absence of serum or albumin. The specificity of the action of the factors was tested on homologous and heterologous, on transformable and non-transformable strains. Two different conditions were applied in the tests: (1) the provoking factor acted on bacteria grown in the absence of serum or albumin, (2) the factor acted on bacteria grown in the presence of serum. In the latter case, the cultures started with small initial inocula and the provoking factors were added before natural competence was achieved. Table III illustrates the specificity of action of the provoking factors tested in the first condition. Strains Wicky, E91/46, Channon, Blackborn and H4 are group H streptococci and were received from the *Streptococcal Reference Laboratory of the Central Public Health Laboratory, Colindale, London*. They all have to be regarded as non-transformable because, when tested, they could not be transformed by any means in various conditions proper for transformation of other streptococci.

As can be seen in Table III, the factor derived from the cultures of strain Challis provokes competence in cultures of the homologous strains and of the heterologous strain 3437/48. The factor produced by strain 3437/48 also

TABLE III

Specificity of the action of the provoking factors tested in media without serum or albumin

Test strains	Transformants in 1 ml of culture			
	Control without provoking factor	Provoking factors from strains		
		Challis	*S. sanguis* Type I/II	3437/48
Challis	$3 \cdot 10^0$	$1 \cdot 10^6$	$9 \cdot 10^1$	$5 \cdot 10^4$
S. sanguis, *Type* I/II..	$1 \cdot 10^0$	$2 \cdot 10^1$	$17 \cdot 10^4$	0
3437/48	0	$21 \cdot 10^4$	0	$22 \cdot 10^4$
Wicky	0	$52 \cdot 10^4$	$7 \cdot 10^0$	$20 \cdot 10^4$
E91/46	0	0	0	0
Channon	0	0	0	0
Blackborn	0	0	0	0
H4	0	0	0	0
S. sanguis, Type I ...	0	0	0	0
S. sanguis, Type II ...	0	0	0	0

acts on bacteria of strain Challis. Both factors provoke competence in cultures of the naturally non-transformable strain Wicky. The provoking factor of *S. sanguis* acts only on homologous cells.

There is some change in this picture when the test is carried out in the presence of serum (Table IV). Under this condition the factor of strain Challis provokes competence in cultures of *S. sanguis*.

Special attention has to be paid to the provocation of competence of the non-transformable strain Wicky. To the best of our knowledge, it is the first example of provoked transformation of a naturally non-transformable bacterium achieved as a result of the application of a transferable factor produced by a heterologous, related strain. The efficiencies of this transformation are of the same order of magnitude as the efficiencies of the provoked homologous transformations observed with strains Challis, 3437/48 and *S. sanguis*, Type I/II (5–15%). In culture supernatants of strain

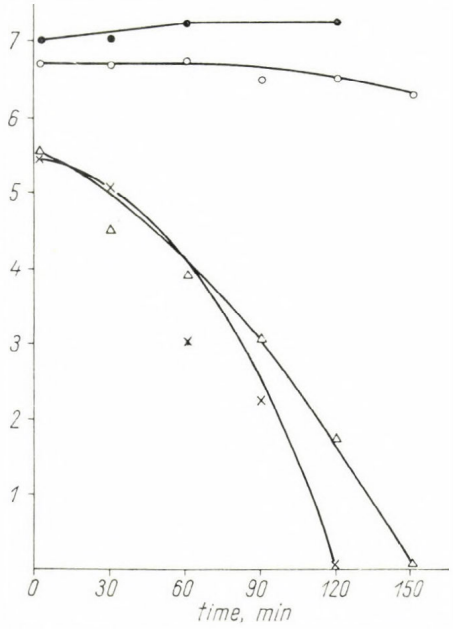

FIG. 6.—Loss of competence at 37°. × = saline, Δ = nutrient medium containing 7.5 mg/ml of chloramphenicol ● and ○ = corresponding curves illustrating viable count

TABLE IV

Specificity of the action of the provoking factors tested in media with serum

Test strains	Transformants in 1 ml of culture			
	Control without provoking factor	Provoking factors from strains		
		Challis	*S. sanguis* Type I/II	3437/48
Challis	$40 \cdot 10^1$	$20 \cdot 10^4$	$4 \cdot 10^2$	$1 \cdot 10^5$
S. sanguis, Type I/II	$5 \cdot 10^1$	$12 \cdot 10^4$	$42 \cdot 10^4$	$4 \cdot 10^1$
Wicky	0	$100 \cdot 10^4$	$2 \cdot 10^1$	$1 \cdot 10^5$
E91/46	0	0	0	0
Channon	0	0	0	0
Blackborn	0	0	0	0
H4	0	0	0	0
S. sanguis, Type I	0	0	0	0
S. sanguis, Type II	0	0	0	0

Wicky no factor-provoking competence was found. We assume, therefore, that strain Wicky cannot be transformed because it does not produce the exocellular factor necessary for the development of competence.

It is also evident from the data given above that serum or albumin are not essential for the development of competence itself, but only for the production of the provoking factor. After the addition of the provoking factor, streptococci are converted into competence in the absence of serum or albumin.

The kinetics of the conversion of non-competent streptococci to competence is consistent with an enzymic reaction and the factor itself seems to be a protein of enzymic activity. It is precipitated by ammonium sulphate, is heat sensitive and does not pass through Visking dialysis tubings. The conversion to competence is time and temperature dependent and very specific. In reciprocal transformations to streptomycin and cathomycin resistance strain Challis does not discriminate between homologous DNA and DNA of strain *S. sanguis*. This is also true for *S. sanguis*, which is transformed with the same efficiency by either homologous DNA or DNA of strain Challis. The strains mentioned, however, can easily be distinguished by the specificity of the action of their provoking factors (Table III).

The findings presented suggest that the development of competence requires the action of an enzyme. The question arises, therefore, what its substrate is. This question cannot be answered until the provoking factor is purified and its action on cell constituents, primarily on the cell wall, tested.

As shown above, chloramphenicol blocks conversion to competence by means of the provoking factor. It also has little effect on cells suspended in non-growth media such as saline. One may conclude, therefore, that conversion to competence requires cells with non-disturbed metabolic activities. Competence, therefore, is the result of the action of the exocellular provoking factor and of one or more factors produced by fully metabolizing cells. This factor (or factors) is probably a protein because conversion to competence

is blocked by chloramphenicol. There is also other evidence that the mechanism of development of competence is rather complicated and that it cannot be explained by the action of a single factor. In Table III five non-transformable strains of group H streptococci are mentioned, of which only strain Wicky can be converted into competence, with the use of the provoking factors produced by related strains. Thus, strain Wicky differs in some respect from the other non-transformable group H streptococci. We could not find the exocellular factor in cultures of strain Wicky but it is possible that, in contradiction to the other non-transformable group H streptococci, it is capable of producing the other components necessary for the development of competence.

DISCUSSION

HOTCHKISS: Certainly the factor seems to be a protein and the reaction between the cell and the factor is a time-dependent process leading to the establishment of the competent state of the cells. However, could not the factor be a substrate acted upon by some enzyme present in the cells, rather than being an enzyme itself?

PAKULA: This is possible, but it is difficult to demonstrate. Whether you are dealing with a substrate or an enzyme, the whole cell in a dynamic physiological state makes the question rather complicated; something must be produced in the cell under some conditions which can bring the cell to competence. The most probable site for this action would seem to be the cell surface.

HOTCHKISS: We have no observation directly supporting and certainly none contradicting Professor Pakula's interesting views on competence in streptococci. I can, however, say that an increase in competence may be caused by a substance in our pneumococcal experiments. In a general way, our views on competence fit quite well with yours, but it will certainly take some time before we know how the recipient cells are altered. We also agree, in particular, about the necessity of understanding competence, in order to do reliable work on transformation. I should like to mention that our work is being done mainly by Dr Alexander Tomasz, who completes this work with some excellent electron-micrographs of thin sections of *Pneumococcus*.

KOHOUTOVÁ: We tried to repeat the experiments done by Dr Pakula in our recipient strain of Pneumococcus, using various amounts of supernatant of competent culture to induce competence in a non-competent culture. The 50 per cent supernatant elicited a 20 min shift of the peak of the competence, but at the same time a tenfold decrease in the incidence of the transformants, in comparison with the control without the supernatant. The presence of 25 and 10 per cent supernatant caused a small reduction of the frequency of transformant in the peak of competence but the position of the peak was not influenced. There was a substantial increase in the frequency of transformant, in the rising part of the competent curve. In this experiment we had the initial density of 4×10^6 of colony forming units in 1 ml of the transformation medium. When, in the next experiment, we used a culture of 1×10^7 colony forming units in

1 ml of the transformation medium, that is the initial density, from which the supernatant was prepared, and we used 25 per cent of it for the initiation of competence, the peak of the competence was shifted from 90 to the 80 min, but at the same time the incidence of transformants decreased to 19 per cent of that of the control without supernatant. The percentage of the supernatant must also be related to the initial density of the culture, from which the supernatant is prepared.

PAKULA: Recently we have really done one experiment with *Pneumococci*. I do not yet know the result, but in Streptococcus we have never used such a great amount either of supernatant or of concentrated provoking factor. What I should like to add is, what we understand under competence. I think, I should agree with Dr Stuy, who in a paper on the competence of *Hemophilus influenzae* writes that the definition of the competent cell is that it is one which can be transformed in the presence of chloramphenicol. We find in our case that once the cell is converted into competence, DNA is taken up in the presence of chloramphenicol, and this means that the transformation of a competent cell is not inhibited by the presence of chloramphenicol.

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PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF GENETIC MARKERS IN TRANSFORMING DNA

By

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The ultimate goal of any research in molecular genetics is to describe the detailed chemical structure of the hereditary material characteristic of a given organism, most desirably in terms of the DNA base sequence, and to determine the mode in which this sequence is transcribed and translated into the multitude of structural, metabolic and regulatory features of the living organism. In attacking this formidable task it is necessary to select some simple organism endowed with several favorable properties, including amenability to genetic analysis possibly by a variety of techniques, among which should be genetic transformation with purified DNA. The organism should be characterized by genetic and physiological simplicity, with its total DNA complement of low molecular size, readily cultivated on chemically defined media, and producing large numbers of mutants (genetic markers), at least some of which synthesize modified proteins, easy to purify and analyse.

The choice of such organisms is at present limited and includes a few bacterial species and several viruses. At the time our studies were initiated we chose *Bacillus subtilis*, since it is a genetically transformable microorganism (Spizizen 1958) which grows on simple inorganic media containing only one organic carbon source and for which many biochemical mutants were isolated. A single exponentially growing *B. subtilis* cell carries, on the average, three distinct nuclei (equal numbers of bi- and tetranucleated cells), each of which contains 2.2×10^{-9} μg DNA, which corresponds to a molecular weight of 1.3×10^9, i.e. 2.2×10^6 nucleotide pairs. This comparatively large size of the DNA complement compares unfavorably with the infectious (transforming) DNA of several viruses, including among others the single-stranded circular DNA of coliphage $\Phi \times 174$ (M.W. of 1.7×10^6), the double-stranded circular replicative form of this phage, and also infectious DNA from the polyoma virus of similar size and configuration (M.W. of $3.0–3.5 \times 10^6$), and the infectious DNA of coliphage lambda (M.W. of 3.1×10^6). All these viral DNAs, which could easily be extracted as single intact molecules, are 30 to 500 times smaller than the *B. subtilis* DNA complement, and thus perhaps more amenable to physical and chemical characterization (Fiers and Sinsheimer 1962, Kleinschmidt et al. 1963, Dulbecco 1963, Weil and Vinograd 1963, Burgi and Hershey 1963, Kaiser and Hogness 1960).

Although *B. subtilis* contains a rather large DNA complement, one should not forget that it is an autonomous organism, endowed with several useful genetic and biochemical features. This report will deal with an attempt to determine several physical and chemical properties of isolated DNA frag-

ments and to correlate them with the genetic characteristics of individual markers. How could this task be undertaken? We tried a few approaches based on comparing the sensitivities of normal and chemically modified transforming DNA to a variety of physical and chemical agents, as measured for several linked and non-linked genetic markers. If the chemical modification affects one purine or pyrimidine base only and at the same time alters the sensitivity of the transforming DNA to the agents tested, one might presume that the markers which are more affected would be associated with DNA fragments containing a larger proportion of the base to be replaced or modified. In conjunction with these studies an attempt was also made to fractionate the marker-bearing molecules.

GENERAL PROPERTIES OF TRANSFORMING DNA

As already stated, a single nucleus of *B. subtilis* contains, on the average, 1.3×10^9 M.W. of DNA. Since the DNA synthesis period occupies the greater part of the bacterial division cycle, most of the nuclei would contain more than one DNA complement, in analogy with the replicating genome of *Escherichia coli*, as depicted by Cairns (1963), which is a circular molecule with part of the circle already reduplicated. The molecular weight of the *B. subtilis* genome should be therefore somewhat below 10^9, but we should use the round figure of M.W. $= 10^9$ (1,600,000 nucleotide pairs). Upon extraction, deproteinization and RNA removal, the molecular weight of the DNA, as determined from the sedimentation data, varies from 20 to 40×10^6, with an average of 25×10^6. Thus the *B. subtilis* genome seems to break up upon isolation into 40 fragments on the average. When assayed with several markers, the specific transforming activity of this DNA, i.e. the number of transformed colonies per 1 μg of DNA (in the proportional range of transformation assay at low DNA concentrations), approaches the value of 6×10^6 transformants per μg of DNA (indole marker), which figure indicates that only 1% of the DNA participates in the transformation process. The latter figure is the product of the weight of DNA per genome (1.6×10^{-9} μg) and of the specific transforming activity (6×10^6), multiplied by 100. Specific transforming activity was assayed under standard conditions, at a concentration of 10^8 recipient cells per 1 ml sample of the vigorously aerated culture. High specific transforming activities were obtained only with DNA fragments of high molecular weight. A decrease in molecular weight resulted in a precipitous decrease in the specific transforming activity. *B. subtilis* DNA of molecular weight lower than 10 million was practically devoid of transforming activity, in deference to the published data for the pneumococcus transforming system (Litt et al. 1958, Rosenberg et al. 1959), although some of the earlier data on molecular weight determination should be taken with 'a grain of salt', especially since the DNA was not exhaustively sheared at the critical rates of hydrodynamic shear, and it was not subsequently fractionated by zone sedimentation or column chromatography. The transforming activity of sheared *B. subtilis* DNA half fragments, separated by zone sedimentation in a sucrose gradient, was recently determined by Nester et al. (1963).

METHODS OF SEPARATING AND DISTINGUISHING INDIVIDUAL DNA FRAGMENTS AND GENETIC MARKERS

The average base composition (per cent guanine + cytosine, % G+C) seems to be rather uniform for all the fragments, since the DNA of *B. subtilis* forms a rather narrow band during CsCl gradient centrifugation (Szybalski et al. 1960). However, it is possible to fractionate individual DNA fragments by applying more refined methods and to follow the individual DNA molecules labeled with genetic markers. Operationally, the wild-type DNA already contains all the prototrophic markers, and it is only necessary to develop the proper auxotrophic receptor strains to be able to detect and quantitatively assay the genetic characteristics of the donor DNA fragments.

Two direct fractionation methods could be used to separate the markers, using small natural differences in the per cent G+C of individual fragments: equilibrium-density-gradient centrifugation in CsCl or similar salts (Meselson et al. 1957, Szybalski et al. 1960, Opara-Kubinska and Szybalski 1962), and chromatography on the methylated albumin column (Mandell and Hershey 1960). In both methods the G+C richer fragments are collected in earlier fractions, since they band at higher buoyant densities (Sueoka et al. 1959), closer to the bottom of the centrifuge tube (fraction collected as described by Szybalski et al. 1960), and they elute earlier from the methylated albumin column (Sueoka and Cheng 1962).

Less direct methods of marker fractionation involve either partial denaturation of the DNA or density labeling of synchronized bacterial cultures. In the first method advantage is taken of the fact that some DNA fragments denature irreversibly upon heating and rapid cooling at slightly lower temperatures than other fragments, all labeled with suitable genetic markers. The irreversibly denatured fragments can then be removed from the residual native (or spontaneously renatured) fragments by virtue of their higher density in the CsCl gradient (by 0.016 g/cm^3 for *B. subtilis*), or very high affinity to the methylated albumin column.

This method could be further improved by employing very mild treatment with chemicals which preferentially crosslink G+C-rich DNA fragments, e.g. chemically or enzymatically reduced mitomycin (Iyer and Szybalski 1963, 1964) or nitrogen mustards (Lawley and Brookes 1963), since separation of denatured G+C-poor fragments and native G+C-rich fragments should be more pronounced under these conditions.

The other method takes advantage of the fact that DNA synthesis in the *B. subtilis* genome is most probably unidirectional, starting at the hypothetical 'swivel point' (Cairns 1963) and proceeding along the DNA circle. If a short pulse of a 'heavy' label (e.g. N^{15}_4Cl, D_2O, or 5-bromodeoxyuridine) were added to the synchronously growing cells, only a short section of the DNA genome would incorporate the density label, the same section in all the cells. The exact timing of the 'density' pulse would determine which section would be labeled. The labeled fragments produced during the extraction of the DNA could be separated by density-gradient centrifugation.

All these methods are complicated by the fact that the DNA fragments are the product of random breakage of the genome during the extraction process and, consequently, have undefined ends and poorly defined lengths. This limitation could be partially overcome by fractionating the fragments

according to their length and molecular weight by zone sedimentation in the sucrose gradient, or by chromatography on long columns of methylated albumin.

Other methods which distinguish between genetic markers located on different DNA fragments, but do not physically separate the fragments, are based on the differential lethal effects of a variety of agents, e.g. various wavelengths of UV light, X-rays, critical and subcritical thermal treatment, hydrodynamic shear and others (cf. Zamenhof et al. 1957).

COMPARATIVE CHARACTERIZATION OF GENETIC MARKERS ON THE BASIS OF BUOYANT DENSITY AND TEMPERATURE OF INACTIVATION

If two genetic markers are located on separate DNA fragments which differ somewhat in average G+C content, they should be separable on the basis of the difference between their buoyant densities. When wild type *B. subtilis* DNA was centrifuged for 72 hours at 30,000 rpm in the CsCl

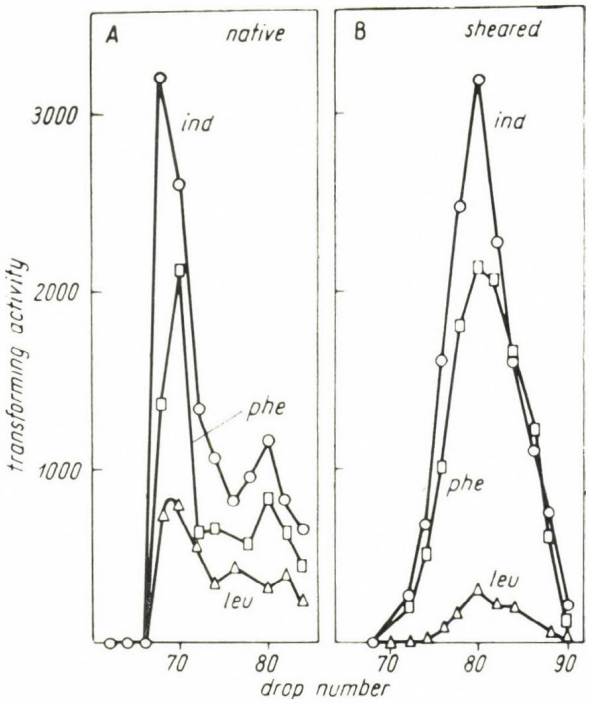

FIG. 1. — Transforming activity (number of transformed colonies per 10^8 receptor cells) requiring indole, leucine, and phenylalanine of 1-drop fractions (10 μl) containing prototrophic *B. subtilis* DNA, and collected according to Szybalski (1960) after 72 h of CsCl density-gradient centrifugation at 35,000 rpm. *A* = mean M.W. of approximately 31×10^6, *B* = mean M.W. of approximately 15×10^6

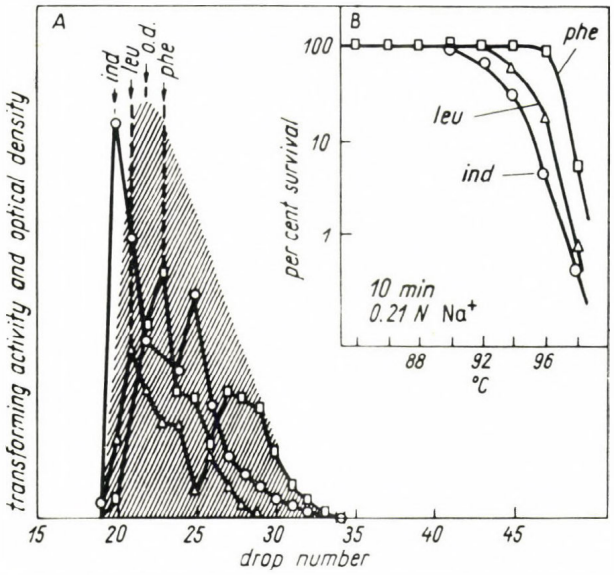

FIG. 2.—*A* = transforming activity (solid lines) and optical density measured at 260 mμ (shaded area) of 2-drop (20 μl) DNA fractions collected after 72 h of CsCl density-gradient centrifugation, under conditions outlined in the caption to Fig. 1, but with a double total amount of DNA (40 μg) used. The banding patterns of single *his* and linked *his–ind* markers are not included in this diagram. *B* = transforming activity of DNA samples heated for 10 min at indicated temperatures in SSC (0.15 M NaCl + 0.015 M Na_3 · citrate), rapidly chilled at 0° C, and assayed with receptor strains requiring indole leucine and phenylalanine

density gradient, with subsequent collection of 1 or 2-drop (0.01—0.02 ml) samples (Szybalski et al. 1960), and then assayed for linked indole (*ind*$^+$) and histidine (*his*$^+$) markers and unlinked leucine (*leu*$^+$) and phenylalanine (*phe*$^+$) markers, using the corresponding auxotrophic receptor strains, the bulk of the DNA bearing the linked *ind*$^+$ and *his*$^+$ markers banded at the highest density, followed by the *leu*$^+$ and *phe*$^+$ markers, with one to two fraction differences between the corresponding peaks (Opara-Kubinska and Szybalski 1962). The separation between the main peaks was clear and reproducible (Figs 1*A* and 2*A*) only with carefully prepared high molecular weight DNA (M.W. over 30×10^6). As the molecular weight of the DNA decreased, secondary peaks became predominant with resulting broadening of marker distribution (Fig. 1*B*). These data indicate that since the *ind*$^+$ and *his*$^+$ markers did not separate during centrifugation of high molecular weight DNA, they must be located on the same fragment, which has a higher average G+C content than the fragments carrying the *leu*$^+$ and *phe*$^+$ markers, the *phe*$^+$ bearing fragment having the lowest average G+C content. Upon breakage of these fragments, the average G+C content of the still smaller sub-fragment carrying the marker in question need not be and often

is not the same as that of the parental fragment. This might be one among the reasons why the markers band in more than one peak when partially sheared transforming DNA is fractionated by CsCl density gradient centrifugation. The comparatively clean separation of the markers experienced with carefully prepared, high molecular weight DNA seems to indicate that the genome either breaks up into fragments at predetermined points, perhaps characterized by single-strand breaks or other weaknesses, or that the variation in the G+C content is very gradual along the DNA genome. However, the density changes upon further fragmentation of the DNA, as already discussed, are inconsistent with the second alternative. Marker separation during preparative CsCl gradient centrifugation was also reported by Rolfe and Ephrussi-Taylor (1961), by Guild (1963) and by Ganesan (1963).

If a particular marker, e.g. *ind*$^+$, is associated with a DNA fragment of comparatively high G+C content, the critical temperature of its thermal inactivation might be exceptionally high, since it is well known that the 'melting' temperature of DNA is proportional to its G+C content (Marmur and Doty 1959). Thermal inactivation of transforming DNA has been studied extensively in several laboratories, as reported by Dove and Davidson (1963), Freese and Freese (1963), Ginoza and Zimm (1961), Guild (1963), Marmur et al. (1963), Roger and Hotchkiss (1961) and others. The results obtained with the *ind*$^+$, *leu*$^+$ and *phe*$^+$ markers (10 min heating in 2×10^{-3} M NaCl · versene, $p_H = 7.8$, or in 0.15 M NaCl + 0.015 M Na_3 · citrate, $p_H = 7.5$, followed by rapid chilling at 0°C) were quite paradoxical, at least on first sight: the *ind*$^+$ marker was inactivated at the lowest temperature followed by *leu*$^+$ and *phe*$^+$ (Opara-Kubinska and Szybalski 1962) (Fig. 2*B*). This result indicates that the critical inactivation temperature of a marker need not be governed only by the average G+C composition of the carrier fragment, especially since it corresponds to the temperature at which the last of the interstrand bonds breaks down, with resulting complete strand separation and irreversibility of the denaturation process. In this respect the 'melting' temperature of the DNA, which indicates about 50% hydrogen bond breakage and which falls well within the reversible denaturation range governed by the *cooperative* process depending on the average G+C content, is different from the critical temperature of inactivation, which depends mainly on a *single*, most temperature-resistant region of the DNA molecule. Exceptionally long runs of G+C pairs (and also some covalent crosslinks in DNA, cf. Iyer and Szybalski 1963, Szybalski, in press) could be responsible for the high inactivation temperatures for particular genetic markers, which on the average are several degrees higher than the 'melting' temperatures determined from the optical density versus temperature measurements.

One might conclude that the buoyant density and the critical temperature of inactivation measure two different properties of the DNA fragments on which given genetic markers happen to be located: buoyant density is governed by the *average* G+C content of the fragment, whereas the inactivation temperature depends on a *particular* temperature-resistant and relatively short region on the same fragment. If the latter region is characterized by long runs of G+C deoxynucleotide pairs, the inactivation temperature and buoyant density should be statistically interrelated, although such correlation is not required for each particular genetic marker. The *phe*$^+$ marker,

e.g., seems to be associated with a DNA fragment which has, on the average, a low G+C content but still seems to contain one comparatively long run of G+C pairs accounting for its relatively high resistance to critical thermal inactivation (Opara-Kubinska and Szybalski 1962). Similar conclusions were reached by Guild (1963).

Although the described experiments provide some general information on the properties of marker-bearing DNA fragments, they reveal very little about the chemical properties of the markers themselves, i.e. about the comparatively short DNA regions governing the synthesis of the particular proteins (enzymes). Several other properties of the markers were therefore evaluated in the hope that these could shed additional light on the structure of the carrier molecules and perhaps the marker regions themselves.

EFFECT OF DENSITY LABELING ON MARKER SEPARATION

At the time the first separation of markers was obtained by CsCl density gradient centrifugation, we attempted to increase or modify this fractionation procedure by labeling the DNA with various density markers. When auxotrophic *B. subtilis* is grown in a medium containing the N^{15} isotope as sole nitrogen source ($N^{15}H_4Cl$), all the nitrogens in the newly synthesized purine and pyrimidine bases are replaced by the heavy isotope. This results in a molecular weight increase for the G+C pair by 8, and only by 7 for the adenine + thymine (A+T) pair. Unfortunately, this preferential density labeling of the G+C deoxynucleotide pairs contributes very little to the density differential between fragments of varying G+C content (approximately 2%) and does not improve the separation significantly.

Preferential labeling of the A+T pairs with 5-bromodeoxyuridine (BUdR), which selectively replaces the thymidine residue in DNA, contributes an increase of 65 in the molecular weight of the A+T deoxynucleotide pairs. However, extensive BUdR-labeling results in wider spreading of the bands and compensates (or even slightly overcompensates by up to 20%) for the higher density of the G+C-rich DNA fragments.

Although extensive BUdR or N^{15} density labeling proved not to be practical for improving marker separation, short-term density labeling of the DNA resulted in differential transfer of the markers into 'hybrid' DNA molecules banding at higher densities in the CsCl gradient. This phenomenon is a reflection of two processes: (1) upon preparation of the inocula and transfer to density-labeling media *B. subtilis* cells become partially synchronized, and (2) since DNA synthesis appears to be a unidirectional process, the markers located closer to the synthesis initiation points are replicated sooner with concurrent change in their density. This phenomenon was utilized by Yoshikawa and Sueoka (1963) for the determination of the sequence of marker replication in *B. subtilis* and consequently of the genetic map of this microorganism (Fig. 3).

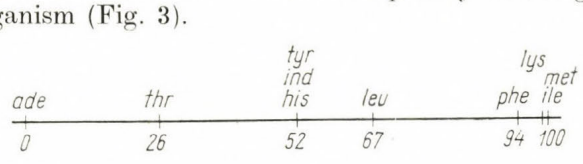

FIG. 3.—Genetic map of *B. subtilis* (Yoshikawa and Sueoka)

ULTRAVIOLET LIGHT AND X-RAY SENSITIVITY OF TRANSFORMING DNA AND ITS RELATIONSHIP TO SPECIFIC TRANSFORMING ACTIVITY AND MOLECULAR SIZE

The relative sensitivities of the markers toward x-rays and short, medium and long wave-lengths of UV light (254 mμ, 300–340 mμ and 350–390 mμ) seem to be arranged in the same order. The *phe*$^+$ marker is the most resistant, with *ind*$^+$ and *leu*$^+$ progressively more sensitive. What could be the reason for this correlation between sensitivities to very different types of radiation? To answer this question we tried to determine which properties of transforming DNA govern its radiosensitivity. We soon found that the size of the DNA fragment was directly related to radiosensitivity. Both for X-rays and for short or medium wavelength UV light the radiation sensitivity decreased with the size of the progressively sheared DNA fragments, as did also the specific transforming activity. Hydrodynamic shear was exerted by passing the DNA (suspended in 0.15 M NaCl + 0.015 M Na_3 . citrate, $p_H = 7.5$, at 10 μg DNA/ml) through a fine needle (gauge No 27) under high pressure generated with a 1 cc tuberculin syringe. With long-wave UV somewhat unequivocal results were obtained, indicating a possible inverse relationship between radiosensitivity and molecular weight. This result, however, might be questionable, since the effects of long-wave UV depend strongly on minute medium contamination with photodynamically active substances. The dependence of short-wave UV sensitivity on the molecular size of transforming DNA was reported by Marmur et al. (1961).

These combined results could be interpreted as follows. For an efficient transformation process DNA fragments of various lengths (and/or fragility) are required for different markers. Markers which must be located on exceptionally long (and/or fragile) molecular fragments transform with lower efficiency, since long (and/or fragile) fragments are comparatively rare, being more often broken during the extraction process. The larger (and more fragile) DNA fragments would also be more sensitive to X-rays and UV light.

SENSITIVITY TO SUBCRITICAL THERMAL INACTIVATION AND HYDRODYNAMIC SHEAR

If the above interpretation is correct, the markers associated with longer DNA fragments should also be more sensitive to prolonged exposures at temperatures just below the melting temperature of DNA, since the chance of depurination (Greer and Zamenhof 1962) followed by ester linkage break, the mechanism most probably involved in this type of inactivation, would be a function of molecular size. Similarly, these markers should also be more sensitive to hydrodynamic shear.

Experiments have corroborated these predictions, since there was almost perfect correlation between the radiosensitivities and the sensitivities to hydrodynamic shear or to prolonged heating at 85° C in a buffer consisting of 0.15 M NaCl + 0.015 M Na_3 · citrate, $p_H = 7.5$. The *phe*$^+$ marker was always the most resistant one, with *ind*$^+$ and *leu*$^+$ being progressively more sensitive (Fig. 4). In agreement with predictions based on the relationship

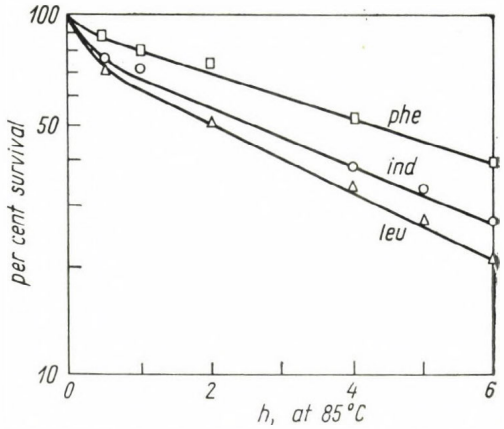

FIG. 4.—Thermal inactivation of transforming DNA at a temperature (85° C) below the 'melting' point for *B. subtilis* DNA in SSC (cf. Fig. 1)

between the genetic properties and molecular structure of DNA, the linked markers were more sensitive to hydrodynamic shear than the single markers (Nester et al. 1963).

DIFFERENTIAL SENSITIZATION OF GENETIC MARKERS BY BUdR LABELING OF DNA

When *B. subtilis* cells are grown in BUdR-containing media, the newly synthesized DNA retains its transforming activity, although it contains BUdR in place of thymidine (Szybalski et al. 1960). Such BUdR-labeled DNA is more sensitive to radiation (Opara-Kubinska et al. 1961). Since molecular fragments characterized by a higher A+T content should incorporate a larger amount of BUdR, and since radiosensitization was shown to depend directly on the degree of thymidine replacement (Erikson and Szybalski 1963), we attempted to determine whether genetic markers located on these A+T-rich fragments would be preferentially sensitized by BUdR. These experiments, which were presented in detail elsewhere (Szybalski and Opara-Kubinska, in press), do not provide a clear-cut answer to this question on account of several technical difficulties. The differences in the degree of sensitization for the three markers were not great and fluctuated somewhat from experiment to experiment. With proper corrections for specific transforming activity of different samples of DNA (Szybalski and Opara-Kubinska, in press), the sensitization factor, calculated as the ratio of the initial slopes of the survival curves, amounted to 4.1–4.7 (154 mμ UV), 6.8–8.7 (300–340 mμ UV) and 1.4–2.0 (X-rays) for unifilarly BUdR-labeled DNA, and 8.9–20.9 (254 mμ UV), 23.2–26.2 (300–340 mμ UV) and 2.5–4.8 (X-rays) for bifilarly BUdR-labeled DNA, in which up to 100% of the thymidine residues were replaced by BUdR. There seems to be some tendency for the *ind*$^+$ marker to become slightly more radiosensitized in almost all of the cases, which result does not seem to be compatible with the idea that radiosensitization is related to the average base composition of the DNA fragments, since the *ind*$^+$ marker seems to have the lowest average A+T content

as indicated by its comparatively high buoyant density in the CsCl gradient. This result is not too surprising, since the sensitivity and radiosensitization of a particular marker should depend more on the base composition in the proximity of the marker rather than on the average base composition of the entire DNA fragment. The problem of postirradiation repair upon entry of the irradiated DNA into the receptor cell could also be involved, since repair might play a differential role in the marker radiosensitivities, and since BUdR incorporation is known to interfere with the repair mechanism (Stahl et al. 1961, Sauerbier 1961, Howard-Flanders et al. 1962). Thus the originally most sensitive marker should be the least radiosensitized, which seems generally to be the case for the *leu*$^+$ marker irradiated with short-wave UV light and X-rays.

CONCLUSIONS AND SUMMARY

During gentle extraction of DNA from *B. subtilis* cells the total DNA complement breaks up into approximately 40 fragments, each of a molecular weight in the neighborhood of 25×10^6 and carrying various genetic markers. Several physico-chemical properties of a few marker-bearing fragments were studied by following the fate of the genetic marker during the fractionation or inactivation procedures (cf. Table I). One could conclude that many of these experiments provided only a gross characterization of the DNA fragments on which the particular markers happened to be located, and supplied little information on the properties of the small marker regions themselves. The buoyant density characterized the average base composition of the whole DNA fragment, whereas the critical temperature of inactivation indicated the properties of the most heat-resistant small region on this molecule, probably an uninterrupted run of G+C deoxynucleotide pairs. Halving of the marker-bearing DNA fragments by hydrodynamic shear modified both the buoyant density and the inactivation temperature of the markers. From parallel centrifugation runs in CsCl and Cs_2SO_4 density gradients* it was possible to establish that the density heterogeneity is caused by less than 4% variation in the base composition between the individual fragments. This heterogeneity is much lower than that between the ends of the DNA molecule extracted from virulent lambda phage, where the differences exceed 10% G+C (Hershey, personal communication), although with some defective lambda dg (kindly supplied by Dr G. H. Echols) we have observed a much lower degree of heterogeneity, than determined by band splitting during centrifugation of sheared phage molecules in the CsCl gradient, at low temperature or in the presence of formaldehyde to prevent secondary cohesion of the DNA fragments.

Sensitivity to hydrodynamic shear, to subcritical thermal inactivation, and to radiation all seem to be related to the specific transforming activity of the DNA for particular markers, which in turn is the function of the actual size and/or fragility of the marker-bearing DNA molecules and of the size requirement for an efficient transformation process. If the UV and X-ray sensitivities are partially governed by the A+T (UV) or G+C (X-ray)

* In Cs_2SO_4 the buoyant density is much less dependent on the G+C content than in CsCl (Erikson and Szybalski 1964).

content of the DNA (Kaplan and Zavarine 1962, Haynes, in press), the differences in base composition seem not to be large enough to overcome other factors contributing to radiation sensitivity, since both for UV and X-ray the same order of marker sensitivities was found. Radiosensitization by the BUdR-for-thymidine substitution in DNA varies only slightly between the markers and does not seem to be related to the average base composition of the marker-bearing DNA fragments.

This contribution is partially based on published and unpublished studies carried out in this laboratory, and partially on data in the literature. No

TABLE 1

*Methods for fractionation of DNA fragments**

Property of transforming DNA fragments	Method of fractionation	Rationale behind methods of fractionation
(1) Average G+C content	(a) Preparative CsCl gradient centrifugation	Fragments with higher G+C content are denser and sediment closer to bottom of tube
	(b) Methylated albumin column chromatography (short column)	Fragments with higher G+C have less affinity for column and elute at lower salt concentration
(2) Localized G+C stretches (runs) or crosslinks	Critical denaturation followed by (a) preparative CsCl gradient centrifugation or by (b) methylated albumin column chromatography (could be preceded by mild treatment with G+C directed crosslinking agent, e.g. nitrogen mustard, or reduced mitomycin)	Fragments with longer G+C runs (or with crosslinks) do not denature irreversibly and (a) band at lower density than denatured fragments, or (b) do not stick to column material. Irreversibly denatured DNA (a) bands at a buoyant density higher by 0.012–0.017 (CsCl) or 0.020–0.026 (Cs_2SO_4), or (b) adsorbs rather firmly to column material
(3) Localized density labeling	Pulse of, or longer exposure to, density label delivered to synchronized cells, followed by preparative CsCl gradient centrifugation of extracted DNA	Density-labeled fragments sediment closer to bottom of tube
(4) Size	(a) Zone centrifugation in sucrose gradient	Larger fragments sediment faster
	(b) Methylated albumin column chromatography (long column)	Smaller fragments have less affinity for column and elute at lower salt concentration

* References: (1) Meselson et al. (1957), Szybalski et al. (1960), Rolfe and Ephrussi-Taylor (1961), Opara-Kubinska and Szybalski (1962), Guild (1963), Sueoka and Cheng (1962), Ganesan (1963). (2) Lacks (1962), Ganesan (1963), Iyer and Szybalski (1963), Marmur et al. (1963). (3) Yoshikawa and Sueoka (1963). (4) Nester et al. (1963), Lacks (1962), Ganesan (1963), Mandell and Hershey (1960).

comprehensive review of the literature was attempted. The authors are greatly indebted to many of their colleagues in the nucleic acids field for stimulating discussions and prepublication communications, and also to Dr E. H. Szybalska for editorial help.

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REFERENCES

Burgi, E. and Hershey, A. D. (1963) *Biophys. J. 3*, 309
Cairns, J. (1963) *J. molec. Biol. 6*, 208
Dove, W. F. and Davidson, N. (1963) *J. molec. Biol. 5*, 479
Dulbecco, R. (1963) *Proc. nat. Acad. Sci. (Wash.) 50*, 236
Erikson, R. L. and Szybalski, W. (1963) *Radiat. Res. 20*, 252
Erikson, R. L. and Szybalski, W. (1964) *Virology 22*, 111
Fiers, W. and Sinsheimer, R. L. (1962) *J. molec. Biol. 5*, 424
Freese, E. B. and Freese, E. (1963) *Biochemistry, 2*, 707
Ganesan, A. T. (1963) *Ph. D. Thesis*, Stanford University
Ginoza, W. and Zimm, B. H. (1961) *Proc. nat. Acad. Sci. (Wash.) 47*, 639
Greer, S. and Zamenhof, S. (1962) *J. molec. Biol. 4*, 123
Guild, W. R. (1963) *J. molec. Biol. 6*, 214
Haynes, R. H. (in press) in *International Symposium on Physical Processes in Radiation Biology*, Academic Press, New York
Howard-Flanders, P., Boyce, R. P. and Theriot, L. (1962) *Nature 195*, 51
Iyer, V. N. and Szybalski, W. (1963) *Proc. nat. Acad. Sci. (Wash.) 50*, 355
Iyer, V. N. and Szybalski, W. (1964) *Bact. Proc.* 1964
Kaiser, A. D. and Hogness, D. S. (1960) *J. molec. Biol. 5*, 392
Kaplan, H. S. and Zavarine, R. (1962) *Biochem. biophys. Res. Commun. 8*, 432
Kleinschmidt, A. K., Burton, A. and Sinsheimer, R. L. (1963) *Science 142*, 961
Lacks, S. (1962) *J. molec. Biol. 5*, 119
Lawley, P. D. and Brookes, P. (1963) *Exp. Cell Res. 9*, Suppl., 512
Litt, M., Marmur, J., Ephrussi-Taylor, H. and Doty, P. (1958) *Proc. nat. Acad. Sci. (Wash.) 44*, 144
Mandell, J. D. and Hershey, A. D. (1960) *Analyt. Biochem. 1*, 66
Marmur, J., Anderson, W. F., Matthews, L., Berns, K. I., Gajevska, E. and Doty, P. (1961) *J. cell comp. Physiol. 58*, Suppl., 33
Marmur, J. and Doty, P. (1959) *Nature (Lond.) 183*, 1427
Marmur, J., Rownd, R. and Schildkraut, C. L. (1963) *Progr. Nucl. Acid Res. 1*, 231
Meselson, M., Stahl, F. W. and Vinograd, J. (1957) *Proc. nat. Acad. Sci. (Wash.) 43*, 581
Nester, E. W., Ganesan, A. T. and Lederberg, J. (1963) *Proc. nat. Acad. Sci. (Wash.) 43*, 581
Opara-Kubinska, Z., Lorkiewicz, Z. and Szybalski, W. (1961) *Biochem. biophys. Res. Commun. 4*, 288
Opara-Kubinska, Z. and Szybalski, W. (1962) *Abstr. Biophys. Soc. 6th Ann. Meeting*, p. WA8
Roger, M. and Hotchkiss, R. D. (1961) *Proc. Natl. Acad. Sci. U. S. 47*, 653
Rolfe, R. and Ephrussi-Taylor, H. (1961) *Proc. nat. Acad. Sci. (Wash.) 47*, 1450

Rosenberg, B. H., Sirotnak, F. M. and Cavalieri, L. F. (1959) *Proc. nat. Acad. Sci. (Wash.) 45*, 144
Sauerbier, W. (1961) *Virology 15*, 465
Spizizen, J. (1958) *Proc. nat. Acad. Sci. (Wash.) 44*, 1072
Stahl, F. W., Craseman, J. M., Okun, L., Fox, E. and Laird, C. (1961) *Virology 13*, 98
Sueoka, N. and Cheng, T. Y. (1962) *J. molec. Biol. 4*, 161
Sueoka, N., Marmur, J. and Doty, P. (1959) *Nature (Lond.) 183*, 1429
Szybalski, W. (in press) *Abhandl. Deutsch. Akad. Wiss. Berlin, Klasse Med.*
Szybalski, W. and Opara-Kubinska, Z. (in press) in *Cellular Radiation Biology*, University of Texas Press, Austin
Szybalski, W., Opara-Kubinska, Z., Lorkiewicz, Z., Ephrati-Elizur, E. and Zamenhof, S. (1960) *Nature (Lond.) 188*, 743
Weil, R. and Vinograd, J. (1963) *Proc. nat. Acad. Sci. (Wash.) 50*, 730
Yoshikawa, H. and Sueoka, N. (1963) *Proc. nat. Acad. Sci. (Wash.) 49*, 806
Zamenhof, S., Leidy, G., Greer, S. and Hahn, E. (1957) *J. Bact. 74*, 194

DISCUSSION

WATANABE, T.: I would like to ask you, Dr Szybalski, how many per cent of G+C are contained in the fractions of DNA carrying different markers.

SZYBALSKI: I would show you this particular slide, with the exact G+C differences (and he is showing the peak of T, L, and P, and drop number on the slide). So the first curve, the heaviest is T, which is the indole-tryptophane-plus locus on the outside. Second is leucin. There are 2–3 drop differences and they correspond to about 2–3 per cent in G+C differences. Now the melting curves: the highest is T, then come L and P types.

PAKULA: May I ask a question? What is the accuracy of determination of G+C base content in DNA measured by the CsCl density gradient centrifugation? I think about the base content you can measure, Professor Szybalski. I think you have done it, using the chromatographic method of determination of base content. What is the accuracy of the method? You have mentioned differences of 2–3 per cent.

SZYBALSKI: They are only from the density scale.

PAKULA: So they are calculated from the density gradient.

SZYBALSKI: They are obtained not by analytical determination, which would result in much lower accuracy.

PAKULA: And what is the estimation of accuracy according to the method of Marmur?

SZYBALSKI: We use the analytical centrifuge data. By taking a marker DNA and an unknown DNA, you could measure with accuracy of about 0.01 mm or even a little better on upper tracing from the actual framing of the cell, and this would correspond to about 0.1 per cent of G+C. You could see differences in one per cent G+C by the same analytical data.

STAHL: Certainly, even by this method; although its accuracy may often not be good, the sensitivity is very,very good. So, there can be no doubt about the right order of the G+C contents. You can be sure that T has more G+C than L has, which has more than has P, but it may be hard to say whether the difference is one per cent or two per cent.

SZYBALSKI: The question is distinctly very complicated since, as already discussed, there are secondary peaks. After all, if you cut the whole molecule in a half, it still has a marker and transforms, but if it has lost all his big arm, which had very high G+C, it is a different molecule from the standpoint of base content. Once you sheared a molecule, all our picture gets smeared and nothing comes out.

KAPLAN: May I have a question? Is this the unlabeled DNA?

SZYBALSKI: This is the unlabeled DNA.

KAPLAN: What happens to the bromouracil labeling which, of course, spreads out?

SZYBALSKI: First, the bromouracil-labeling gets spreading. As a result of this, the definition is very poor and separation is very difficult. We tried something else, i.e. to label with ^{15}N, assuming that it would increase the difference, because G+C would give heavier labeling with nitrogen, which could increase the difference. In calculation, it gives as little increase as is really not worthwhile running the experiment. But we still run it and found another difficulty, namely, the sequential labeling.

KAPLAN: I understand, when you add bromouracil and it is only the thymine which gets heavier, it could be that the different thymine content in these different markers could lead to this shift also in the peaks.

SZYBALSKI: Yes, it would be a very good idea as long as labeling itself would be extremely clean, so that you would not loose resolution, but as a result of bromouracil-label you are facing, in addition, a terrific error of uneven label.

SIK: When measuring the base proportions and ratios from density, Marmur found that the melting point was proportional to the G+C content. He found the higher the G+C content the higher the melting point, and even the density.

SZYBALSKI: Yes, it is absolutely correct for a whole molecule. But now we are confronted with the measurement of the melting point of a marker and that was which I tried to explain. It is really not the same, and it does describe two different properties. You see, what Marmur measured is the T_m, the transition temperature, or maybe, what he measured is the irreversibility of melting. I think, it is a slightly different thing.

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EFFECT OF X-RAYS ON THE TRANSFORMATION FREQUENCY OF *ESCHERICHIA COLI*

By

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There are many studies on the effect of ionizing radiation, i.e. on the biological activity of transforming DNA (Ephrussi-Taylor and Latarjet 1955, Defilippes and Guild 1959, Hutchinson and Arena 1960, Lerman and Tolmach 1959). It appeared interesting to investigate the effect of radiation on the recipient cells. We studied the effect of x-rays on the genetical transformation frequency of *Escherichia coli*. The prototrophic strain of *E. coli McLeod* was used as a donor and the auxotrophic strain *E. coli* methionine (vitamin B_{12}-less, 113-3 *Davis)* as a recipient. Both were received from B. M. Mehta. Transformation was carried out by the method described also by him (Mehta et al. 1962). The preparation of DNA was made according to Marmur's method (1961). The cells in the logarithmic growth phase were suspended in cold double-concentrated minimum medium, irradiated at 0° C by means of a Stabilivolt apparatus (180 kV, 10 mA, 0.5 mm Cu filter, 124 r/min dose measured in air), then transformed.

In our experiment we used 10 μg DNA and 2.5×10^7 recipient cells in 1 ml minimum medium. The mean value of survivors, transformants and the transformation frequencies corrected for the percentage of survivors are shown in Table I. The data are corrected for the radiation-induced back-mutation frequency. As can be seen from the table, irradiation changed the frequency of prototrophic colonies compared with the unirradiated control. Transformation frequency as a rule was increased by the x-ray pre-treatment, after which it began to decrease in inverse proportion to the dose. It reached the highest value after irradiation with 2.5 kr, and was lower for 5 kr. However, in both cases it exceeded the transformation frequency of the unirradiated control, undoubtedly. No uniform result was obtained after irradiation with 10 kr. Though, considering the mean value of the experiments, their transformation frequencies were lower than that of the control, but in

TABLE I

Treatment	Number of		Transformation	
	survivors	transformants	frequency	per cent
Control	5.0×10^7	2.4×10^4	0.048	100
2,500 r	3.1×10^7	2.1×10^4	0.066	141
5,000 r	1.2×10^6	6.9×10^1	0.061	119
10,000 r	2.5×10^5	9.9×10^1	0.039	82

some cases higher values were obtained, too. It is of interest that greater variability in experimental data was observed at the higher doses.

Summarizing our results it may be stated that the number of prototroph transformants in the methionine-less auxotroph population of *E. coli*, incubated with prototroph DNA, increases upon irradiation. On the basis of present knowledge, the mechanism of this phenomenon cannot be satisfactorily explained since, in addition to transformation and the mutagenic effect of x-rays, other selective factors may also interfere with the increased ratio of occurrence of prototrophs.

REFERENCES

Defilippes, F. M. and Guild, W. R. (1959) Irradiation of solutions of transforming DNA, *Radiat. Res. 11*, 38–53

Ephrussi-Taylor, H. and Latarjet, R. (1955) Inactivation par les rayons X, d'un facteur transformant du Pneumococque, *Biochim. biophys. Acta (Amst.) 16*, 138–97

Hutchinson, F. and Arena, J. (1960) Destruction of the activity of deoxyribonucleic acid in irradiated cells, *Radiat. Res. 13*, 137–47

Lerman, L. S. and Tolmach, L. J. (1959) Genetic transformation, II, The significance of damage to the DNA molecule, *Biochim. biophys. Acta (Amst.) 33*, 371–87

Marmur, J. (1961) A procedure for isolation of deoxyribonucleic acid from microorganisms, *J. molec. Biol. 3*, 208–18

Mehta, B. M., Rege, D. V. and Sreenivasan, A. (1962) Transformation to prototrophic condition in *Escherichia coli* induced by deoxyribonucleic acid, *Nature (Lond.) 193*, 296–7

DISCUSSION

PAKULA: What have been radiated, the recipient cells or the DNA?

IGALI: The recipient cells.

PAKULA: Could you say some details about the transformation of *Escherichia coli*, can this strain be transformed also, let us say, to drug resistance?

IGALI: No, I know only the case which was described by Mehta in Bombay and have worked with his strain and I used only the methionine-less marker. I do not know about transformation where drug resistance was transformed.

PAKULA: What about the DNA, was it a sterile preparation of DNA you were using?

IGALI: It was prepared according to Marmur's method.

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DNA COMPOSITION OF TRANSFORMANTS OF THE ENTERIC BACTERIA GROUP

By

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Bacteria, as well as other groups of microorganisms, are characterized by high contents of nucleic acids. It is known today that nucleic acids of microorganisms are subjected to considerable changes depending on the phase of development, conditions of cultivation, physiological state of cells and strain variety.

The composition of nucleic acids is very constant: the invariability of DNA in the process of growth of *Pseudomonas hydrophila* culture has been proved (Reddi 1954); no changes of DNA composition in respect to age have been noticed in *E. coli* (Spirin et al. 1956), in *Azotobacter agile* (Zaytseva and Belozersky 1957), in *Sarcina lutea* (Dutta et al. 1956), etc. Different compositions of media and sources of nitrogen do not change the DNA composition of *Azotobacter agile*.

However, hereditarily changed forms of microorganisms, which are obtained experimentally under various conditions and which differ morphologically, culturally and biochemically from their initial forms, change their DNA composition. Spirin and Belozersky (1956) studied the composition of DNA and RNA in some bacteria of the enteric group and their atypical forms obtained as a result of experimental changeability; it was found that 'neutral' as well as 'alkali-producing' forms of the changed bacteria had different DNA composition compared with the initial cultures. The authors claim that alkali-producing forms obtained under various conditions have a similar DNA composition. The same can be observed in neutral forms.

The specificity index of DNA composition of alkali-producing forms is markedly G+C-type, while neutral forms are characterized by A+T-type. RNA composition of these variants changes insignificantly.

Blokhina et al. (1961) noted DNA composition changes of 'water' and 'pigmentary' variants of Enterobacteria with changed metabolism.

Dutta et al. (1956) when studying DNA and RNA compositions of a streptomycin-resistant culture of *Sarcina lutea* found that DNA composition did not change while that of RNA changed a little.

Jones et al. (1957) found changes in DNA composition of a streptomycin-resistant strain, *Aerobacter aerogenes*, but they did not find any considerable changes in RNA composition.

Guberniyev and Ugoleva (1960) did not find any changes of DNA composition in Staphylococcus 209-P which is resistant to streptomycin and actinoxantine. Changes of specificity index of DNA composition were noted only in the variant which had 1000 times enlarged resistance to actinoxantine.

The examples taken from the literature and cited here are few and deal with variants obtained in conditions different from transformation. Our presentation contains information on the study of DNA composition in variants obtained by transformation with the help of DNA. Variants, henceforth called transformants, were obtained by V. Ya. Romanstova in the Department of Genetics of Microorganisms, Institute of Microbiology of the Academy of Sciences of the Ukrainian S.S.R. when she was cultivating the initial cultures of recipients *B. typhimurium* 8407, *B. paracoli* 63/1,

TABLE I

Composition of the DNAs of the cultures of the recipient, donor and transformants

Culture	N-content of bases mol%				$\frac{G}{C}$	$\frac{A}{T}$	$\frac{G+A}{C+T}$	$\frac{G+C}{A+T}$
	G	A	C	T				
B. paracoli 63/1 recipient	25.5	23.8	26.5	24.2	0.96	0.98	0.97	1.08 ±0.0086
B. paracoli 63/1 donor	24.5	24.9	25.2	25.4	0.96	0.98	0.97	0.99 ±0.015
Transformant 21	24.6	25.8	24.8	24.8	0.98	1.03	1.01	0.98 ±0.021
Transformant 24	24.7	25.6	24.7	25.0	0.97	1.02	0.99	0.98 ±0.02

B. typhi abdominalis 319 on MPB with the addition of DNA obtained from the donor *B. paracoli* 63/1 resistant to 10,000 units of streptomycin. The biological properties of the transformants obtained differed from those of the initial cultures only in the degree of streptomycin resistance.

The methods of Spirin et al. (1956) were employed to study the nucleotide composition, and the methods of Yevreinova et al. (1958) were used for hydrolysis.

Our data agree with Chargaff's results for high-polymer DNA. The specificity index G+C/A+T of DNA composition of the donor culture, that is, a streptomycin-resistant variant obtained by inoculation in media with gradually increasing concentration of an antibiotic amounts to 0.99 which is somewhat lower than that of the initial culture. The DNA composition of transformants 21 and 24 is identical with that of the donor, and their specificity index equals 0.98 (Table I).

Changes in DNA composition occur as a result of a decrease in guanine and cytosine and an increase in adenine and thymine.

The DNA composition of hetero-transformants X-21 obtained from *B. typhimurium* 8407, and 60 and 57 obtained from *B. typhi abdominalis* 319, practically does not alter.

In this way, the changes of the specificity index of DNA were observed in transformants obtained from *B. paracoli*, that is, under the conditions of an isotransformation test, when the donor and recipient were subcultures of the same strain. It should be noted that the specificity indices of streptomycin-resistant variants *B. paracoli* obtained by serial transfers as well as by transformation coincide. Thus, irrespective of the methods of getting the variants, their DNA composition changes similarly.

Besides the single transformants described, double transformants were separated in the same experiments. These double transformants differed from the initial cultures in their different degrees of resistance to streptomycin and ability to be agglutinated with the antiserum of the donor (Table II). We studied DNA-12 composition of the double transformants obtained from *B. typhimurium* 8407 and *B. typhi abdominalis* 319 and 1203. The specificity index of DNA composition of all the double transformants studied changes approaching the specificity index of DNA composition of the donor culture.

TABLE II

Characteristics of the double transformants

Culture	Resistance to streptomycin U/ml medium	Degree of agglutinability with antiserum of the donor	Specificity index of DNA composition $\frac{G+C}{A+T}$
B. paracoli 63/1 donor	10,000	1/12,800	0.99
B. typhimurium 8407 recipient	80	—	1.16
Transformant 32	10,000	1/200	0.97
2	1,000	1/200	0.99
30	2,000	1/100	0.99
12-1	5,000	1/200	1.02
3	2,000	1/200	0.99
9-1	5,000	1/200	0.99
B. typhi abdominalis 319 recipient	50	—	1.09
Transformant 13	10,000	1/200	1.01
35	5,000	1/100	0.94
10	2,500	1/200	0.97
B. typhi abdominalis 1203 recipient	750	—	1.06
Transformant 2a	4,500	1/100	0.97
3a	1,500	1/100	0.97
5	3,000	1/100	0.97

Thus, the DNA composition specificity indices of the transformants obtained from *B. typhimurium* 8407 and *B. typhi abdominalis* 1203 equal 0.97–0.99, except transformant 12-1; the initial culture of *B. typhimurium* 8407 had 1.16, and *B. typhi abdominalis* 1203 had 1.06; the transformants 13, 35 and 10 of *B. typhi abdominalis* 319 have different indices, i.e. 1.01, 0.94, and 0.97.

It should be mentioned that we failed to find any correlation between the change of DNA composition and the degree of resistance to antibiotics acquired by the transformants. Thus, transformants *B. typhimurium* 30 and 3 which are resistant to 2,000 units of streptomycin, and transformant 2 which is resistant to 1,000 units of streptomycin, have a specificity index of DNA composition similar to that of transformant 9-1, which is resistant to 5,000 units of streptomycin. The DNA composition specificity indices of three transformants of *B. typhi abdominalis* 319 and the degree of their

resistance to antibiotics are different but again in this case no correlation between them has been observed.

We also failed to find a definite correlation between the changes of the specificity index of DNA composition and the degree of agglutinability with the antiserum of the donor culture; possibly, this can be explained by the fact that the agglutinability of these cultures of transformants was low in solutions (1/100 and 1/200).

CONCLUSIONS

(1) The specificity index of DNA composition of streptomycin-resistant transformants obtained under conditions of isotransformation is subject to changes, whereas no noticeable changes have been found in the heterotransformants.

(2) The DNA composition of the studied double transformants, their resistance to streptomycin and their capability of agglutinating with the antiserum of the donor are identical with the DNA composition of the donor.

(3) The change of specificity index of DNA composition under the conditions of heterotransformation is possibly connected with the changes of the antigen structure of the transformants.

REFERENCES

Blokhina, I. N., Morozova, G. F., Perova, R. S. and Ustova, M. V. (1961) *Book of Works on Biochemistry and Physiology of Microbes*, Gorki, U.S. S.R.

Dutta, S. K., Jones, A. S. and Stacey, M. J. (1956) *J. gen. Microbiol. 14*, 160

Guberniyev, M. A. and Ugoleva, I. A. (1960) *Dokladi Akad. Nauk S. S. S. R. 2*, 133

Jones, A. S., Marsh, C. E. and Rizvi, S. B. H. (1957) *J. gen. Microbiol. 17*, 586

Reddi, K. K. (1954) *Biochim. biophys. Acta (Amst.) 15*, 585

Spirin, A. S. and Belozersky, A. N. (1956) *Biokhimiya 21*, 6

Spirin, A. S., Belozersky, A. N. and Pretal-Martines, A. (1956) *Dokladi. Akad. Nauk S. S. S. R. 111*, 1297

Yevreinova, T. N., Yermolayeva, L. P. and Gerasimova, A. M. (1958) *Dokladi Akad. Nauk S. S. S. R. 118*, 2

Zaytseva, G. N. and Belozersky, A. N. (1957) *Mikrobiologiya 26*, 722

DISCUSSION

SZYBALSKI: If I have understood correctly, I think it would be a basis for a discussion, if you have a donor and a recipient and the donor has a little less G+C content than the recipient has, and the transformant has a similar amount of G+C as has the donor, which, I understand is about two per cent difference. May I ask Dr. Pakula to translate my question, whether it is correct?

PAKULA: Dr Dekhtyarenko, as Dr Szybalski explains, if the donor strain and the recipient one were different in their G+C content, one should know what difference there was between them. (After that Professor

Pakula has translated the question, Dr Dekhtyarenko gives the explanation in Russian.)

SZYBALSKI: Then it is less than two per cent.

PAKULA: Are you satisfied with the answer, Dr Szybalski? Then I should like to ask a question. I have never done any transformation with gram-negative bacteria and I should like to know some details of the method of transformation just used, because here you deal with interspecific transformation and with intraspecific one. The interspecific transformation is between *Salmonella typhimurium*, *S. typhi* and *E. paracoli* bacteria. So, I should like to know some details, just about the technique of transformation. (Dr Dekhtyarenko gives her explanation in Russian.) Thank you, I shall not translate more, because it was a question of my personal interest.

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INFECTION OF THE RECIPIENT CELL BY TRANSFORMING DNA. THE STIMULATION AND INHIBITION OF INFECTION

By

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The infection of the recipient cell by biologically active transforming DNA *in vitro* can be prevented or reduced by a number of factors; a much more difficult task for the investigator is, however, to obtain a regular high infection which finally results in a transformed cell, and this is because we still do not know the basis of competence.

The problem of the basis of the physiological state at which the recipient cell becomes competent interested us right from the beginning of our work on the transformation of the character in *Pneumococcus*. In the present study, using the method of transformation of the well-evaluated character of streptomycin resistance, we started from certain premises based on our results obtained during the transformation of the character of the polysaccharide capsule in our recipient strain of *Pneumococcus* (Kohoutová 1961, 1962, Kohoutová and Kopecká 1963).

The premises from which we started were as follows. (1) When using the saturation concentration of DNA, the limiting factor which determines the number of the developed transformants is the number of competent cells (Hotchkiss 1954). If we use the saturation concentration of DNA and add salts with univalent kations to the complete transformation medium (so that in fact we have a hypertonic medium) and they regularize the results of the transformation reaction and increase the frequency of the transformants, we may assume that somehow they participate in the reactions which are responsible for the competence, or that they partake directly in the competent reaction. (2) Provided that in the presence of a strong inhibitor of DNAse small quantities of active DNAse, which act in a short time interval, are required for the transformation reaction to proceed, then it is probable that in the normal transformation reaction mechanisms also exist which at the right moment activate and inhibit the DNAse. The most likely moment in the sequence of reactions leading to the transformed cell where DNAse could participate in the reaction, appears to be again the competent state of the recipient cell, when the cell is permeable for large molecules (Thomas 1955, Ottolenghi and Hotchkiss 1962). Recently, Lacks published an interesting paper (1962) which also supports this hypothesis.

In order to come somewhat nearer to the question of the basis of competence and so to gain control over the infection of the recipient cell by transforming DNA, in our study we followed up the factors which had a considerable influence on the frequency of the transformants, which either increased or reduced it and again regularized it to the level of the control. As time is short, I shall mention here only some of the results.

If we add KCl salt at a final concentration of 0.2 M to the NS transformation medium, prepared after Ephrussi-Taylor (a modification of 1951) and follow up its effect on the origin and course of competence by taking samples of 0.1 ml of the growing recipient culture every 10 min and diluting it into 1 ml of the pre-cooled transformation medium with DNA, we see (Fig. 1) that in the first cycle of competence its effect is minimal and sometimes rather inhibiting, in particular in the declining phase of competence.

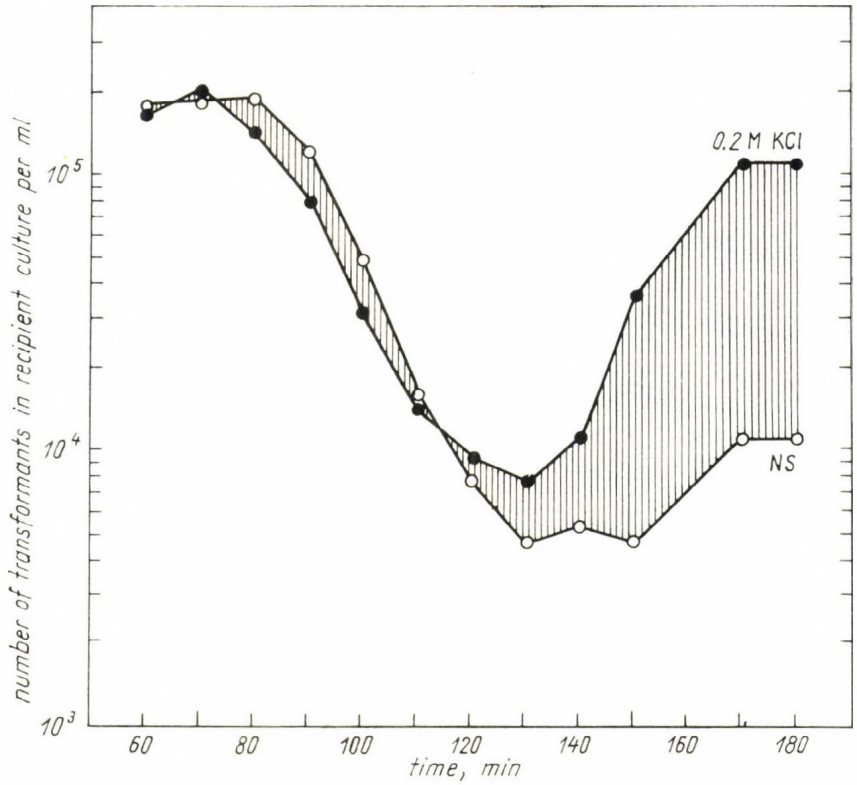

FIG. 1.—Cyclical development of competence. Influence of KCl 0.2 M

In contrast, in the second cycle, where a rapid drop of the transformants occurs in the control in comparison with the first cycle, the effect of KCl is always stimulating and causes a multifold increase in frequency of the transformants. The concentrations of 0.2 M, 0.15 M and 0.1 M did not substantially influence this course. The concentration of 0.2 M always seemed to be the most effective for stimulation of transformation. Figure 2 shows that the size of inoculum in the transformation medium, in the limits of 3×10^6, 6×10^6, and 1.2×10^7 colony forming units per ml of the inoculated transformation medium, and of the same physiological condition, also seems to have no influence on the course of the competence in the presence of KCl. We see that KCl again unusually increased the number

of transformants only in the second cycle of competence. In contrast, in the first cycle it acted as inhibitor.

It is, however, interesting that in the actual transformation experiments, when we used 1 ml of non-diluted ice-cooled recipient culture for 3 min, which was added to DNA also in ice-cooled test tubes, there occurred a substantial increase in the transformants in the presence of KCl and that was independent of the degree of competence of the recipient culture. The in-

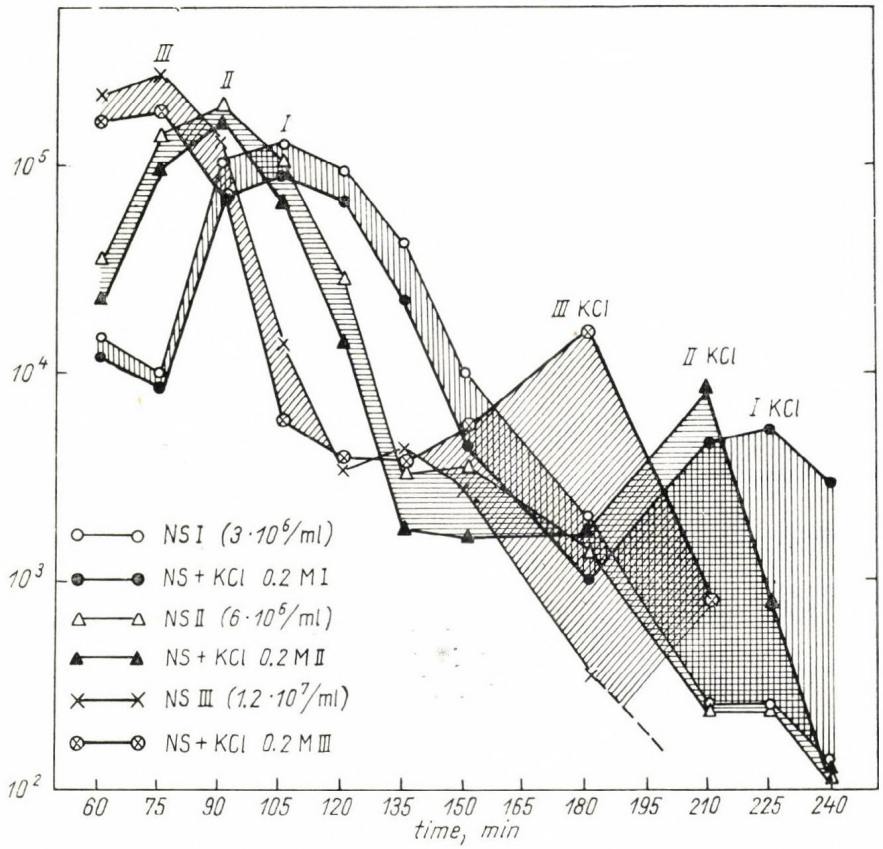

FIG. 2. — Effect of KCl on three different initial densities of recipient culture

crease always appeared whether we used the recipient culture in its maximal competence or whether before or after the peak of maximal competence.

In Fig. 3 we see the number of transformants in 1 ml of the recipient culture, when the recipient cultures of 80 min, i.e. 10 min prior to peak of the competence, as well as the cultures of 120, 180, and 230 min, i.e. far behind the peak of the competence, are added to DNA with 0.2, 0.15, and 0.1 M KCl in the final concentration. In contrast to the controls in the NS medium, the relative increase is approximately 4, 25, 40, and 90fold in the corresponding time in the presence of 0.2 M KCl in the final concentration. We see that

the highest relative effect of KCl is found at the low state of competence, i.e. if we use the recipient culture when it is far past the state of the maximal competence and when the frequency of the transformants in the NS medium is very low.

Figure 4 represents the follow-up of the kinetics of the effect of KCl as a function of time. In a series of equally prepared test tubes with DNA, in the NS medium inoculated with 1 ml of a highly competent culture which

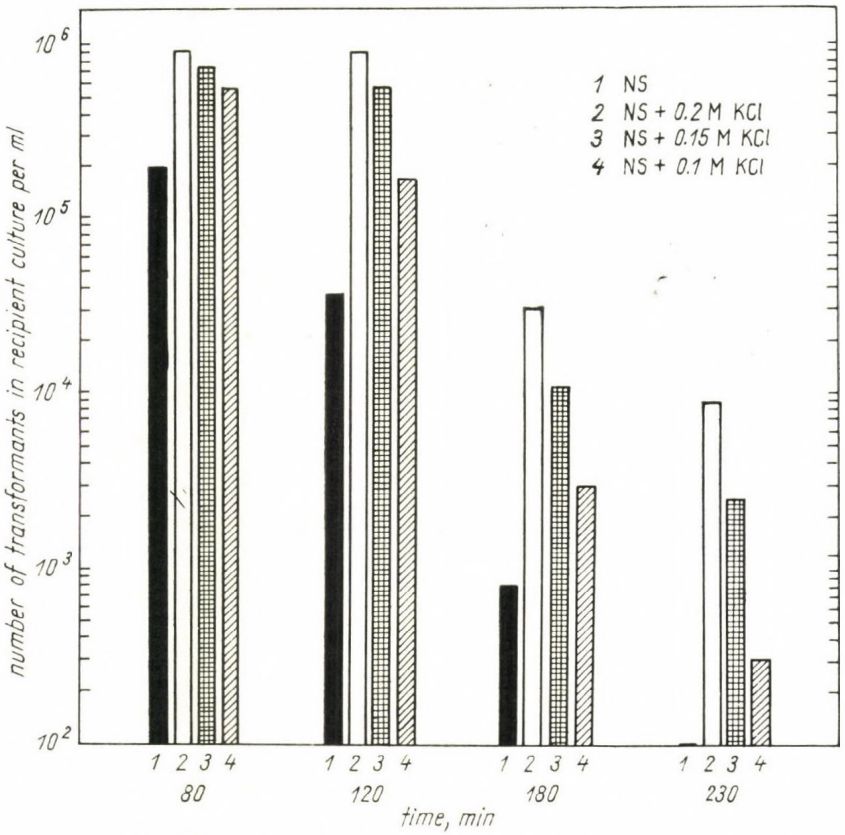

FIG. 3.—Effect of various concentrations of KCl on transformation

had been cooled for 3 min in ice, we added KCl to achieve the final concentration of 0.2 M. At 0, 1, 2, 3, 5, 10, 15, 20, and 30 min KCl was added to the DNA-bacterial complex and kept in all test tubes for 30 min in a bath at 37° C and then put into ice and suitably diluted on the plates. The control in the NS medium without KCl was treated in the same way. As can be seen in Fig. 4, the highest effect of KCl was in the 3 min old DNA-bacterial complex. From 0 min up to 3 min the effect increased, then fell again rapidly and in the 30th min it sank below the value of its own control.

Figure 5 expresses the follow-up of the penetration of DNA into the recipient cells as a function of time. In a series of identically prepared test tubes

with DNA-bacterial complex either in NS or NS with KCl medium DNAse was added at zero time, then after 1, 2, 3, 5, 10, 20, and 30 min and after a further incubation of 1 min with DNAse the samples were put into ice and after proper dilution they were plated. Both experiments were carried out in the course of a 30–40 min interval on the same recipient culture.

It is evident that in the first 10 min the number of the competent cells per 1 ml in both systems gradually increased. However, the increase in the pure NS medium was steeper than that in the NS medium with KCl. Ten minutes later the competent state of the cells had obviously terminated, the DNAse could no longer influence the penetrated DNA. A steep increase in the trans-

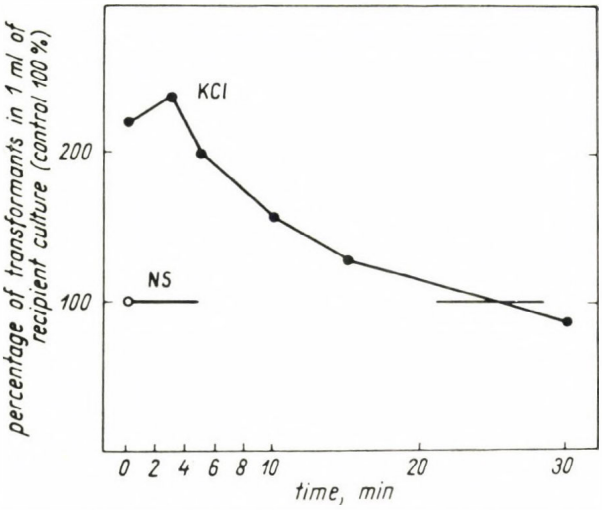

FIG. 4.—Kinetics of KCl as a function of time

formants occurred in the NS with KCl medium, many times higher than in the pure NS medium, and the reaction seemed to be concluded in the 30th minute.

The results of our experiments seem to indicate that, if we add 0.2 M KCl or NaCl to the NS medium at the moment when the actual transformation reaction proceeds, the effect of the hypertonic transformation medium, which we use in our work, is most intense when it is added to a 2–3 min old DNA-bacterial complex and not to a 0–2 min old complex. This seems to suggest that KCl does not act at the period when the maximal adsorption of DNA onto the cell receptors takes place, but rather at a period of maximal penetration and fixation of DNA by the competent cells. The effect of KCl salt added to a DNA-bacterial complex which is older than 3 min decreased rapidly though the competent state under our experimental conditions lasted for 10 min. We may, however, assume that competence also decreased in these 10 min.

Figure 5 seems to show that in the presence of 0.2 M KCl the DNAse enters the competent cell along with the DNA (Thomas 1955), because in the first 10 min in the medium with KCl we obtained a substantially lower

number of transformants than in the control without KCl, but after the termination of the competence, i.e. when DNAse was added to a 10 min old DNA-bacterial complex, when the DNAse could no longer affect the DNA which had penetrated into the cell, a 10fold increase of transformants per 1 ml occurred in the presence of KCl, in contrast to the control without KCl. Fox (1959) and Fox and Hotchkiss (1960) found that the amount of the DNA fixed by the transformed population of bacteria is proportional to the number of bacteria transformed. We may, therefore, deduce that KCl by some mechanism leads to an increase in the irreversible fixation of DNA.

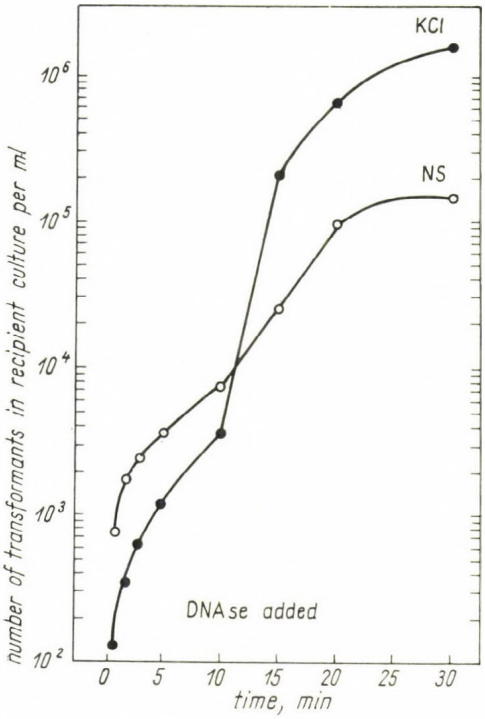

FIG. 5.—Kinetics of KCl under termination of DNA-bacterial complex reaction with DNAse

Because the effect of KCl, in comparison with the control without KCl, increased not towards the peak of the competence but exactly opposite, at a time when the conditions for competence were unfavourable, it seems probable that KCl participates in the reactions which are responsible for the origin of the competence. It is likely that the hypertonic medium stabilizes the temporary one, as if it were the protoplastic state of the dividing recipients, and thus increases the amount of the penetrated DNA, or at a certain instant increases the total of the modified competent cells in the population. Both these explanations would indicate that the competent state, in the sense of the hypothesis of a localized protoplast, includes the increased permeability for macromolecules. At the same time, the results of our experiments show that the transformation was stimulated only in those instances when we added the recipient cells to DNA with KCl in an undiluted state. If we diluted the competent cells into the transformation medium with DNA in the presence of KCl salt, it inhibited the transformation. This would suggest that KCl acts as a stimulator provided that some factor carried in by the recipient cells exists in a surplus, but as an inhibitor as soon as this factor drops below a certain probably optimal level. This implies that it might be a question of the quantitative relations between the substrate and the enzyme during the contact of DNA with the competent culture. The factor whose amount is either small or insufficient at the beginning of the competent phase, probably optimal in the maximal competence, and excessive in the decreasing phase of the competence and in the second cycle of the competence seems to be the enzyme DNAse.

Ottolenghi and Hotchkiss (1962) have shown that the filtrates of young pneumococcus cultures produce a significant effect on the biological activity of DNA in the sense of its inactivation, and that these filtrates reduce the viscosity of the pneumococcal DNA. They are of the opinion that small quantities of DNAse, which are produced by pneumococci, result in the loss of the capacity of the aging culture to carry out the transformation by the added DNA.

We have obtained analogous, as yet unpublished, results from the experiments carried out in our laboratory. We think, however, that the small quantities of DNAse, if they are in an optimal concentration for the given reaction, participate actively in the reactions responsible for the state of competence in the recipient population and that the salts which we use for the process of the transformation reaction, apart from the functions already mentioned, inhibit the DNAse which is present in a surplus in the growing culture, so that they substantially help to increase the transformants even in cultures with an originally low competence, but act as inhibitors if there is suboptimal concentration of DNAse in the reaction system, i.e. on dilution of the recipient culture from the first cycle of competence.

REFERENCES

Ephrussi-Taylor, H. (1951) *Exp. Cell Res. 2*, 589
Fox, M. S. (1959) *J. gen. Physiol. 42*, 737
Fox, M. S. and Hotchkiss, R. D. (1960) *Nature (Lond.) 187*, 1002
Hotchkiss, R. D. (1954) *Proc. nat. Acad. Sci. (Wash.) 40*, 49
Kohoutová, M. (1961) *Nature (Lond.) 190*, 1171
Kohoutová, M. (1962) *Folia microbiol. (Praha) 7*, 33
Kohoutová, M. and Kopecká, H. (1963) *Nature (Lond.) 198*, 711
Lacks, S. (1962) *J. molec. Biol. 5*, 119
Ottolenghi, E. and Hotchkiss, R. D. (1962) *J. exp. Med. 116*, 491
Thomas, R. (1955) *Biochim. biophys. Acta (Amst.) 18*, 467

DISCUSSION

STAHL: Upon what evidence do you identify the 'competence substance' as DNAse?

KOHOUTOVÁ: I should not speak of DNAse as a 'competence substance'. Our previous experimental results and the results presented at this Symposium and those I shall present at the *Congress of Genetics at The Hague* strongly indicate that DNAse takes an active part in the transformation reaction, specifically in the reaction or the reactions connected with competence. Therefore, we do think that DNAse is not only a depolymerizing enzyme, but at a certain moment of the competent reaction and at optimal concentrations of the substrate and the enzyme, in the presence of specific inhibitors, which might be bound in the cell, the DNAse might act on the DNA at the moment of penetration.

HOTCHKISS: None of our own work with Pneumococcus is in serious conflict with the work Dr Kohoutová has presented, although we have not been

convinced that nuclease-like activity from outside the cell is involved in activating DNA for transforming effectiveness. I am again impressed how essential it is to investigate the condition for competence, as Dr Kohoutová has so carefully and thoroughly done, before one can do reliable quantitative work with a new system. It is very encouraging to see that such difficult work has been carried forward so well.

KOHOUTOVÁ: We, of course, do not think of an effect of DNAse from outside but from inside the cell or somewhere in the space between the cytoplasmic membrane and the cell wall, from where the DNAse comes off into the culture medium.

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INFECTIVITY OF ISOLATED *B. SUBTILIS* PHAGE DNA

By

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Phenol-extracted DNA from bacteriophage SP3 has been reported to be infective for competent cells of *B. subtilis* (Romig 1962). Since the efficiency of this system was low, it seemed of interest to test whether higher yields of infectivity, which would allow a quantitative investigation of such a phage DNA infection, could be obtained with another phage and under different conditions for infection.

A virulent *B. subtilis* phage, termed SP50, was isolated from soil. Phenol extracts from purified stocks of this phage, which did not contain any complete virus, were found to initiate the production of complete phage particles, when incubated with cells of *B. subtilis* strain 168, which were competent for bacterial transformation.

The following results indicated that the infective material in the phenol extract is DNA. (1) Treatment of the phenol extract with DNAse or shearing it by passage through a capillary pipette eliminated infectivity. (2) No infectivity was observed, when non-competent cells were used for infection by the phenol extract. (3) The infectivity was drastically reduced, when foreign DNA was present during the incubation of competent bacteria with the phage extract.

Neither the treatments mentioned, nor the choice of plating bacteria (competent or non-competent cells) influence the infectivity of intact SP50 phage.

It can be assumed that the uptake of the *complete* genome of the phage is required to initiate phage production in a cell. This would represent an amount of DNA roughly 100 times greater than that taken up in transformations of single bacterial markers. The following peculiarities of infection of *B. subtilis* with SP50 DNA may be attributable to the necessity for this large amount of DNA. (1) A more than linear increase in plaque number is observed, when increasing amounts of DNA are used for infection. This relationship is tentatively interpreted as being due to *several* fragments of phage DNA rather than *one integer* DNA molecule comprising the infective unit. (2) The efficiency of infection is low as compared with the transformation of bacterial markers. With a final DNA concentration in the incubation mixture of 1 μg/ml and a cell concentration of 2×10^7/ml, approximately 100

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cells will give phage progeny. (3) The uptake of a whole phage DNA equivalent is completed by the majority of cells only at 3 hours after the mixing of competent cells and phage DNA, although an 'attachment' reaction between DNA and cells is comparatively fast. This was concluded from the following experiment. A series of identical mixtures of phage DNA and cells were incubated for 25 min at 30° C ('attachment' period). All mixtures were then plated, and the incubation continued at 37° C. DNAse was layered on top of individual plates at varying times during the incubation period. No infected cells were observed in samples treated during the first hour of incubation after plating. With samples treated later, the number of infected cells rose to the level observed with untreated plates, which was reached after 3 hours.

A detailed account of this work is being published elsewhere (Földes and Trautner 1964).

REFERENCES

Földes, J. and Trautner, T. A. (1964) Infectious DNA from a newly isolated *B. subtilis* phage, *Z. Vererbungsl. 95*, 57

Romig, W. R. (1962) Infection of *Bacillus subtilis* with phenol-extracted bacteriophages, *Virology 16*, 452—9

DISCUSSION

PAKULA: May I ask you about the molecular weight of the phage DNA you use and the technique?

TRAUTNER: The indirect evidence from the irradiation studies seems to show that it is somewhat smaller than that of the T2 or T4 DNA.

PAKULA: This, perhaps, is smaller than the usual molecular size of transforming DNA. In other words, the transforming DNA's size is bigger.

TRAUTNER: I think, it is smaller.

WATANABE: I consider the bacterial piece would be larger than the one of the phage.

TRAUTNER: Yes, that is the question.

SZYBALSKI: This must be about one hundred million.

CLOWES: Have you ever tried in your transformation studies to use phage resistant recipients?

TRAUTNER: No.

CLOWES: This might not only restrict the plaque formers in transformants, but could insure against any side-effects by other phage fragments, involving normal adsorption with possible lethality.

TRAUTNER: No, we have not yet tried this.

HOTCHKISS: I am impressed that both the contributory effect of higher DNA concentration and that of additional time of reaction are quite parallel to the effects in pneumococcal transformation which we have analysed as evidence of multiplicity (reaction with two DNA particles). Is it not more significant to know how many DNA particles are required for plaque induction than what mass of DNA is needed? Just because you are dealing with a multiparticle transformation.

TRAUTNER: Yes, it is a multiparticle transformation, but it must be that even more than two particles are involved.

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TRANSFORMATION OF STREPTOMYCIN MARKERS IN ROUGH STRAINS OF *RHIZOBIUM LUPINI*

By

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Dependence on streptomycin (str-d) is one of three alternative genetically determined responses of bacteria to the presence or absence of streptomycin in its environment, the two others being sensitivity and resistance (str-s and str-r). The genetic analyses of differences between the single-step and multi-step types of mutation to streptomycin resistance at a high level have been made by means of genetic transformation with *Diplococcus pneumoniae* (Hotchkiss 1952, Hashimoto 1955a), conjugation with *Escherichia coli* (Ushiba et al. 1957) and transduction with *Salmonella typhimurium* (Watanabe and Watanabe 1959a,b). The genetic analyses of streptomycin dependence have been made by conjugation with *E. coli* K12 (Demerec et al. 1949, Newcombe and Nyholm 1950, Szybalski and Cocito-Vandermeulen 1958), transduction with *E. coli* (Lennox 1955, Hashimoto 1960) and transformation with *D. pneumoniae* (Hashimoto 1955b). An indirect way of analysis of streptomycin dependence by means of the estimation of suppressor mutations was chosen with *Proteus mirabilis* (Böhme 1961).

The study reported here attempts to answer the following questions. (1) Is it possible to transform streptomycin dependence (Balassa, G. 1963, Balassa, R. and Gábor, M. in press) and independence, that means streptomycin resistance or sensitivity in Rhizobium? (2) Whether or not single-step, highly streptomycin-resistant and dependent mutants of independent origin do result from mutations at the same gene locus (or at very closely linked loci)? (3) Whether the streptomycin-sensitive 'revertants' (mutants or transformants) from str-d were true revertants, due to reverse mutation at the same locus or to change in some other part of the genome (at a suppressor locus which modified the expression of the str-d mutant gene)?

The strains used were the following: streptomycin sensitive rough strain H-13-3 (3 str-s) of *Rhizobium lupini* (Balassa, R. and Gábor, M. 1961), its single-step mutants toward resistance and dependence on a high concentration of streptomycin, and streptomycin-sensitive revertant strains obtained by a single-step selection of spontaneous mutants or of transformants on streptomycin containing or lacking medium, respectively.

The transformation system used consisted of a competent recipient culture, about $(1–5) \times 10^6$ bacteria per ml or $(1–5) \times 10^5$ bacteria per ml. The fresh transformation medium was prepared with 300 μg/ml streptomycin in the case of transformation of streptomycin independence, or without, in the case of transformation of streptomycin dependence (in the latter case the recipient was a str-s culture) (Balassa, R. 1957). In most experiments, the contact between the donor DNA and the recipient cells was not

interrupted for 24 hours. In the control DNAse was used. The number of transformants was determined on selective medium containing or lacking streptomycin, respectively, and the total number of cells was scored on appropriate nutrient medium. The transformation frequency was expressed as a percentage of the total number of cells. When the recipient culture used was streptomycin-dependent, the number of transformants and the total number of cells were determined before and after washing twice with saline. The developed transformant colonies were picked up from nutrient agar, suspended in saline and plated on nutrient agar with different concentrations of streptomycin (0, 10, 30, 50, 100, 300, 1000 μg/ml). Our results are as follows. (1) The transformation frequency of streptomycin resistance in the str-s recipient is 0.05–1 per cent. (The more detailed data about transformability, intra- and interspecific transformations, levels of resistance transferred, single and complex mutations are described in Balassa, R. and Gábor, M. 1961.) (2) The transformation frequency of str dependence, when different donors (1-str-$d_{(1000)}$ 3-str-$d_{(1000)}$ and T3-str-$d_{(300)}$) were used, was very low (0.0001–0.00001 per cent). Spontaneous mutation occurs from 1 to 4.5 $\times$ 10^9. (3) The DNA of a str-d strain is able to transfer either resistance to a low concentration of streptomycin, or the entire resistance of the donor strain, or, more rarely, dependence itself. This suggests that the mutation responsible for dependence is found at the same locus as the complex streptomycin resistance mutation, or in two closely linked loci. (4) There was some correlation between the size of transformant colonies and their character, i.e. in all cases the large colonies were resistant, the medium size and little ones being either resistant or dependent. (5) The transformation frequency of streptomycin independence varied from 0.039 to 0.074 per cent using different donors.

In order to decide whether dependence and single-step resistance to high concentration of streptomycin are determined by a single locus or at very closely linked loci, it was necessary to compare the kind of transformants obtained by transformation with the DNA of str-r strains of different origin and the transformants obtained with the DNA of 'wild' (str-s) ones. It was expected that when these markers are determined by one single locus the results would be quite different from those obtained if these markers are closely linked or are, though linked, not closely linked. The donor marker of resistance or sensitiveness can be recovered only in the case of a single locus or in the case of close linkage, but if dependence and resistance are not linked, the results of such experiments would be similar to those in which the donor is the sensitive 'wild type'. Table I shows the results of experiments in which the recipient was str-d-15 strain and the donors either T-str-$r_{(1000)}$ or str-r-$4b_{(1000)}$ or 'wild type' 3-str-s. It became apparent that in the experiment in which the str-s was the donor 2900 transformants, 100 per cent of the transformant colonies tested was sensitive. Thus, the result of this test makes it probable that the *str-d and str-r are determined by one or two very close loci.* In the experiment when T-str-$r_{(1000)}$ strain (a highly resistant one, obtained by transformation of str-r) or str-r-$4b_{(1000)}$ strain (a highly resistant 'revertant' obtained from the str-d-15) were donors, *none* of the transformant colonies proved to be *sensitive*, the *great majority* of them represented the *donor* (resistant) type. Thus, it could be supposed that str-d-15, T-str-$r_{(1000)}$ and str-r-$4b_{(1000)}$ represent *closely linked loci.* The control

shows the distribution of high-resistant, low-resistant and sensitive 'revertants' when no DNA or the DNA prepared from the recipient itself were given to the recipient. It is evident that a large number of tested colonies, 39 and 55 (75 per cent and 64 per cent) were resistant to low concentrations of streptomycin (10–50 μg/ml), and only 3 and 6 colonies (5.6 per cent and 6.5 per cent) to high concentrations of streptomycin (100 to 1000μg/ml), whereas very few colonies (1 and 7, i.e. 1.9 per cent and 7.6 per cent) were sensitive to streptomycin. So the experiments with the two donors (T-str-$r_{(1000)}$ and str-r-$4b_{(1000)}$) indicate that the majority of *high-resistant*

TABLE I

Results of transformation experiments with str-d-$15_{(1000)}$ strain as recipient and str-r, str-s or str-d-$15_{(1000)}$ strains as DNA donors

	Donors					Control	
	T-str-r (1000)	str-r-4b (1000)	3-str-s (wild-type)	str-s-81	T-str-s-112	with DNA	without DNA
Transformant colonies tested	218	105	2900	276	153	52	91
Dependent high degree	1	13	0	1	4	2	0
Dependent low degree	3	1	0	3	5	7	23
Resistant high degree	213	91	0	0	0	3	6
Resistant low degree	1	0	0	68	64	39	55
Sensitive	0	0	2900	204	71	1	7

colonies represents a transformation by the donor marker, and that the high-resistant 'revertants' from str-d-15 form only a small fraction. All *low-resistant* types in str-d-15, str-s-81 and T-str-s-112 were presumably due to 'revertants'. *Sensitive* types could have originated either by reversion (see data of control), or by transformation of the marker in 3-str-s and str-s-81. It is remarkable that some streptomycin-sensitive colonies appeared between the transformants or in the control, which proved to be str-dependent after retests, but at a lower level of streptomycin concentration. We were able to find such kinds of str-d colonies in almost all cases. This type could have originated after further reversions.

The third question was whether the streptomycin-sensitive and the streptomycin-resistant 'revertants' were due to reverse mutation at the same locus to which the original change that caused the streptomycin-dependence also belonged, or to mutation at a suppressor locus which modified the expression of the str-d mutant gene.

The genotype of the sensitive 'revertants' of the str-d strain can be determined by transformation experiments, using the sensitive revertants as recipients, and the sensitive 'wild type' as donor. In our experiments we

could not prove the presence of suppressor locus, so we have to suppose that all of seven str-s revertants estimated were originated by a reverse mutation (Balassa, R. and Gábor, M. in press).

REFERENCES

Böhme, H. (1961) Über Rückmutationen und Supressormutationen bei Proteus mirabilis, *Z. Vererbungsl. 92*, 197–204

Balassa, G. (1963) Genetic transformation of Rhizobium. A review of R. Balassa's work, *Bact. Rev. 27*, 228–41

Balassa, R. (1957) Durch Desoxyribonukleinsäuren induzierte Veränderungen bei Rhizobien, *Acta microbiol. Acad. Sci. hung. IV*, 77–84

Balassa, R. and Gábor, M. (1961) Transformation of Rhizobium, *Mikrobiologiya 30*, 457–63

Balassa, R. and Gábor, M. (in press) Transformation of streptomycin-dependence

Demerec, M., Wallace, B., Witkin, E. M. and Bertani, G. (1949) The gene. *Carnegie Inst. Wash. Ybk. 48*, 154–66

Hashimoto, K. (1955a) Transformation of streptomycin indifference and resistance, in 'Studies on the transformation of streptomycin resistance in Pneumococci. I', *Jap. J. Bact. 10*, 933–8

Hashimoto, K. (1955b) Transformation of streptomycin dependence, in 'Studies on the transformation of streptomycin resistance in Pneumococci. II', *Jap. J. Bact. 10*, 1049–53

Hashimoto, K. (1960) Streptomycin resistance in *Escherichia coli* analysed by transduction, *Genetics 45*, 49–62

Hotchkiss, R. D. (1952) The role of desoxyribonucleates in bacterial transformations, in *Phosphorus Metabolism, 2*, edited by W. D. McElroy and Bentley Glass, Johns Hopkins Press, Baltimore, 426–36

Lennox, E. S. (1955) Transduction of linked genetic characters of the host by bacteriophage P1, *Virology 1*, 190–206

Newcombe, H. B. and Nyholm, M. H. (1950) The inheritance of streptomycin resistance and dependence in crosses of *Escherichia coli*, *Genetics 35*, 603–11

Szybalski, W. and Cocito-Vandermeulen, J. (1958) Neamine and streptomycin dependence in *Escherichia coli*, *Bact. Proc. 1958*, 37–8

Ushiba, D. T., Watanabe, T., Hashimoto, K. and Hsu, Y. (1957) Genetic studies on streptomycin resistance in bacteria, *Proc. int. Gen. Symp. 1956*, 445–7

Watanabe, T. and Watanabe, M. (1959a) Transduction of streptomycin resistance in *Salmonella typhimurium*, *J. gen. Microbiol. 21*, 16–29

Watanabe, T. and Watanabe, M. (1959b) Transduction of streptomycin sensitivity into resistant mutants of *Salmonella typhimurium*, *J. gen. Microbiol. 21*, 30–9

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SEDIMENTATION STUDIES OF THE DEOXYRIBONUCLEIC ACIDS OF *RHIZOBIUM MELILOTI* AND OF ITS LYSOGENIC FORM

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The DNA of *Rhizobium meliloti* was shown to be biologically different from that of its lysogenic form for phage 16–3 (Szende et al. 1961). With the DNA isolated from lysogenic Rhizobia immunity was transferred to non-infected cells. The transformant cells were immune against a weak virulent mutant of the phage 16–3. The DNA of non-infected cells did not show such activity. These results promoted the search for chemical, physico-chemical or biochemical differences between the two types of DNA. The zone centrifugation method was used for comparative studies, being suitable for characterizing well-defined molecular species differing in size, shape or density.

Rhizobium meliloti strain 41 and its lysogenic form 41(16–3) were isolated and characterized by Szende and Ördögh (1960). The bacteria were grown in glucose-inorganic medium containing yeast-extract and P^{32} 10 mC/ml in Tris buffer (p_H 7.2). After 22 hours of cultivation in rotating test tubes at 30° C, the cells were spun down, washed and disrupted with one per cent sodium deoxycholate in 0.1 M sodium chloride. The extracted DNA was deproteinized with phenol and treated with RNAse. After a final phenol treatment and several other washings, the DNA was precipitated with ethanol. This was the method worked out for the preparation of transforming DNA.

The zone centrifugation was carried out according to Hershey et al. (1963). The DNA preparations were dissolved in 0.1 M sodium chloride (p_H 7.5 with Tris) and 50 μl of each of these solutions containing 10–20 μg DNA were layered on the sucrose gradient. The gradient was made of 25 per cent sucrose in 0.1 M sodium chloride (p_H 7.5) continuously diluting it to 6 per cent with the saline into the 5 ml cellulose tube of the SW 39 rotor of Spinco Mod. L 50 centrifuge. After running for 4.3×10^9 (rpm)^{2}h, 40 serial samples were collected on planchets trough, the hole punctured at the bottom of each tube. The distribution of the DNA was determined by measuring the radioactivity on the planchets with an end-window GM-tube.

The sedimentation distributions of the prepared native DNA of Rhizobium 41 and of its lysogenic 41(16–3) are shown in Fig. 1. In the direction of sedimentation (from right to left) the first smaller peak represents the part of DNA degraded during the preparation. The peak with higher sedimentation rate corresponds to the really native DNA. Comparing the sedimentation distributions of the two types of DNA, the native part of the non-infected cells seems to be more homogeneous than that of the lysogenic cells. In the case of the slower components this part of the lysogenic DNA sedimented faster than did the 41 type. The maximum is shifted towards the sucrose gradient of higher specific gravity.

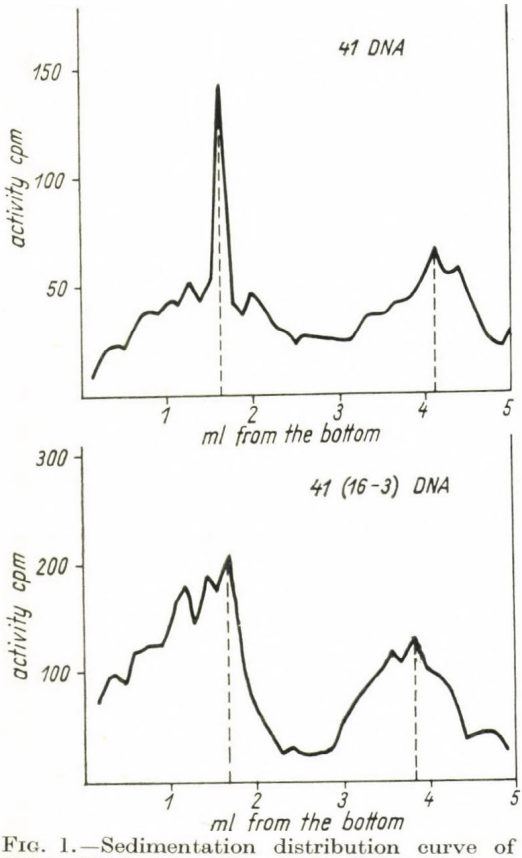

FIG. 1.—Sedimentation distribution curve of DNA of Rhizobium 41 and of its lysogenic form 41(16–3)

In order to calculate the molecular weight of the components, the approximation equations of Burgi and Hershey (1963) were used. The maximum of the native part corresponds to a molecular weight in the order of 10^8, whereas the slower peak in the order of 10^6. The slower component of DNA 41 was calculated to have a molecular weight 3×10^6, that of 41(16–3) to have a 6×10^6 one. To understand this great difference, the sedimentation distributions of heat-denatured DNA of both non-infected and lysogenic cells were also studied. The native DNA samples were denaturated by heating over the T_m. The T_m of Rhizobium DNA was taken as 95° C from the curve of Schildkraut et al. (1962) according to the G+C content (61 per cent) of our preparations. The native solutions were heated in a boiling water bath for one hour and then abruptly chilled. After thawing, 50 μl samples were layered on sucrose gradient and centrifuged like the native ones ($5.2 \times \times 10^9$ (rpm)^{2}h).

The distribution curves of the denatured DNA specimens are shown in Fig. 2. Both DNA 41 and the lysogenic 41(16–3) are sedimenting in one band resulting in one maximum on the distribution curve. The shape of the curve shows homogeneous DNA molecules. Using the approximate calculations

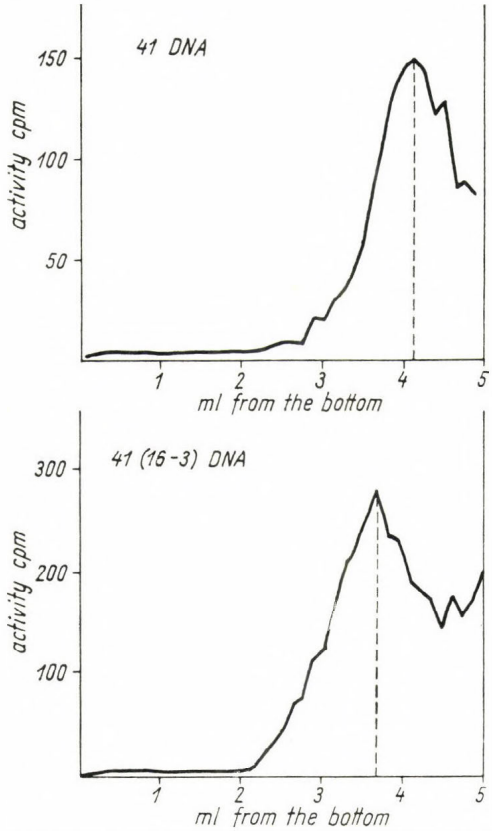

FIG. 2.—Sedimentation distribution curve of heat-denatured DNA of Rhizobium 41 and of its lysogenic form 41(16–3)

mentioned above, the molecular weight for DNA 41 was found to be 1.5×10^6 and for DNA 41(16–3) to be 4.7×10^6. The DNA of the lysogenic cells is faster, showing a higher molecular weight than does the denatured DNA 41.

Although the sedimentation distribution was reproduced in repeated runs for each sample and rechecked with freshly prepared DNA both from Rhizobium 41 and its lysogenic form 41(16–3), the values for the molecular weights can be taken only as informative because of the approximate equations used in the calculation. At this early stage of the experiments the conclusions are more reliable qualitatively. In the preparations taken as native two parts were found: the larger with higher molecular weight and a slower sedimenting one. In the case of lysogenic DNA, the inert part of DNA preparations found in transformation experiments by Szende et al. (1961) can be explained on this base.

The other question, whether or not the prophage attached to the DNA of lysogenic cells can only be the cause of the difference in the distribution of the native faster running part of DNA and is responsible for the shift of maximum with the slower part or heat-denatured DNA, needs further experiments before it can be answered.

REFERENCES

Burgi, E. and Hershey, A. D. (1963) *Biophys. J. 3*, 309

Hershey, A. D., Goldberg, E., Burgi, E. and Ingraham, L. (1963) *J. molec. Biol. 6*, 230

Schildkraut, C. L., Marmur, J. and Doty, P. (1962) *J. molec. Biol. 4*, 430

Szende, K. and Ördögh, F. (1960) *Naturwissenschaften 47*, 404

Szende, K., Sik, T., Ördögh, F. and Győrffy, B. (1961) *Biochim. biophys. Acta (Amst.) 47*, 215

DISCUSSION

STAHL: By what mechanism do you suppose that the presence of prophage increases the sedimentation rate of Rhizobium DNA? The lysogenic cell shows a slight shift in the molecular weight. The prophage performs some function, for instance the glucosylation throughout the entire host DNA.

SIK: We believe that only a part of the prophage is attached to the host DNA. Our first hope was that we can cut off the prophage.

STAHL: Oh, yes. The prophage is very small compared with the host chromosome. It seems that all the DNA of the host chromosome is slightly shifted in its molecular weight. Do you get this chromosome in one piece? Do you get it out intact?

SIK: As it is shown on the first slide, we have some degradation. Perhaps the phenol treatment degraded our DNA. So we do not have the completely native DNA.

STAHL: What would the approximate molecular weight of the undegradated DNA be?

SIK: Using the zone centrifugation technique it is hard to determine the molecular weight accurately. The first peak is of the magnitude $2\text{–}4 \times 10^6$.

SECTION II

BACTERIOCINOGENY

Symp. Biol. Hung. 6, 85–95 (1965)

COLICIN FACTORS AS SEX FACTORS

By

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The stable, heritable ability of a bacterial strain to produce colicin is termed *colicinogeny*, and this state show many similarities with *lysogeny*, the ability to produce a temperate phage. One of the most striking of these is the demonstration by Ozeki et al. (1959) that colicin production is due to a lethal synthesis within a small minority of cells, the state of colicinogeny being expressed in the rest of the population only as a potential to produce colicin in a similar minority of their progeny.

Colicinogeny has been presumed to be due to the presence in the colicinogenic cells of genetic elements termed *colicin factors* or *C* factors. These factors were shown some years ago by Frédéricq and Betz-Bareau (1953) to be capable of infectious transfer from one strain to another in the absence of any other known genetic determinant and were therefore suggested to exist as cytoplasmic entities.

Since that time the tendency has been to extrapolate to *C* factors, the results and conclusions found using other well-documented cytoplasmic elements, such as the F sex factor. Although this has undoubtedly been of great value, there are dangers in pushing these analogies too far within systems, which in spite of their striking similarities have undoubted but less well-known differences. The intent of this paper is to re-examine the genetic status of colicin factors and to show the other side of the coin, how these factors may be exploited to clarify some of the complexities of other genetic elements including the classical F sex factor of K12.

For obvious reasons, we have limited our investigations mainly to the behaviour of two colicin factors that have been most extensively employed by other workers, namely *colE1* and *colI*. Let us first consider the transfer of these two factors in the *E. coli* K12 Hfr mating system. Table I shows the results of 'interrupted matings' (Monk and Clowes, unpublished) using a series of Hfr donor strains, each colicinogenic for *colI*, which inject their chromosome as shown on the circular map (Fig. 1). These donors are each crossed with the same recipient strain J62, selecting in each instance for *pro*$^+$, *his*$^+$ and *try*$^+$ recombinants, and testing both the recombinants and the recipient population for the acquisition of colicinogeny. After 120 minutes, *colI* transfer to the F$^-$ recipient is found to be between 1.4 to 7.7%, depending on the Hfr strain used. However, the transfer to the recombinants is in all cases greater, and, irrespective of the marker selected or the donor strain used, the transfer is greater as the time of entry of the selected marker is later. Thus, in selecting for *his*$^+$, *try*$^+$ and *pro*$^+$ recombinants from *AB311* where the respective times of entry are about 11, 36 and 56 minutes, trans-

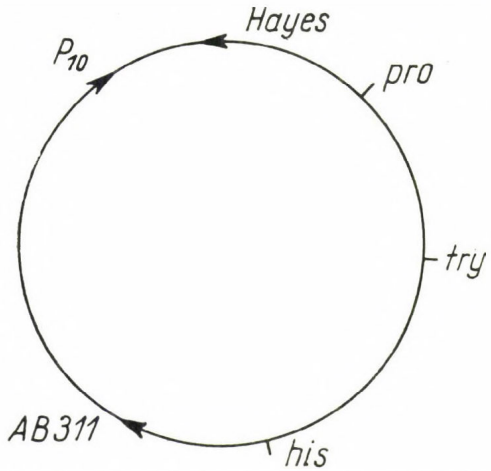

FIG. 1.—Circular chromosome map of *Escherichia coli* K12

fer of *colI*$^+$ is found to be 6, 7 and 41%. With *P10*, where this particular order of entry is preserved, but now where each marker enters about 50 min later, these frequencies are found to be 11, 16 and 55 per cent. In contrast, using Hfr Hayes, where the order of entry is reversed, *pro*$^+$ entering at 14′, *try*$^+$ at 34′ and *his*$^+$ at 59′, the proportion of these recombinants becoming *colI*$^+$ are 14, 17 and 27 per cent. These results are interpreted to mean that *colI* is transferred independently of chromosomal transfer. However, by selecting for late markers, we also select for pairs that have been held together for periods greater than the time of entry of the marker in question, and so increase the time during which the *colI* transfer may take place.

Figures 2 and 3 show the results of similar experiments, crossing five Hfr strains now carrying the *colE1* factor, with the *thr. leu* recipient W677, similarly streptomycin resistant and resistant to the colicin of the donor (Clowes 1963a). In these crosses, it will be apparent that much higher levels of *C* factor transfer obtain, and here we have focussed our attention on the kinetics of transfer. As reported in previous investigations by Alföldi et al. (1958), the maximum level of *colE1* transfer varies with the donor. However, with *these particular* Hfr strains the relative levels of transfer can be seen to be unrelated to any specific chromosomal region which might be trans-

TABLE I

ColI transfer in crosses of Hfr colI$^+$ *strains with the same recipient strain J62 (try.his.pro.colI str-r F*$^-$*)*

Hfr colI$^+$*.str-r* × *pro.his.try.str-r F*$^-$

Mix at ratio 1 : 1 at 37°. Select after 120′. See Fig. 2 for caption and further details

Hfr strain	% *colI*$^+$ among F$^-$ recipients (*str-r*)	Selected markers: histidine (*his*$^+$) Time of entry (*his*)	% rec	% *colI*$^+$	tryptophan (*try*$^+$) Time of entry (*try*)	% rec	% *colI*$^+$	proline (*pro*$^+$) Time of entry (*pro*)	% rec	% *colI*$^+$
AB311	1.4	11	50	6	36	5	7	56	0.16	41
P10 = Type 4	3.2	58	0.3	11	83	0.03	16	103	0.003	55
Hayes (H) ..	7.7	59	0.03	27	34	0.9	17	14	40	14

ferred with the same relative frequencies by all Hfr strains. More important, however, is the finding that the *time of entry* of the *colE1* factor is approximately the same, irrespective of the donor, entering between 7 and 12 minutes. The most satisfactory explanation is, by again assuming a cytoplasmic transfer of *colE1*, unrelated to chromosomal transfer.

It has also been found that the reverse crosses where the *colE1* factor is now present in the F^- parent which were shown by Alföldi and his collaborators to produce killing of the recipient, can be reproduced qualitatively in the absence of any colicin factor (Clowes 1963a). We now have reason to believe that this lethality is associated with the anomalous behaviour of a male specific, cell wall enzyme (Clowes, Silver and Moody, unpublished).

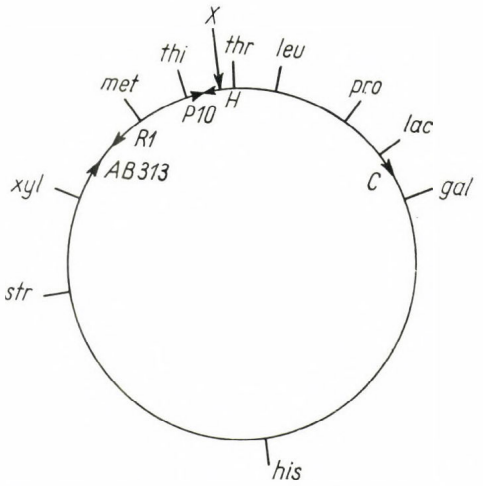

FIG. 2.—Circular chromosome map of *Escherichia coli* K12. The symbols on the outside of the circle represent bacterial markers, symbol *X* denoting the suggested location of the *colE1* factor by Alföldi et al. (1958). The symbols inside the circle denote Hfr strains whose origins and directions of transfer are shown by the arrows on the circle

In other crosses using F^+ donors, the transfer of *colI* and *colE1* resembles very closely, both in the level and kinetics of transfer, the transfer by the corresponding Hfr strains, being about 7% for *colI* and 100% for *colE1*, supporting further the idea of extra-chromosomal transfer. Since these experiments were initiated, similar results have been reported for other colicin factors by Nagel de Zwaig et al. (1962), using *colV* which shows a high *colE1*-like rate of transfer, and *colE2* which is transferred at around the same low rate as *colI*, again irrespective of whether Hfr or F^+ donors were used.

It is thus concluded that *C* factor transfer is, in all cases so far investigated, extra-chromosomal, and that there is no direct evidence for a chromosomal

TABLE II

Fertility of crosses with Hfr Cavalli and related donors with recipient J62 (pro.his.try.str-r F^-)

Donor strain	Recombinant fraction		
	pro$^+$	*his*$^+$	*try*$^+$
1. Hfr Cavalli	220×10^{-4}	3.3×10^{-4}	1.2×10^{-4}
2. Cavalli *MS2-r*	1.3×10^{-8}	0.6×10^{-8}	10^{-8}
3. *ColI*$^+$ F^-	c.0.2×10^{-8}	c.0.2×10^{-8}	c.0.2×10^{-8}
4. Cavalli *MS2-r·colI*$^+$	746×10^{-8}	4.2×10^{-8}	5.1×10^{-8}
5. (Corrected for contact)	282×10^{-6}	1.4×10^{-6}	1.6×10^{-6}

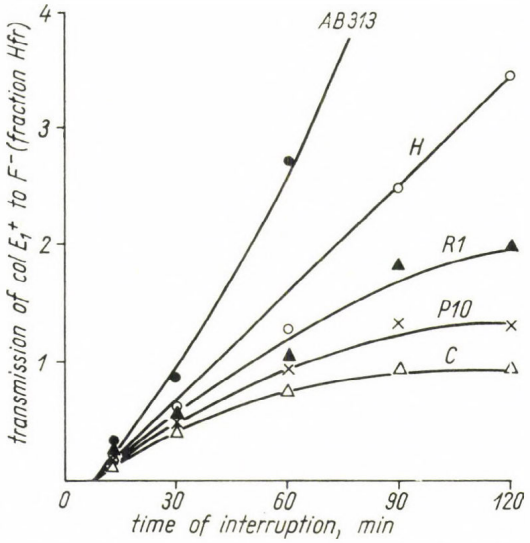

FIG. 3.—Kinetics of transfer of *colE1*$^+$ from Hfr (*colE1*$^+$) strains to W677F$^-$. The individual curves represent the appearance of *colE1*$^+$ in W677F$^-$ when mated with various Hfrs at a Hfr:F$^-$ ratio of 1 : 20

site, which would entitle these *C* factors for inclusion in the class of 'episomes' as defined by Jacob et al. (1960). Nevertheless, the ability of these factors, notably *colI*, to act as sex factors by promoting chromosomal transfer in both Salmonella (Ozeki and Howarth 1961) and *E. coli* (Clowes 1961), might be cited as indirect evidence of a transient chromosomal association. The following experiments have some bearing on this possibility.

Dr Simon Silver has recently isolated from parental Hfr-*E. coli* K12 a series of strains which are resistant to the male specific phage MS2. On examination, these strains are also found to have lost the ability to act as chromosomal donors. However, other reactions, such as their immunity to super infection by F$^-$ prime factors, as shown by Scaife and Gross (1962), suggest that they have not lost the sex factor (and are 'cured'), but may be 'sex mutants' having lost only the ability to produce the male specific, conjugating antigen (Clowes 1963b). This mutation might be either within the sex factor itself, at the locus controlling the male specific antigen, or within the bacterial chromosome suppressing this antigen in some way. These strains may thus be regarded as '*impotent*' males, having lost only their ability to form contacts, and might therefore be revirilized by the presence of an alternate conjugating antigen produced, for example, by the *colI* factor. Table II shows the results of infection of one such strain with *colI*, which demonstrates a form of complementation between the two sex factors *F* and *colI*.

The results show crosses attempted using the same recipient strain J62, using as donor firstly the control parental Hfr Cavalli (Line 1), secondly a male-specific phage resistant mutant (*MS2-r*) of this Hfr (Line 2), and finally this impotent male infected with *colI* (Line 4). For comparison is shown a cross of a colicinogenic F$^-$ strain related to the original Hfr Cavalli also carrying the *colI* factor (Line 3). It will be noted that the efficiency of the impotent male as a donor is reduced very considerably from the level of the Hfr parent. In contrast, when *colI* is introduced into this strain, the transfer of one marker (*pro*$^+$) is increased fivehundredfold, whereas the other markers are little affected. The recombination in this cross (Line 4) differs from that using a *colI*$^+$ F$^-$ donor (Line 3), not only in the level of transfer, but also by the fact that there is a *polarity of transfer* which is similar to that shown by Hfr Cavalli, and which suggests an oriented process

of transfer not characteristic of *colI*. The actual chromosomal transfer therefore may be promoted by the resident F sex factor, although the efficiency is still much reduced. In Hfr crosses it is known that mating contacts reach 100%. On the contrary, contacts in *colI*-mediated conjugation are far lower. A correction for mating efficiency may be made taking this into account, by assuming that contacts are measured by the level of *colI* transfer. Applying this correction, the recombinant fraction per contact, using the $colI^+ \cdot$ *MS2-r* donor is shown in the lower line (Line 5). Even with this correction it appears that chromosomal transfer and integration still reach only a 1 to 2% maximum of those of the parental Hfr Cavalli. This value has a bearing on the recent hypothesis of Jacob et al. (1963), who suggest that the F sex-factor in both F^+ and Hfr strains is located near to the cell surface and directly determines the production of the contact antigen. Contacts are suggested therefore to occur only at these specific F sites, which then trigger off a round of DNA replication and concomitant chromosomal transfer by Hfr cells.

We have found that infection of a cell carrying the F sex factor with the *colI* factor does not prevent the plating of the male specific phage, from which it may be inferred that the specific *colI* conjugating antigen does not occupy the same surface position as the F specific antigen, or its mutated product in the case of MS2 resistant cells. Moreover, the MS2 resistant, $colI^+$ cells do not plate male specific phage and thus do not have the F specific antigen. Nevertheless, specific *colI* mediated contacts can trigger off a type of chromosome transfer which appears to be promoted by the original, chromosomally-located F sex factor. However, since the efficiency of this transfer is at most only a few per cent of the standard Hfr transfer, it does not present unequivocal evidence for or against this hypothesis. It should be noted, however, that in the cross of the $MS2\text{-}r \cdot colI^+$ donor, the colicin factor need not be supposed to interact in any way with the chromosome, but could merely form the contact and conjugation bridge necessary to permit transfer of chromosome mobilized by the resident F sex factor. Similar crosses involving F^- cells made donors by either *colI* or RTF, or, even when cells carrying a sex factor affinity locus such as the Richter female 3 strain are similarly induced to act as donors (Sugino and Hirota 1962), could well involve a similar mechanism in which the function of the introduced *colI* or RTF factor may be merely to produce conjugation bridges.

Various *C* factors have been shown to be transferred to varying extents when present in cells containing F sex factors. The transfer of *C* factors in the apparent absence of other factors has been the subject of several publications by Stocker and his colleagues working mainly with Salmonella strains carrying factors for *I*, *E1* and *E2* (Ozeki et al. 1962, Stocker et al. 1963, Smith et al. 1963). In brief, they find that *colI* can promote its own transfer as well as the transfer of the other two *C* factors, whereas both *colE1* and *colE2* are transferred very infrequently, if at all, in the absence of *colI*.

In K12 we have found a similar situation (Monk and Clowes, unpublished). In the absence of the F sex factor, *colI* can effect its own transfer and that of either *colE1* and *colE2* with equal efficiency, whereas the latter two factors are not transferred to any significant extent in the absence of *colI*. In the presence of F as already noted, *colE1* is transferred with a high effici-

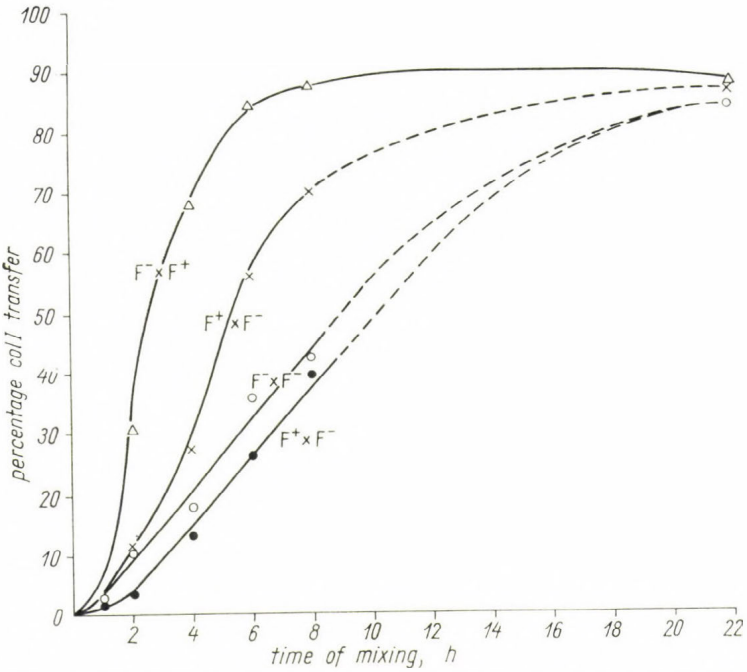

FIG. 4.—Low-frequency colicin factor transfer. Transfer of *colI* between K12 strains. Overnight cultures of the two strains are mixed at about 10^9 cells per ml and incubated at 37° without aeration. In each experiment a *colI*$^+$ str-s strain is mixed with a *colI*$^-$str-r strain. At times, samples are plated on streptomycin and tested to select for the acquisition of *colI* by the str-r strain. The curves represent four mixtures, the first quoted strain being in each instance the colicinogenic and the second strain the non-colicinogenic component. ○ = F$^-$ *colI*$^+$. str-s × F$^-$ *colI*^{r}. str-r, ● = F$^+$ *colI*$^+$. str-s × F$^-$ *colI*^{r}. str-r, △ = F$^-$ *colI*$^+$. str-s × F$^+$ *colI*^{r}. str-r, × = F$^+$ *colI*$^+$. str-s × F$^+$ *colI*^{r}. str-r

ency of near to 100% and *colE2* with a lower efficiency of rather less than 10%. The two sex factors F and *colI* produce distinct patterns of transfer, and this difference is also shown in that the presence of *colI* in an F$^-$ strain does not permit the adsorption and plating of male specific phage as does the presence of the F factor. Moreover, *colI*, unlike RTF (Watanabe 1963), does not interfere with this adsorption when present together with F in an F$^+$ cell. Chromosomal transfer mediated either by F$^-$ or Hfr is also little influenced by the presence of *colI* in either donor or recipient, again in distinction to the RTF factor. However, certain low level interactions between F and *colI* can be noted. Figure 4 shows the effect on the kinetics of *colI* transfer between two K12 strains with the introduction of the F factor. (1) The control F$^-$ × F$^-$ mixture, shown for comparison, indicates that in *E. coli*, transfer of *colI* is more efficient than found in Salmonella by Ozeki et al. (1962). This is supported by the microscopic observation of increased

numbers of specific contacts, and also that the number of 'effective donors' in a stabilized *colI* population of *E. coli* is of the order of 1% compared with a figure of 1 in 5000 in Salmonella. (2) When F is introduced into the *colI* strain, initial transfer is reduced. This is supposed to be due to the fact that the specific F surface is epistatic to that of *colI*, although both bring about conjugation of cells, and that the *colI* factor cannot pass through the cell unions mediated by specific F contacts. It is known, however, that in a small proportion of F^+ cells the F antigens are not expressed (known as F^- phenocopies) and we suppose that *colI* is able to produce specific *colI* contacts and transfer only in these cells. Later transfer rises to that of the control which is presumed to be due to the increasing part played by newly infected *colI* F^- recipient as donors of *colI*. (3) When F is present in the non-colicinogenic parent, however, initial transfer is enhanced. This is interpreted as being due to the part played by specific F contacts between the two strains, which either stabilize or increase the rate of formation of specific *colI* contacts between the pairs. We conclude that an F^+ cell is as good a recipient for *colI* as an F^- cell. This high rate of transfer is maintained, which suggests that when F^+ cells are newly infected with this factor they are more efficient *colI* donors than is a stably infected *colI* F^+ cell, and that $F^+ \times F^+$ contacts are still available to assist the *colI* contacts. (4) When F is present in both cells, there is little initial effect; if anything, a small enhancement. This can again be interpreted by assuming that the *colI* surface is expressed only by the F^- phenocopies as before, but now these *colI* F^- phenocopies have the added advantage that they can form stable contacts with most of the non-colicinogenic F^+ cells, which enhances the rate of *colI* contact formation and stability. This assistance presumably almost outweighs the reduction in the numbers of original *colI* F^+ cells able to produce the *colI* surface.

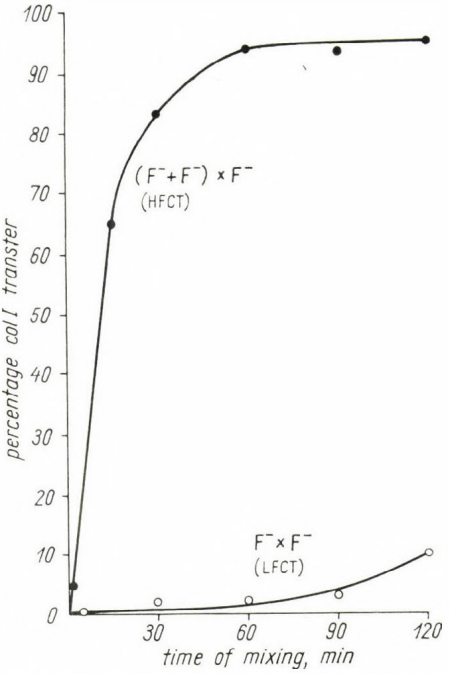

FIG. 5.—Low and high frequency colicin transfer (LFCT and HFCT) in *E. coli* K12. A colicinogenic (donor) and a non-colicinogenic (intermediary) strain of *E. coli* are mixed at 10^5 cells per ml and incubated overnight at 37°. They are then diluted 1 : 20 into fresh broth and re-incubated for a further 1 1/2 h, when they are mixed with the recipient strain at about 10^7 cells per ml. After further incubation, samples are withdrawn and assayed for *colI* transfer

It is obvious from the kinetics of these curves, that in K12, as in Salmonella, we find that newly infected cells have an enhanced ability to transfer, as discovered by Stocker and his associates, and termed by them high frequency colicin factor transfer of HFCT (Stocker et al. 1963). Figure 5 demon-

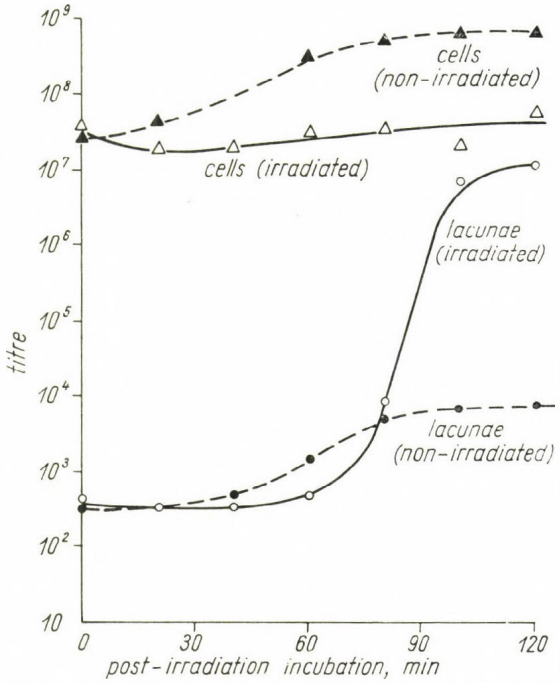

FIG. 6. — UV induction of *colI* production in *E. coli* K12. Log cultures of cells at about 5×10^7 cells/ml are washed and resuspended in buffer and irradiated in 5 ml vols by a UV source at a dose of 5 erg/mm²/sec. They are then rapidly resuspended in broth and reincubated. Samples are removed at intervals and assayed for viable cells and *colI*

strates HFCT in K12. The HFCT system is analogous to Salmonella and is produced by mixing two strains, one *colI*$^+$ and the other non-colicinogenic, overnight and then subculturing for 2 hours. A large proportion of the originally non-colicinogenic strain has been recently infected with *colI*, and high efficiency transfer can now be observed from this strain to a third strain, which is the recipient, also non-colicinogenic. It can be seen that whereas normal transfer in *E. coli* (LFCT) reaches about 10% in 2 hours, in HFCT this value is about 80% after only 40 minutes.

Why are newly transferred *C* factors so much more infective? Smith and Stocker (1962) have suggested that some time after transfer they are stabilized by what they call a 'change of state'. This, as we have seen from Table I and Fig. 3, is not likely to involve a change from a cytoplasmic to a chromosomal localization. A more plausible explanation which has been independently suggested by Clark and Adelberg (1962) and by Marilyn Monk is that established *C* factors probably set up a system of self-regulation with the cell which limits their rate of replication and the level of their specific syntheses. Newly introduced factors may need some time to set up this

system of repression, and during this period their replication and specific syntheses would occur at a higher rate than normal. Either a higher rate of specific *colI* surface antigen synthesis, or a greater number of *C* factor per cell would be likely to lead to increased transfer, by increasing the rate and/or the stability of contacts, and the efficiency of *C* factor transfer during contact, respectively. The hypothesis that newly introduced *C* factors may be unrepressed would predict that in HFCT systems other colicin directed syntheses, such as the lethal synthesis leading to colicin production, should be enhanced. Table III shows that this prediction is fulfilled and that, in an HFCT system, the number of cells undergoing lethal colicin synthesis, which can be demonstrated by the formation of 'lacunae' (Ozeki et al. 1959), is increased by a factor of two hundred over the control (LFCT) system. The

TABLE III

Enhancement of colicin production in HFCT preparations

	Donor mixture		Recipient	% *colI* transfer to recipient in one hour	Numbers of lacunae per 10^8 recipient cells	Limiting colicin titre of donor mixture
	Donor	Intermediate				
HFCT	*colI*$^+$	*col*$^-$	*col*$^-$	98	4×10^5	1/16
LFCT	*colI*$^+$	*colI*$^+$	*col*$^-$	6.9	2×10^3	0

amount of free colicin released is also increased during this period by at least sixteenfold. A further prediction would be that *derepression* might lead both to enhanced colicin production and infectivity. This prediction is also fulfilled to some extent. Figure 6 shows the induction of colicin production in *colI* cells by UV irradiation, which results in an absolute increase of a thousandfold in the numbers of lacunae, which could be even tenfold greater per viable cell. During this period there is a reproducible, but less well marked, increase in the transfer efficiency, which increases about twofold from about seven to fifteen per cent.

It may thus be concluded that HFCT is the result of a state of derepression, which may be produced both by recent transfer of the *C* factor, or by UV irradiation of stably colicinogenized cells. It is pertinent to ask whether such derepression may exist in other systems, particularly in the case of the F sex factor. So far, we have been unable to demonstrate enhanced transfer by a newly introduced F factor, but since in this system the rate of contact formation and actual transfer is ordinarily much higher than in the case of the *colI* factor, increases in F transfer are more difficult to measure. However, when the effect of UV irradiation on F$^+$ cells is considered, the classical work of ten years ago by Hayes (1953) comes to mind. Here UV irradiation was shown to enhance the frequency of F$^+$ mediated recombination by a factor of at least twentyfold. This enhancement has since been shown by Hayes (unpublished) to be of a transient nature, persisting only for two generations, and which is unaccompanied by any increase in the rate of formation of *stable* Hfr mutants. We have repeated these experiments using as an F$^+$ donor a strain carrying also the *colE1* factor, and have

studied the kinetics of F and *colE1* transfer to a recipient after an exposure of the donor strain to UV irradiation which resulted in a twentyfold increase in the numbers of recombinants. At all times, and in several experiments, the transfer of both F and *colE1* were found to be greater from irradiated donors, F transfer after 2 hours contact with the recipient being about 80% by the unirradiated and 95% by the irradiated donor, the respective transfers of *colE1* at 2 hours being 50% and 75%.

Such increases are consistent with the hypothesis that UV irradiation leads to a derepression of both the F and the *colE1* factors, resulting in enhanced numbers of both entities per cell and/or an increase in the amount of F conjugating antigen, both effects leading to a higher probability of transfer per cell contact. Whether this derepression could entirely account for the observed increases in chromosomal transfer is uncertain, but clearly an effect of UV, restricted entirely to the chromosome or to the interaction of the F factor with the chromosome does not by itself appear to explain the enhanced transfer of these extra chromosomal entities.

Self-regulation is obviously necessary in all autonomous cellular entities. However, even when these entities are potentially lethal, lack of regulation may not necessarily lead to lethality, but may result merely in a temporary alteration in the metabolism of the cell which may disappear as regulation is again self-applied.

REFERENCES

Alföldi, L., Jacob, F., Wollman, E. L. and Mazé, R. (1958) *C. R. Acad. Sci. (Paris) 246*, 3531

Clark, A. J. and Adelberg, E. A. (1962) *Ann. Rev. Microbiol. 16*, 289

Clowes, R. C. (1961) *Nature (Lond.) 190*, 988

Clowes, R. C. (1963a) *Genet. Res. 4*, 162

Clowes, R. C. (1963b) *Biochem. biophys. Res. Commun. 13*, in press

Frédéricq, P. and Betz-Bareau, M. (1953) *C. R. Soc. Biol. (Paris) 147*, 2043

Hayes, W. (1953) *Cold Spr. Harb. Symp. quant. Biol. 18*, 97

Jacob, F., Brenner, S. and Cuzin, F. (1963) *Cold Spr. Harb. Symp. quant. Biol. 28*, in press

Jacob, F., Schaeffer, P. and Wollman, E. L. (1960) *Symp. Soc. gen. Microbiol. 10*, 67

Nagel de Zwaig, R., Anton, D. N. and Puig, J. (1962) *J. gen. Microbiol. 29*, 473

Ozeki, H. and Howarth, S. (1961) *Nature (Lond.) 190*, 986

Ozeki, H., Stocker, B. A. D. and Smith, S. (1962) *J. gen. Microbiol. 28*, 671

Ozeki, H., Stocker, B. A. D. and Margerie, H. de (1959) *Nature (Lond.) 184*, 337

Scaife, J. and Gross, J. D. (1962) *Biochem. biophys. Res. Commun. 7*, 403

Smith, S., Ozeki, H. and Stocker, B. A. D. (1963) *J. gen. Microbiol.* in press

Smith, S. M. and Stocker, B. A. D. (1962) *Brit. med. Bull. 18*, 46

Stocker, B. A. D., Smith, S. and Ozeki, H. (1963) *J. gen. Microbiol. 30*, 201

Sugino, Y. and Hirota, Y. (1962) *J. Bact. 84*, 902

Watanabe, T. (1963) *Bact. Rev. 27*, 87

HOLLAND: In connection with the surface properties of strains carrying colicin factors *I*, what are your views of the function of the colicin itself? Especially, in view of recent evidence that some colicins affect DNA synthesis, is it possible that the colicin is concerned in formation of recombinants in some strains?

CLOWES: I do not think the colicin is itself involved in any aspect of conjugation, for the simple reason that *col* factor-mediated conjugation and transfer take place even in the presence of trypsin (200 μg/ml), which is sufficient to eliminate all detectable levels of lethal colicin activity.

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MATING INDUCED BY COLICINOGENIC FACTOR B IN AN F⁻ STRAIN OF *ESCHERICHIA COLI* K12

By

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Chromosome transfer in *Escherichia coli* is induced by several episomes, including *F* (see Jacob and Wollman 1961), *R* factors (Watanabe 1963) and colicinogenic factor *I* (Ozeki et al. 1961). Colicinogenic factors other than *I* are known to be unable to confer fertility upon *E. coli* cells. We have recently found that colicinogenic factor *B* (*col. B*) induces chromosome transfer in an F⁻ strain of *E. coli* K12, which had originated from an Hfr strain. We have been led to this discovery through using a virulent phage W31 which has a specificity to an autonomous F in *E. coli* K12. This phage forms large, clear plaques on F⁻ and Hfr strains with high efficiencies of plating (*eop*) but small, turbid plaques on F⁺ strains with low *eop*. A spontaneous F⁻ segregant was isolated from an Hfr strain W2252. This F⁻ segregant acted only as females in mating like usual F⁻ strains but, by F infection, it was converted to Hfr and not to F⁺. This converted Hfr strain was found to transfer its chromosome with the same frequency and in the same kinetics as the original Hfr W2252. Thus it is suggested that this F⁻ segregant has the so-called *sfa* locus (sex factor affinity locus) (Adelberg and Burns 1960). *Col. B* in usual F⁻ strains reduces the *eop* of phage W31 and makes the plaques of this phage small and turbid. However, it was found that the above segregant W2252 (F⁻) with *col. B* gives a high *eop* and large, clear plaques of phage W31 unlike usual F⁻ strains. Since neither the original Hfr strain nor the converted Hfr strain, where F is assumed to be in an integrated state, restricts the progeny formation of phage W31, it was suspected that *col. B* is also integrated in strain W2252 (F⁻). The following studies were undertaken and the above assumption, that *col. B* can induce chromosome transfer in this particular F⁻ segregant, was proven.

Materials and methods. — *Col. B* was transferred to substrains of *E. coli* K12 by mixed cultivation with *Salmonella typhimurium* LT2 (*col. B*) (kindly supplied by H. Ozeki). Crosses were made between colicinogenic F⁻ strains and non-colicinogenic F⁻ strains with our standard procedure (Watanabe and Fukasawa 1962). Donor strains W2252 (Hfr), W2252 (F⁻) and CSH-2 (F⁻) were methionine-requiring and the recipient strain W677/Pro⁻T6^{r}Sm^r col. B^r (F⁻) was requiring threonine, leucine, vitamin B_1 and proline and unable to ferment mannitol, xylose, maltose, galactose and lactose and resistant to phage T6, high concentrations of streptomycin and *col B*. Various types of possible recombinants were selected on minimal medium enriched with proper nutrients. Kinetics of chromosome transfer was studied by the interrupting of the mating with phage T6 as was studied by Hayes (1957).

Results.—According to the results shown in Table I, the genetic recombination occurred with W2252 (F^-) (*col. B*) as a donor, although the frequency was rather low. W2252 (F^-) and CSH-2 (F^-) (*col. B*) were completely infertile. The kinetics of chromosome transfer were almost equal with W2252 (Hfr) and W2252 (F^-) (*col. B*) as donors. The genetic constitutions of recombinants by the original W2252 (Hfr) and W2252 (F^-) (*col. B*) are shown in Table II. As seen in this table, they are practically identical to each other.

TABLE I

Frequencies of recombination by various derivatives of Escherichia coli K12

Donor strain*	Frequency of recombinants per introduced donor cell
W2252 (Hfr)	2.3×10^{-3}
W2252 (Hfr) (*col. B*)	2.1×10^{-3}
W2252 (F^-)	$<3.7\times10^{-8}$
W2252 (F^-) (*col. B*)	1.2×10^{-4}
CSH-2 (F^-)	$<3.5\times10^{-8}$
CSH-2 (F^-) (*col. B*)	$<3.8\times10^{-8}$

* W2252 and CSH-2 are both methionine-requiring. They were crossed with W677/Pro$^-$T6^{r}Sm^{r}col.B^r (threonine, leucine, vitamin B_1 and proline-requiring, F^-) with our standard procedure. Pro$^+$, Sm^r recombinants were selected on minimal agar containing 50 μg per ml each of threonine and leucine, 5μg per ml of B_1 and 1,000 μg per ml of streptomycin.

TABLE II

Genetic constitutions of Pro$^+$, Sm^r recombinants in the crosses of W2252 (Hfr) and W2252 (F^-) (col. B) with W677/Pro$^-$T6^{r}Sm^{r}col. B^r

Unselected marker	Genetic constitutions of Pro$^+$, Sm^r recombinants* with	
	W2252 (Hfr)	W2252 (Hfr) (*col. B*)
Lac$^+$	34/50	45/76
T6^s	26/50	23/52
Thr$^+$Leu$^+$	5/50	16/76
B_1^+	5/50	9/76
Man$^+$	0/50	0/76
Xyl$^+$	0/50	0/76
Mal$^+$	0/50	0/76
Gal$^+$	0/50	0/76

* The Pro$^+$, Sm^r recombinants obtained in mating for 2 hours in the experiment shown in Table I were studied for their unselected markers with a replica plating method.

Summary.—*Col. B*, like an autonomous F, was found to restrict the progeny formation of a virulent phage W31 in F^- strains of *E. coli* K12. However, it did not restrict phage W31 in an F^- strain derived from W2252 (Hfr). This F^- segregant is assumed to have an *sfa* locus, because the infection of this strain with wild type F converted it to Hfr and not to F^+; W2252 (Hfr), which has a stably integrated F, did not restrict phage W31 and hence it was suspected that *col. B* might be also in an integrated state in W2252 (F^-) (*col. B*). It was found that this strain carries out chromosome transfer, although with lower frequencies than the original Hfr W2252. The kinetics of transfer of host chromosomes by W2252 (F^-) (*col. B*) was the same as that by the original Hfr W2252. These results suggest that *col. B* is likely to be integrated at the *sfa* locus. Since W2252 (Hfr) and its derivatives transfer the terminal markers of their chromosomes very rarely (as seen in Table II), it is not easy to study this possibility by making experiments. We are now studying this possibility by transduction with phage *Plkc*.

If the integrated state of *col. B* were proven by the experiments which are now in progress, the genetic homology between F and *col. B* would be disclosed. However, it may also be possible that *col. B* is a complex of a colicinogenic factor and a sex factor.

*

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REFERENCES

Adelberg, E. A. and Burns, S. N. (1960) *J. Bacteriol. 79*, 321—30

Hayes, W. (1957) *J. gen. Microbiol. 16*, 97—119

Jacob, F. and Wollman, E. L. (1961) *Sexuality and the Genetics of Bacteria*, Academic Press, New York and London

Ozeki, H., Howarth, S. and Clowes, R. C. (1961) *Nature (Lond.) 190*, 986—9

Watanabe, T. and Fukasawa T. (1962) *J. Bact. 83*, 727—35

Watanabe, T. (1963) *Bact. Rev. 27*, 87—115

DISCUSSION

WOLLMAN: Did I understand well that in your case you find the transfer of the colicinogenic *B* character from a colicinogenic *B* F^- strain to a non-colicinogenic F^- strain?

WATANABE: Yes, this is indeed what we do find.

CLOWES: Stocker also finds transfer of colicinogeny *B* in Salmonella, in a way rather similar to the transfer of colicinogeny *I*. There are two comments that I should like to make. The first is that Dr Watanabe's observation that some *R* factors prevent adsorption of the male specific phage to the male K12 strains, whilst permitting transfer of chromosome, is paralleled in our finding that certain mutants of K12, selected to be

completely resistant to male specific phage, may still preserve a certain level of transfer of chromosome. Secondly, with regard to the observation that when *R* factors are transferred to Proteus two distinct new G+C types are found. This I understand has also been reported with regard to the F factor in *E. coli*. However, one must not assume in either case that both G+C types represent two types of particles which characterize the F or R factors, since both systems would be capable of transferring other cytoplasmic entities accidentally present in the donor cell and unrelated to the sex factor. Such particles appear to exist in K12 which influence the efficiency of plating of certain phages. Some auxotrophs, which are F^+, are able to plate T3 with an efficiency of only 1 in 10^5 of that shown on other strains some of which are F^-. Drs Shell and Glover in our laboratory have shown that, if these two types of strains are mated, among the F^- cells which have been converted to F^+ there exists a whole variety of responses to the plating of T3, clearly showing the independent transfer of other factors independent of F.

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EFFECT ON DNA SYNTHESIS OF PREPARATIONS OF A BACTERIOCIN FORMED BY *B. MEGATERIUM*

By

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In preliminary studies on episomal transfer experiments in *B. megaterium*, 18 megacinogenic (M^+) strains were isolated from soil samples by methods described by Iványovics and Nagy (1958). When these M^+ strains were grown in mixed culture with M^- bacteria it was found in several cases that the M^- organisms were killed. Figure 1 summarizes these results when *B. megaterium* De Bary was the non-producing M^- bacteria. The M^+ strains were divisible into three groups (only one representative of each being included in the figure): Group 1 (non-killers), did not kill the M^- bacteria; in the presence of Group 2 (delayed killers), the M^- bacteria multiplied for 30–45 min before being killed; Group 3 (rapid killers), caused killing of strain De Bary immediately on mixing.

DIFFERENT TYPES OF MEGACIN

Subsequently it was found that the megacin produced by Groups 2 and 3 M^+ strains was liberated by lysis after induction with UV irradiation whereas Group 1 megacin was not UV inducible. Further studies, including determination of activity spectra, indicated that Groups 2 and 3 megacin (*megacin Type A*) had in fact several different properties from those of Group 1 or *megacin Type B*.

The killing effects described above could not however be attributed to megacin A, since the latter is not usually produced in young growing cultures, and moreover M^- strains resistant to megacin A were still killed in mixed culture. After further investigation the production of a third megacin, megacin C, was confirmed when megacin A^- mutants were isolated which no longer lysed on UV induction but which still produced megacin C and were killed in mixed culture.

FORMATION OF MEGACIN C

Samples were taken from growing cultures of MA^- and MA^+ strains, centrifuged and assayed with a megacin A resistant indicator organism (Fig. 2). In both strains megacin C is liberated by the young growing cultures

* Part of this work was carried out in collaboration with Dr C. F. Roberts and whilst the author was an I. C. I. Biochemistry Research Fellow. Presentation of the communication was facilitated by the award of a grant from the Welcome Trust.

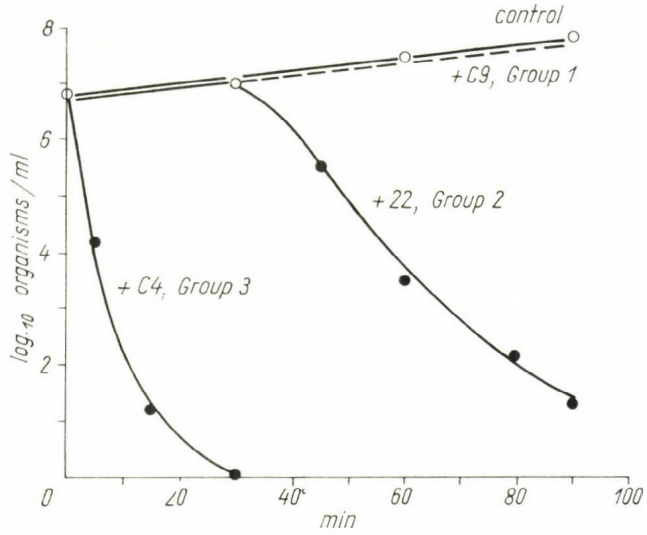

FIG. 1.—Young exponentially growing cultures of M$^-$ strains harvested, washed and mixed with a similar concentration (10^7 organisms/ml) of the tre-r M$^-$ strain, *B. megaterium* De Bary. Control mixed with broth alone

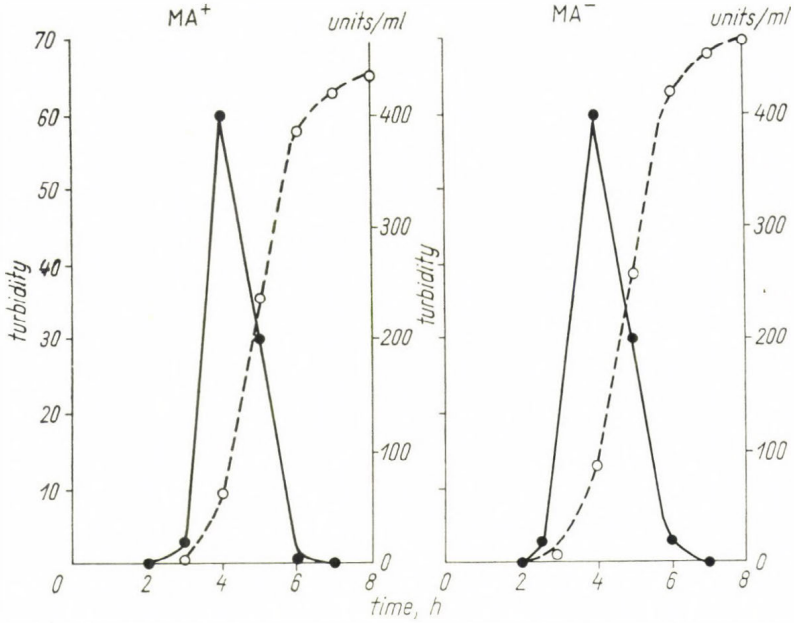

FIG. 2.—*B. megaterium* strain C4 and its MA$^-$ derivative grown in broth; samples were taken at intervals and the turbidity determined. They were also centrifuged and the supernatant assayed against *B. megaterium* strain Mut. as indicator

but after reaching a maximum value in mid-growth it disappears, possibly by re-adsorption. Megacin C, like other bacteriocins, has been found to contain protein and has a characteristically very specific antibacterial spectrum. Unlike megacin A it has apparently no effect upon the cytoplasmic membrane of sensitive bacteria (Ivánovics et al. 1959, Holland 1962).

MODE OF ACTION OF MEGACIN C

So far only crude preparations of megacin C have been used, since the bacteriocin is rather unstable. These preparations were simply obtained by centrifuging cultures, in peptone broth, of the producer strain containing maximum amounts of megacin. The preparation was finally sterilized and kept overnight before use.

Like colicins, megacin C was found to be adsorbed by sensitive bacteria which are rapidly killed. Thus, with a growing culture treated with 100 units megacin/ml (Holland 1961) the viable count falls to a survival of 10^{-5} in 20 min. Using such conditions, after treatment with megacin, samples were taken at intervals and DNA, RNA and protein extracted; DNA was

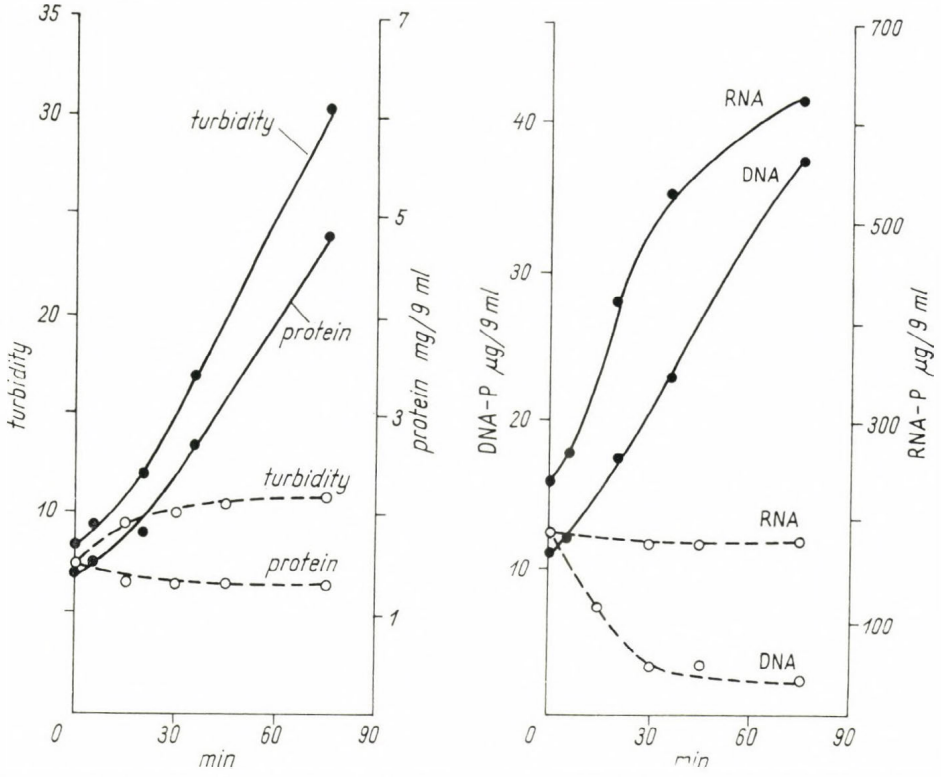

FIG. 3.—Effect on DNA and protein synthesis of megacin C (final concentration 100 units/ml) added at time 0 to growing cultures of strain Mut.; 9 ml samples were taken at intervals, mixed with 1 ml ice-cold TCA and rapidly cooled to 0° to precipitate nucleic acids and protein

then estimated with diphenylamine, RNA with orcinol and protein with Folin reagent. In treated cultures net RNA and protein synthesis is immediately halted, as shown in Fig. 3, whilst, in contrast, the amount of DNA in the culture markedly decreases, usually to about 25% of the initial value. Other work has indicated that although megacin C is adsorbed by both growing and non-growing cultures, DNA breakdown occurs only in cells exposed to conditions permitting growth in the absence of megacin. This suggests that DNA breakdown may depend upon the formation of an induced DNAse, and/or, that only DNA being rapidly synthesized is affected by adsorption of megacin C.

INDUCTION OF PHAGE FORMATION BY MEGACIN C

Further indications that megacin C preparations specifically affect DNA synthesis were obtained when it was found that treatment of a lysogenic strain induced phage formation (Fig. 4). The lysogenic strain *B. megaterium* 899 was treated with different concentrations of megacin C and in each case after residual growth some lysis occurred. With 50 units/ml the culture lysed after 45 min and the phage titre increased from 2.6×10^6 to 2.6×10^9 particles/ml using strain Mut. as indicator. The results obtained with megacin C were in fact identical with those obtained with UV induction of strain 899.

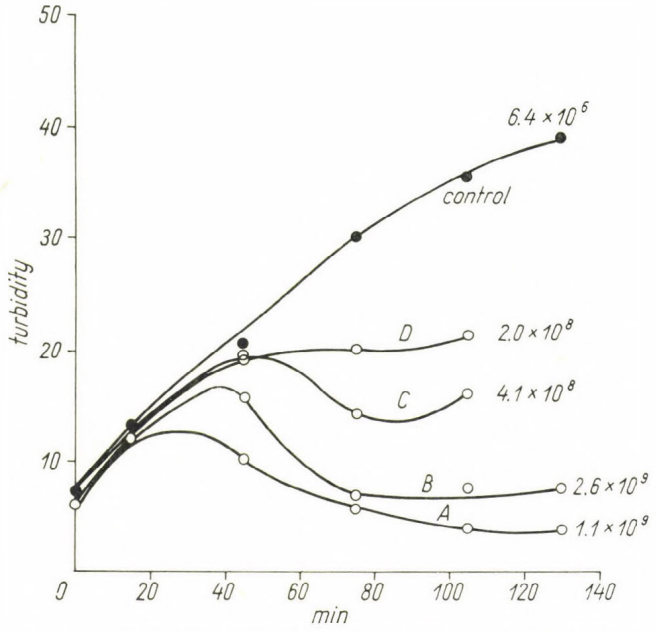

FIG. 4.—Induction of lysogenic strain 899 with megacin C. Extracellular phage was determined with strain Mut. as indicator and the titres appear beside the curves. Control = no megacin, A = 100 units/ml megacin, B=50 units/ml, C=25 units/ml, D =10 units/ml

It is now hoped to isolate megacin C and then to investigate in more detail the mechanism of DNA breakdown, especially in relation to the normal system of control of DNA replication in this organism.

REFERENCES

Holland, I. B. (1961) *Biochem. J. 78*, 641
Holland, I. B. (1962) *J. gen. Microbiol. 29*, 603
Iványovics, G. and Nagy, E. (1958) *J. gen. Microbiol. 19*, 407
Iványovics, G., Alföldi, L. and Nagy, E. (1959) *J. gen. Microbiol. 21*, 51

DISCUSSION

SCHAEFFER: What is the evidence that megacin C is not a phage?

HOLLAND: You can assume that it is a defective phage, you can call it as you like, but surely it is not a complete phage.

IVÁNOVICS: The megacin A referred to by Dr Holland is inducible by UV light. If you take, for instance, that this is the growth in optical density, here we find that the UV induction begins in about 10–15 minutes and then there is a very rapid lysis, and it follows that the bacteriocin further affects on the lysis of the cells. The bacteriophage titre of the lysis increases and a new protein appears. This protein has been identified and also purified by Dr Holland. One of the strangest strains we studied is strain 216 developed by Dr Holland. It has been studied quite carefully. But we have always found that it has an inactive band of defective chromosome, that is a prophage. We suspected that, besides the incomplete phage particles, also infective phage particles appear at lysis. Neither the infective particle nor any structure resembling bacteriophage appeared. I believe that this kind of megacinogenic strain carries a defective protein. If one takes a typical lysogenic strain, A99 for instance, carrying a different bacteriophage, one finds just the same phage as in the case of the megacinogenic strain. Here is, of course, AU1 liberated now. But this megacin A can be induced by UV light and so can be A99. We have found induction by some chemicals. We have tried, of course, also nitrogenmustard, but we never found any induction. Recently, we have been doing some further trials of induction using cytostatic substances, recommended for the treatment of cancer. These substances and antibiotics have been tested and we also found them effective in inducing a typical lysogenic strain and a megacinogenic strain. We do not have any evidence that this megacin is induced to a defective protein. We made further attempt to isolate a megacinogenic clone from a lysogenic one. But we were not able to recover a megacinogenic clone, however, sometimes we found more blocks on a phage-resistant indicator. One can have other results, we have, however, never been able to pick up a bacterium-producing megacin. — Now I go into speculation, if you do not mind. We know that the infective bacteriophage is always an enzymatically active phage. In the case of T2 phage a lysozym-like substance has been isolated and now it is believed that it penetrates by the enzyme and then DNA is injected. However,

if we consider the cytoplasmic membrane, it should also be penetrated by DNA. It is very characteristic, how this penetration is done. This membrane is denser and more elastic than the cell wall itself. Therefore, I believe that there is another enzyme bringing forth the penetration of DNA, but this enzyme has not been found so far. In the very moment, perhaps, when it is acting on a cytoplasmic membrane, somehow it is inactivated. My idea is that the megacin produced by a megacinogenic A strain is probably nothing else but an enzyme which destroys, at least partly, the cytoplasmic membrane. In fact, *B. megaterium* appears to be a particularly interesting subject for studying bacteriocin or bacteriocin-like systems. As Dr Holland pointed out, we have already found a second megacin and a third megacin as well and according to our assumption, recently we have also found a new one.

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ON THE GENETIC DETERMINATION OF COLICINOGENY

By

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This symposium being devoted to transformation and to bacteriocins, at first I did not intend to give a paper, since I have never worked on the first of these subjects and not worked recently on the second. Upon the insistance of the organizers of this meeting, I was however induced not only to speak but also to write the present paper. The work reported is not mine, but work done by Drs Nagel de Zwaig, J. Puig and D. N. Anton at what was once the *Laboratory of Bacterial Genetics of the Instituto Nacional de Microbiologia (Buenos Aires)*. Some of this work has already been published, some has been the subject of a university thesis and is in the process of publication.

*

The ability to produce colicins is a widespread property among *Enterobacteriaceae* (Frédéricq 1948). Strains that produce colicins are called colicinogenic and colicinogeny is indeed a stable, hereditary property of such strains. For many years, the similarities between the specificities of colicins and of bacteriophages, on the one hand (Frédéricq 1953) and between lysogeny and colicinogeny, on the other (Jacob et al. 1953), have been emphasized. The hereditary property of colicinogenic bacteria to produce colicins was therefore ascribed to a genetic structure or co'icinogenic factor, in the same way as the heritable property of lysogenic bacteria to produce bacteriophage is under the control of a hereditary structure, the prophage. It was Frédéricq (1954) who first showed that colicinogeny could be transferred from colicinogenic to non-colicinogenic bacteria in mixed cultures. Furthermore, using strains of *Escherichia coli* K12 of different sexual types, he was able to demonstrate that colicinogeny could only be transferred from donor to recipient bacteria and that this transfer was independent of that of chromosomal markers. The conclusion was that colicinogenic factors were extrachromosomal genetic structures.

The understanding of the mechanism of bacterial conjugation (Wollman et al. 1956) allowed a genetic analysis of lysogeny (Jacob and Wollman 1957) and opened the way to apply similar methods to the study of colicinogeny. Preliminary results obtained in the case of colicinogeny E_1, although showing that no linkage existed between this colicinogenic factor and chromosomal markers, led to the premature conclusion that colicinogenic factor E_1, like the genetic material of temperate bacteriophages or the sex factor of bacteria, could have the properties of an episome (Jacob and Wollman 1958). This hypothesis was based on two lines of evidence. The first one

was that in a series of crosses involving different non-colicinogenic Hfr donor strains and the same colicinogenic recipient, killing of the zygotes occurred in some cases and the extent of this lethal zygosis varied according to the type of Hfr strain used (Alföldi et al. 1957). The second one was that in a series of crosses involving different Hfr donor strains, all colicinogenic for E_1, and the same non-colicinogenic recipient, the extent of transfer of colicinogeny varied according to the Hfr strain used (Alföldi et al. 1958). It was the apparent correlation between these two series of facts that led to the above-mentioned hypothesis.

In view of the indirect character of the evidence concerning the episomic nature of colicinogenic factors, it was realized that more systematic work was desirable. At the occasion of a stay at the *Instituto Nacional de Microbiologia (Buenos Aires)* in 1960, this problem was therefore suggested as a working project to the group interested there in bacterial genetics. The genetic behaviour of colicinogenic factors E_1,E_2, I, V and B was thus analyzed. It was found that the observations made in the case of colicin E_1 had been purely coincidental. The effect of lethal zygosis observed when an excess of non-colicinogenic Hfr was put in the presence of F^- colicinogenic recipients is also found when both strains are non-colicinogenic and only with certain types of Hfr strains (Nagel de Zwaig et al. 1962). This effect has since been studied in greater details by Clowes (1963) who showed that the extent of lethal zygosis varies according to the conditions of the experiment and to the types of Hfr and F^- strains used.

EXPERIMENTAL CONDITIONS

The investigations to be summarized bear on colicins E_1, E_2, I, V and B. For each of these colicins, *E. coli* K12 recipient F^- bacteria, F^+ donors and several Hfr strains chosen for their differences in chromosomal polarities were made colicinogenic either by mixed culture with a colicinogenic donor or by transduction. In all cases, in order to avoid distortions in the results of crosses, the non-colicinogenic as well as the colicinogenic strains were rendered resistant to the particular colicin under study. Background knowledge on bacterial conjugation may be found in Wollman and Jacob (1959) and Jacob and Wollman (1961). In each case, the reciprocal crosses were made:

♂ col^+ × ♀ col^- (proportion ♂/♀ = 1/20)
♂ col^- × ♀ col^+ (proportion ♂/♀ = in general, 20/1)

as well as the control crosses: ♂ col^+ × ♀ col^+ and ♂ col^- × ♀ col^-.

When an excess of males was used, precautions were taken to minimize zygotic killing. The F^- recipient was a polyauxotroph for threonine, leucine, vitamin B_1, tryptophan, histidin and arginin called PA309 which is also streptomycin-resistant. The F^+ donor was strain 112, a double auxotroph for cysteine and histidine. The different Hfr types are represented in Fig. 1. All donor strains were streptomycin-sensitive. Recipient bacteria having acquired the colicinogenic character of the donor as well as genetic recombinants for any of the auxotrophic characters of the recipient are therefore selected on appropriate media containing streptomycin in order to eliminate

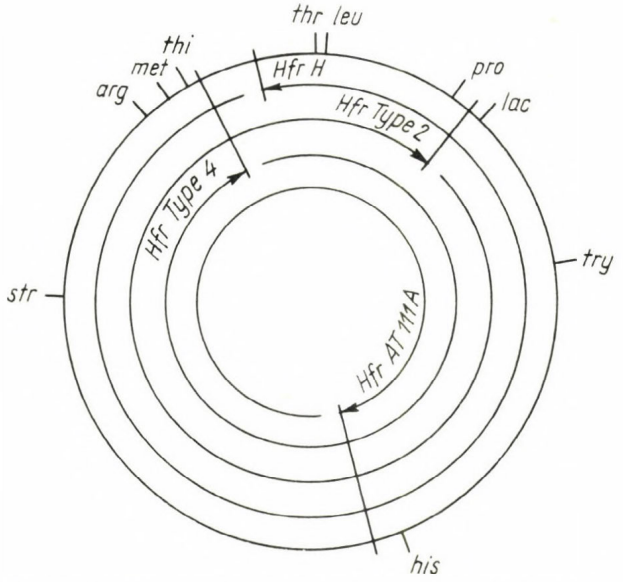

FIG. 1.—Representation of the Hfr donor types. The outer circle represents the genetic map of *E. coli* K12, with the relevant markers. The inner circles correspond to the linkage groups of the different Hfr males indicated, with their origin and polarity of transfer. *leu* = requirements for leucine, *thr* = threonine, *pro* = proline, *try* = tryptophan, *his* = histidine, *arg* = arginine, *met* = methionine, *thi* = thiamine, *lac* = utilization of lactose, *str* = resistance or sensitivity to streptomycin

donor bacteria. Experimental details may be found in the original papers of the authors: for colicins E_2, I and V in Nagel de Zwaig et al. (1962), for colicin E_1 in Nagel de Zwaig (1963) and for colicin B in Puig (1963) as well as in forthcoming publications.

FREQUENCIES OF TRANSFER OF COLICINOGENY

In crosses where the male colicinogenic donor is the minority parent and the female non-colicinogenic recipient the majority parent (ratio ♂/♀ = 1/20), the frequency of transfer of colicinogeny is expressed by the ratio of recipients having acquired colicinogeny to the initial number of donor bacteria. The results of such crosses are summarized in Table I, where the five types of colicinogenic properties are arranged according to the frequencies of their transfer. It may be seen that, for a given colicin, the frequency of its transfer varies to a certain extent with the type of colicinogenic donor used. For different colicins, however, the frequency of transfer differs much more widely, and this is, so to say, independent of the type of colicinogenic donor used, whether F^+, or Hfrs of very different origin and polarity have been used. The difference in efficiency of transfer of colicinogeny V and colicinogeny I, for instance, is of the order of 10^4.

In reciprocal crosses between non-colicinogenic males and colicinogenic females, the transfer of colicinogeny from females to males has not been observed in all cases in which it has been investigated and could be hoped to be detected, i.e. in the cases of colicins E_1, B and V.

In these experiments, therefore, transfer of colicinogeny has only been observed to occur from males to females and never from females to males. It may also be added that such transfer has not been observed from females to females either.

TABLE I

*Frequencies of transfer of colicinogenic factors in crosses ♂str^S col^+ /col × ♀ F^- PA309 str^r col^-/col**

Types of donor strains	Types of colicinogenic factors in donor strains				
	V	E_1	B	E_2	I
F^+ 112	×11	× 6	14	1	0.3
Hfr *H*	× 8.5	× 7.8	29	0.3	0.2
Hfr Type 2	× 4.8	× 6	50	1.3	—
Hfr Type 4	—	×10	11	3	0.2
Hfr AT 111 A	×21	× 9	—		

* Crosses were performed in the proportion of 1 donor for 20 recipients, except for the crosses involving col I where the proportions were reversed (♂/♀ = 20/1). Platings were made after 90′ for crosses involving colicins E_1 and B, after 120′ for crosses involving colicins V, E_2 and I. The results are expressed as the ratio (in %) of the F^- having become colicinogenic for the factor indicated to the initial number of bacteria of the minority type (generally the donor type). When this ratio exceeds 100%, the factor by which the number of recipients having acquired colicinogeny exceeds the initial number of donor bacteria is given.

As for the transfer of non-colicinogeny from non-colicinogenic males to colicinogenic females which has been thoroughly investigated, it has never been found to occur.

From these results, it may be concluded that the colicinogenic factors examined behave as extrachromosomal factors the frequencies of transfer of which vary from one colicinogenic factor to the other but are relatively independent of the type of donor strain employed.

KINETICS OF TRANSFER OF COLICINOGENY

This conclusion is strengthened by the study of the kinetics of transfer of colicinogeny. It can already be seen in Table I that for those colicinogenic factors which are transferred with high efficiency, the number of recipient cells that become colicinogenic exceeds the number of donor bacteria present in the mating mixture, which indicates that, unlike chromosomal markers, acquisition of colicinogeny does not require chromosomal integration. It is already known from single cell isolation that colicinogenic factors replicate in the recipients faster than the recipient's genome do (Alföldi et al. 1958). This is confirmed by kinetic experiments done in the cases of colicinogenic factors V, E_1 and B such as that represented in Fig. 2 for the case of colicin V. It is seen that whereas the number of chromosomal recombinants

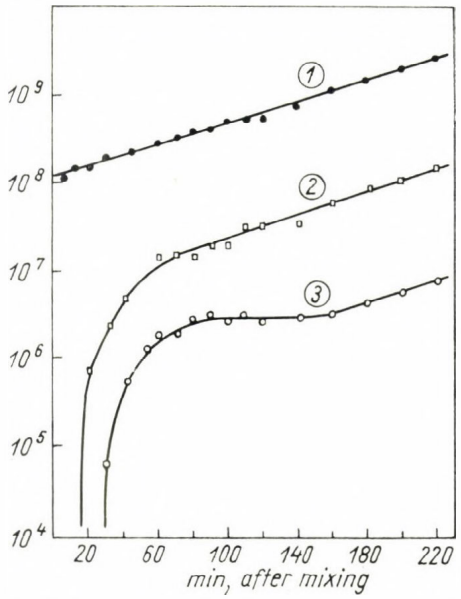

FIG. 2.—Kinetics of transfer of colicin V. In a cross Hfr Type 2 col$^+$ V $\times$ F$^-$ PA309 *str*^{r} col$^-$V, performed in the proportion of 1 donor for 20 recipient bacteria, samples were withdrawn at different time intervals, treated in a Waring Blendor, diluted and plated on appropriate media. The ordinates represent the numbers (per ml) of the mating mixture, of 1 = total recipient bacteria, 2 = recipient bacteria having become colicinogenic for V, 3 = genetic recombinants *thr*$^+$ *leu*$^+$*str*^{r} (Nagel de Zwaig et al. 1962)

TABLE II

*Frequencies of col$^+$ bacteria among different types of genetic recombinants formed in crosses Hfr str^s col$^+$/col $\times$ F$^-$ PA309 str^r col$^-$] col bacteria**

Donor strains	Colicinogenic factors	% col$^+$ recombinants			
		T$^+$L$^+$str^r	try$^+$str^r	his$^+$str^r	arg$^+$str^r
Hfr H	V	79	91	100	100
	E_1	56	58	64	68
	B	48	80	100	100
	E_2 (1/20	0	0	0	2
	E_2 (20/1	0	0	2	7
		T$^+$L$^+$str^r	arg$^+$str^r	his$^+$str^r	try$^+$str^r
Hfr Type 2	V	80	84	100	100
	E_1	41	42	69	75
	E_2 (1/20	0	2	1	3
	E_2 (20/1	0.7	1.3	1.2	1.5
		arg$^+$str^r	arg$^+$str^r	his$^+$str^r	
Hfr Type 4	E_1	58	62	73	
	B	35	90	98	
	(1/20	0.3	0	1.5	
	(20/1	0.4	0	0	

* Crosses are identical with those in Table I. Unless otherwise indicated, the proportions of Hfr to F$^-$ are 1/20. For each type of Hfr donor, the genetic recombinants are given according to the decreasing frequencies of their formation, i.e. to the order of transfer of the Hfr markers.

reaches a plateau before starting to increase again at the same rate as the recipients, the number of recipients that become colicinogenic does not reach such a plateau but increases as the rest of the recipient population.

In interrupted mating experiments it is found that, whatever the colicin under study, be it V, E_1 or B, and whatever the donor type, be it F^+ or of any of the Hfr types represented in Fig. 1, transfer of colicinogeny always starts within 5 to 10 min after the onset of the cross. Similar results have been found for colicin E_1 by Clowes (1963). These results again demonstrate the extrachromosomal nature of colicinogenic factors.

A similar conclusion is reached when recombinants selected in the same crosses are analyzed for colicinogeny. Results of such analysis are given in Table II where it is seen that no linkage is ever observed between a given colicinogenic factor and genetic markers distributed along the bacterial chromosome. Colicinogeny is found in almost the same proportions among recombinants for proximal or for distal markers. There is, however, a tendency to find more colicinogens among recombinants for distal markers than among recombinants for proximal ones, and whatever the polarity of the Hfrs used. This is interpreted to mean that the longer the duration of conjugation, the higher the probability for a colicinogenic factor to be transferred.

EFFECT OF COLICINOGENY ON THE FORMATION OF GENETIC RECOMBINANTS

In general the colicinogenic character of either the donor or the recipient or both has no effect on the frequency of recombinant formation or on the genetic constitution of the recombinants. This has been generally the case for crosses involving colicins E_1, E_2, I or V. The formation of recombinants is closely similar to that observed in control crosses involving non-colicinogenic strains, provided, needless to say, that in crosses involving one colicinogenic strain, both parents are resistant to the colicin under study.

The situation is completely different for crosses involving colicin B as already observed by Frédéricq and Betz-Bareau (1956). Whereas the crosses between non-colicinogenic males, F^+ or Hfr, and colicinogenic females, yield as many recombinants as the control crosses between non-colicinogenic bacteria, the reciprocal crosses between colicinogenic males and non-colicinogenic females as well as the crosses between colicinogenic males and colicinogenic females exhibit a drastically reduced frequency of recombinants. The extensive results obtained by Puig (1963) are particularly striking. He has found that crosses involving an F^+ donor colicinogenic for B are completely sterile. Crosses where the Hfr donor is colicinogenic for B exhibit a frequency of recombinants reduced by a factor 10^3 to 10^4, although in such crosses the gradient of transmission of Hfr markers appears to be preserved.

This is not the place to elaborate on this phenomenon which is still under study by Puig. It is clear that the presence of colicinogenic factor B in the donor has no effect on the process of conjugation proper since colicinogenic factor B itself is transferred with high frequency from donor to recipient bacteria. It is not known, however, yet, whether the effect of colicinogeny B is on chromosomal transfer or on chromosomal integration.

SUMMARY AND CONCLUSIONS

The experiments summarized here show that in the five cases studied, i.e. colicinogeny E_1, E_2, I, V and B, bacteriocinogeny is under the control of genetic structures, i.e. the colicinogenic factors, which all are non-chromosomal. (1) Colicinogenic factors are transferred from male donors to female recipients but not from females to males, or from females to females. (2) Each colicinogenic factor is transferred with a certain efficiency, which differs widely for different colicinogenic factors but which, for a given colicinogenic factor, is within the same order of magnitude, whatever the type of male used. (3) Transfer of colicinogeny begins early, whatever the type of male, F^+ or Hfr, with different polarities. (4) Colicinogenic factors replicate faster than the bacterial chromosome. (5) The presence of a colicinogenic factor in either the male, the female or both is without effect on the different steps leading to the formation of genetic recombinants in the cases of colicinogeny E_1, E_2, V or I. This is not the case for colicinogeny B where the colicinogenic character of the donor greatly reduces the frequency of genetic recombinants.

If these facts clearly demonstrate the non-chromosomal location of colicinogenic factors, a conclusion also reached by Frédéricq (1963) and Clowes (1963), they do not solve all the problems raised by the inheritance and transferability of these genetic elements.

One problem is that of the location of colicinogenic factors. It seems unikely that colicinogenic factors are randomly distributed in the cytoplasm. The high frequency of transfer of some of them, and the early time after the onset of conjugation at which they start penetrating into the recipient, speak against such a hypothesis. A similar problem is raised by the behaviour of the sex factor of donor F^+ bacteria (Wollman and Jacob 1958). In this case, it may be assumed that the sex factor resides at certain specific sites of the bacterium, perhaps at the cytoplasmic membrane, in the vicinity of sex-determined receptors of the bacterial surface. Conjugation occurring at these sites, the sex factor would be transferred preferentially (Jacob et al. 1963).

The problem of the location of colicinogenic factors is intimately linked to the problem of their transferability. It could be assumed that colicinogenic factors are also located at the bacterial surface and also connected to certain surface receptor sites. The immunity of colicinogenic bacteria to the colicin they are able to produce might perhaps result from specific modifications of the surface of the bacterium. To account for the differences in the frequencies of transfer of different colicinogenic factors, the hypothesis could be made that the closer the location of a colicinogenic factor to a male receptor site, the greater the frequency of its transfer. Colicinogenic factors generally located at sites remote from the site of location of the sex factor would have only very low probabilities of passing through the conjugation bridge.

The fact, however, that colicinogenic factors, whatever the frequency of their transfer from males to females, do not detectably pass from females to males (or from females to females) calls for additional hypotheses. Obviously, and this is just restating the facts, the presence of a sex factor in a bacterium confers a 'polarity', direct or indirect, to other genetic elements present in the male cells. In the present status of our knowledge, it seems however more useful to state the problem than to propose precise models.

A problem related to the one just mentioned is that posed by the behaviour of colicinogenic factor B. Here the presence of the sex factor in the male results in a high frequency of conjugation and a high frequency of transfer of colicinogenic factor B. Conversely, however, the presence of colicinogenic factor B reduces drastically the transmission of chromosomal markers. Frédéricq has found a relationship between colicinogenic factor B and the sex factor (1963). Such linkage between these genetic elements has not hitherto appeared in the system investigated by Puig (1963).

It may be concluded from this brief discussion that if a few, well-established facts have arisen from the comparative study of the genetic behaviour of colicinogenic factors, many more problems concerning these genetic elements remain to be investigated.

REFERENCES

Alföldi, L., Jacob, F. and Wollman, E. L. (1957) *C. R. Acad. Sci. 244*, 2974

Alföldi, L., Jacob, F., Wollman, E. L. and Mazé, R. (1958) *C. R. Acad. Sci. 246*, 3531

Clowes, R. C. (1963) *Gen. Res. 4*, 162

Frédéricq, P. (1948) *Rev. belge Path. 19*, Suppl. 4

Frédéricq, P. (1953) *Ann. Inst. Pasteur 84*, 294

Frédéricq, P. (1954) *C. R. Soc. Biol. (Paris) 148*, 399

Frédéricq, P. (1963) *J. theor. Biol. 4*, 159

Frédéricq, P. and Betz-Bareau, M. (1956) *C. R. Soc. Biol. (Paris) 150*, 615

Jacob, F., Brenner, S. and Cuzin, F. (1963) *Cold Spr. Harb. Symp. quant. Biol.* (in press)

Jacob, F., Siminovitch, L. and Wollman, E. L. (1953) *Ann. Inst. Pasteur 83*, 295

Jacob, F. and Wollman, E. L. (1957) in *The Chemical Basis of Heredity*, Johns Hopkins Press, Baltimore, p. 468

Jacob, F. and Wollman, E. L. (1958) *C. R. Acad. Sci. (Paris) 247*, 154

Jacob, F. and Wollman, E. L. (1961) *Sexuality and the genetics of bacteria*, Academic Press, New York and London

Nagel de Zwaig, R. (1963) *Thesis*, Univ. of Buenos Aires

Nagel de Zwaig, R., Anton, D. N. and Puig, J. (1962) *J. gen. Microbiol. 29*, 473

Puig, J. (1963) *Thesis*, Univ. of Buenos Aires

Wollman, E. L. and Jacob, F. (1958) *C. R. Acad. Sci. (Paris) 247*, 536

Wollman, E. L. and Jacob, F. (1959) *La sexualité des bactéries*, Masson ed., Paris

Wollman, E. L., Jacob, F. and Hayes, W. (1956) *Cold Spr. Harb. Symp. quant. Biol. 21*, 141

DISCUSSION

CLOWES: With regard to the effect shown by the induction of col. B in repressing the fertility of donor K12 strains, is it known whether the col. B specific surface antigen masks the F male specific surface? It might be shown, perhaps, by a reduction in the ability of these strains to plate male specific phages. It can be that col. B, like RTF, but unlike col. I, interferes in some way with the F mating surface.

WOLLMAN: No tests have been made using this phage. However, col. B does not appear to be transferred between two F strains of K12.

WATANABE: We have studied the effect of colicinogenic factor B on the sensitivity of the host cells to male specific phages. We have found that the cells become resistant to these phages.

Symp. Biol. Hung. 6, 115–8 (1965)

BACTERIOCINOGENY IN *PSEUDOMONAS AERUGINOSA**

By

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Bacteriocinogeny and lysogeny in *Pseudomonas aeruginosa* have been found by Hamon et al. to be very widespread (Hamon 1956, Hamon et al. 1961). Those bacteriocins which were examined in detail were each found to show a different spectrum of action towards sensitive strains. The effect of ultraviolet irradiation on bacteriocin and phage production by 31 strains of *P. aeruginosa* of diverse origin has now been investigated, and the action spectrum of the bacteriocin produced by each strain determined against the other 30 strains. The pyocins have been divided into 4 main types on the basis of their action spectra, giving a classification system similar to that initially developed by Frédéricq (1948) for the colicins.

BACTERIAL STRAINS

Pseudomonas aeruginosa strains 1999, 2000, 5083, 5940, 6749, 6750, 6751, 7244, 7771, 8058, 8060, 8203, 8505 and 8506 were obtained from The National Collection of Type Cultures, strains 20, 21, 24/1, 24/2, 73, 117, 118, 119, 130 and 149 were kindly supplied by Dr M. Rhodes, strains C_4, C_6 and C_{10} by Dr L. Dickinson, and strains 1, 2, 3 and 29 by Dr B. Holloway.

EXPERIMENTAL

A 10 ml sample of a log phase tryptone broth culture of each strain ($E = 0.5$) was irradiated in an open petri dish rocked by hand for 30 sec, 1 metre below a low pressure mercury vapour lamp. The rates of growth of the irradiated culture and of an unirradiated control were followed in a nephelometer during a further 2 h incubation at 37°. Bacterial cells and debris were removed by centrifugation, and the supernatant fluids sterilized by heating to 55° for 30 min. Serial tenfold dilutions of the supernatants of control and irradiated cultures were assayed by placing standard drops on peptone agar plates seeded with one drop of an overnight culture of one of the indicator strains. Examination of the morphology of the inhibition zones appearing after overnight incubation indicated the presence of pyocin and/or phage in the supernatant fluids, and gave a quantitative measure

* This work was supported by a grant from the Agricultural Research Council, and forms part of a Ph. D. Thesis to be submitted to the University of Sheffield.

of the production of each by the irradiated cultures compared with the controls. Where the quantity of phage present was sufficient to give confluent lysis at high dilutions, thus obscuring a possible pyocin reaction, samples were irradiated strongly (10 min at 25 cm below the lamp) to inactivate the phage, and reassayed.

In addition to the plate assay method, all strains were tested by the perpendicular streak method of Abbott and Shannon (1958) and by the two-layer technique of Gratia (1947). Pyocin reactions were scored positive only if at least one of these methods showed a positive reaction, in addition to the plate assay. Non-specific inhibition by low molecular weight catabolic products was excluded by assaying dialysed samples of the supernatants of irradiated cultures: the antibacterial activities of dialysed and of non-dialysed samples were the same. In samples treated in a 1 KV ultrasonic disintegrator for 5 min, antibacterial activities were negligible.

RESULTS AND CONCLUSIONS

Although the optimum UV dose for each strain was not determined, and it was evident that the UV sensitivity of different strains varied, all irradiated cultures showed a diminished growth rate as compared with the non-irradiated control cultures, and most irradiated cultures lysed 60 to 90 min

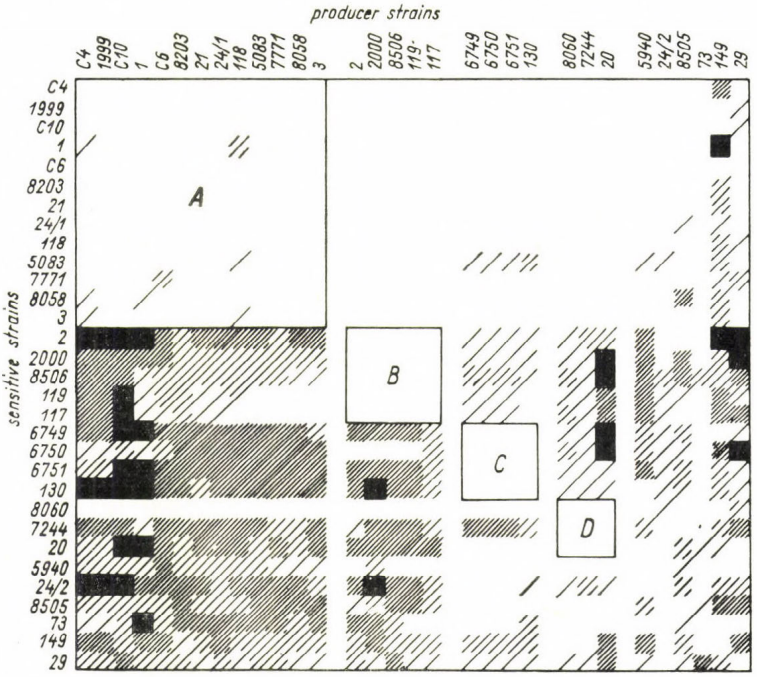

FIG. 1. Sensitivity of each strain to the pyocin from each producer strain. The symbols indicate the greatest dilution of the supernatant from an irradiated culture of the producer giving a discernible inhibition zone. / = 10^{-1}, // = 10^{-2}, /// = 10^{-3}, ■ = 10^{-4} or greater

after irradiation. Every strain, however, showed a 10 to 100fold increase in the level of pyocin in the irradiated culture, as compared with the control, so that all the pyocins appeared to be UV inducible.

While few of the pyocins showed identical action spectra, it was found possible to group them into four main types on the basis of both sensitivity and resistance of the indicator strains. No strain was sensitive to its own pyocin, and in consequence those strains producing similar pyocins should show cross-resistance to one another. In Fig. 1, areas of cross-resistance within each group have been enclosed in squares for clarity, and the pyocin type of each group designated by a capital letter. Cross-resistance in Group A is not complete, and indeed the pyocins which have been placed in this group show rather wide quantitative differences in their spectra, decreasing activity against Group B strains correlating approximately with increasing activity against Group C strains. However, it seems difficult to justify subdivision of this group on the basis of action spectra alone. Groups B, C and D show complete group cross-resistance, but small heterogeneities in spectrum. Six strains could not be fitted into any group, and may either be multiple pyocinogenic, or carry pyocins of a rare type. It has been pointed out by Frédéricq (1953) that the specific resistance of selected bacteriocin-resistant mutants gives a better foundation than sensitivity on which to base a

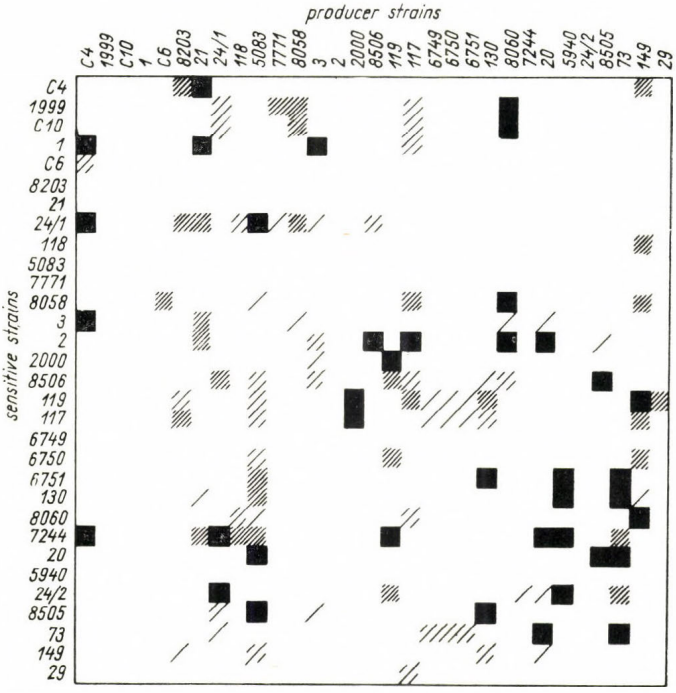

FIG. 2.—The sensitivity of each strain to the phages from each lysogenic strain. The symbols indicate the greatest dilution of the supernatant from an irradiated culture of the producer giving plaques in the area of the assay drop. / = 10^{-1}, // = 10^{-2}, /// = 10^{-3}, ■ = 10^{-4} or greater

bacteriocin-grouping system. Attempts to produce pyocin-resistant mutants have so far been unsuccessful.

Five strains were not found to be lysogenic. The action spectra of the phages produced were very much narrower than those of the pyocins (Fig. 2), and did not show similar groupings, except in the case of three strains producing Type C pyocins, whose phages also showed identical action spectra.

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I am indebted to Dr B. A. Fry for many helpful criticisms and suggestions during the course of this work.

REFERENCES

Abbott, J. D. and Shannon, R. (1958) *J. clin. Path. 11*, 71
Frédéricq, P. (1948) *Rev. belge Path. 19*, Suppl. *4*, 1
Frédéricq, P. (1953) *Bull. Acad. roy. Méd. Belg. 18*, 126
Gratia, A. (1947) *Bull. Soc. Chim. biol. (Paris) 29*, 352
Hamon, Y. (1956) *Ann. Inst. Pasteur 91*, 82
Hamon, Y., Véron, M. and Péron, Y. (1961) *Ann. Inst. Pasteur 101*, 738

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A PROPOS DES BACTÉRIOCINES PRODUITES PAR *KLEBSIELLA PNEUMONIAE* ET *AEROBACTER AEROGENES*

par

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Poursuivant nos recherches sur le pouvoir bactériocinogène des microbes de la tribu des Klebsiella (Hamon, Y. et Péron, Y. 1961, 1962, 1963), nous avons étudié les bactériocines produites par les souches appartenant aux genres Aerobacter (33 souches) et Klebsiella (112 souches parmi lesquelles figurent 96 souches de *K. pneumoniae*, 8 de *K. oxytoca*, 6 de *K. ozenae* et 2 de *K. rhinoscleromatis)*; 35 p. 100 des cultures de Klebsiella et 75 p. 100 des cultures d'Aerobacter aerogenes élaborent des bactériocines qui agissent sur les Klebsiella, les Aerobacter et parfois sur certaines *Enterobacter cloacae*. Elles ne provoquent aucune inhibition des cultures révélatrices des autres familles de bactériocines, en particulier de celles qui permettent de mettre en évidence les colicines (souches B, K12S, 36 d'*E. coli*, Y6R de *Shigella paradysenteriae)*.

Ces bactériocines présentent donc une individualité certaine; elles se différencient notamment des autres entérobactériocines (colicines, cloacines, marcescines et carotovoricines).

SPECTRE D'ACTIVITÉ DE CES BACTÉRIOCINES

Bien que leur sensibilité soit généralement peu étendue, nous avons trouvé quelques souches révélatrices de Klebsiella qui sont sensibles à toutes (ou presque toutes) les bactériocines produites par les cultures de ce genre; au contraire, les souches révélatrices d'*Aerobacter aerogenes* sensibles à toutes les bactériocines de cette espèce sont communément rencontrées. Or, nous avons constaté que: (a) les bactériocines d'*Aerobacter aerogenes* sont fréquemment actives sur les cultures de Klebsiella et notamment sur les cultures révélatrices de *Klebsiella pneumoniae*, (b) les bactériocines des Klebsiella agissent rarement sur les cultures d'*Aerobacter aerogenes*, même sur les souches révélatrices de toutes les bactériocines produites par cette espèce.

Les constatations précédentes (a) et (b) permettent d'affirmer que: (1°) les bactériocines produites par les Klebsiella et celles qui sont élaborées par *A. aerogenes* présentent un certain degré de parenté, (2°) ces bactériocines sont, toutefois, différentes puisque les souches révélatrices d'*A. aerogenes* qui sont sensibles à toutes les bactériocines produites par cette espèce, ne permettent de mettre en évidence qu'un petit nombre de bactériocines de Klebsiella.

*Read by title.

La thermorésistance relativement plus élevée des bactériocines produites par *A. aerogenes* confirme cette différence: en effet, alors que celles-ci résistent habituellement à un chauffage à 60° C pendant 30 minutes, les bactériocines élaborées par les Klebsiella sont généralement détruites par ce traitement.

En nous inspirant de la nomenclature implicitement admise pour désigner les nouvelles familles de bactériocines, nous proposons d'appeler *pneumocines* les bactériocines produites par les diverses espèces du genre Klebsiella et de nommer *aérocines* celles élaborées par *Aerobacter aerogenes*.

INTÉRÊT TAXONOMIQUE DE CETTE ÉTUDE

Les bactéries appartenant aux genres Aerobacter et Klebsiella synthétisent donc des bactériocines qui forment deux familles distinctes. *Aerobacter aerogenes* ne peut donc pas être considéré comme un type de Klebsiella; toutefois, l'action fréquente des aérocines sur les Klebsiella et l'action non exceptionnelle des pneumocines sur les Aerobacter indiquent la parenté de ces deux genres. En outre, l'étude des bactériocines d'*A. aerogenes* confirme les études biochimiques d'Hormaeche et Edwards (1960) qui rapprochent *A. aerogenes* d'*Enterobacter cloacae:* en effet, alors que les pneumocines n'agissent qu'exceptionnellement sur Enterobacter cloacae, les aérocines sont assez fréquemment actives sur ces cultures. Il est donc possible qu'*A. aerogenes* fasse la transition entre les Klebsiella et les Enterobacter.

Les bactériocines produites par les Enterobacteriaceae ou entérobacteriocines, comprennent donc actuellement, en dehors des colicines, les 5 familles suivantes: pneumocines, aérocines, cloacines (Hamon et Péron 1963), marcescines (Hamon et Péron (1961) et carotovoricines (Hamon et Péron 1962).

BIBLIOGRAPHIE

Hamon, Y. et Péron, Y. (1961) *Ann. Inst. Pasteur 100*, 818
Hamon, Y. et Péron, Y. (1962) *C. R. Acad. Sci. 254*, 2868
Hamon, Y. et Péron, Y. (1963) *Ann. Inst. Pasteur 107*, 127
Hormaeche, E. et Edwards, P. R. (1960) *Int. Bull. bact. Nomencl. 10*, 71

SECTION III

DNA SPECIFICITY AND REGULATION

Symp. Biol. Hung. 6, 123–30 (1965)

REGULATION OF RNA SYNTHESIS IN BACTERIA*

By

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More than 80 per cent of the ribonucleic acid (RNA) of *Escherichia coli* is represented by ribosomal RNA molecules, the nucleic acid moiety of the ribosomes that are the site of protein synthesis in the cell, and most of the remainder of the RNA is accounted for by the transfer RNA molecules that function as amino acid 'adaptors' in protein synthesis. Finally, a quantitatively minor, albeit qualitatively most important, fraction of the RNA is represented by the ephemeral *messenger* RNA molecules that harbor the sequence information for the ordered copolymerization of amino acids into specific polypeptides (Jacob and Monod 1961). In this discussion, I should like to summarize briefly some recent ideas that lead to the notion that, besides being the amino acid adaptor, and thus the agency that 'knows' the genetic code, the transfer RNA may also play a role in the regulation of bacterial biosynthesis, in particular, that the transfer RNA may intervene in the general regulation of the synthesis of RNA.

AMINO ACID CONTROL OF RNA SYNTHESIS

There exist numerous observations that indicate that the availability to the bacterium of amino acids governs its differential rate of RNA synthesis. Since most of the experiments relevant to this regulation of RNA synthesis were concerned with measurements of *total* RNA, I shall not attempt to distinguish here between the possibly separate control of synthesis of ribosomal, transfer and messenger RNA. One set of such observations pertains to the *upshift* and *downshift* experiments (Kjeldgaard et al. 1958, Schaechter et al. 1958, Neidhardt and Magasanik 1960). These experiments show that there ensues an immediate acceleration in the synthesis of RNA upon transfer of a bacterial culture from a minimal medium to an amino acid-supplemented one (upshift); this increase in the rate of RNA synthesis occurs well before any later acceleration of other biosynthetic functions becomes manifest (reflected in such properties of the culture as its turbidity, viable cell count, or DNA content). The time lag between the accelerations of the syntheses of RNA and of other cell constituents accounts for the higher relative RNA content of bacteria in an amino acid-supplemented medium than in a minimal one (Maaløe 1960). Conversely, there ensues an immediate halt of RNA

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synthesis upon transfer of a culture from an amino acid-supplemented medium to a minimal one (downshift). The complete halt in RNA synthesis persists after other biosynthetic functions again proceed at the lower rate characteristic of the minimal medium and RNA synthesis finally resumes only after the specific RNA content of the post-downshift culture has fallen to the pre-upshift level.

A second set of experiments pertains to RNA synthesis in amino acid-requiring bacterial auxotrophs. Such auxotrophs stop synthesizing not only protein but also RNA as soon as they are deprived of an exogenous source of the required amino acid. However, if synthesis of protein is inhibited, e.g., by an addition of the antibiotic chloramphenicol to the culture, then a very small concentration of the required amino acid — far below that otherwise necessary for optimal growth—suffices to promote RNA synthesis at a nearly maximal rate (Gros and Gros 1956, 1958, Pardee and Prestidge 1956, Aronson and Spiegelman 1958). That is, the requirement for amino acids in RNA synthesis appears to be *catalytic*, rather than stoichiometric. This catalytic role of amino acids is suggested also by the finding that addition of chloramphenicol to a culture of non-auxotrophic bacteria growing in a minimal medium engenders a temporary acceleration of RNA synthesis. Here the antibiotic appears to prevent the neutralization of free amino acids liberated by the spontaneous breakdown of bacterial proteins and thus to allow the build-up of an intracellular amino acid pool, mimicking the conditions of 'upshift' (Kurland and Maaløe 1962).

The catalytic intervention of amino acids in the control of bacterial RNA synthesis allows the bacterium to adjust its steady state level of RNA in accordance with the nutritional limitations imposed on the over-all rate of protein synthesis: as long as all of the 20 standard amino acids are in abundant supply, the cell can utilize the maximum amount of ribosomal and messenger RNA molecules in order to effect protein synthesis at the maximum rate; as soon as the supply of any one amino acid falls to a suboptimal level, protein synthesis must necessarily decelerate and render supernumerary some of the cellular RNA. It is possible to understand the nature of this regulatory process by supposing that within the bacterium there exists a set of 20 *inhibitors* of RNA synthesis, each inhibitor species subject to neutralization by one particular kind of amino acid. Hence, the lower the intracellular concentration of amino acids, the higher the effective inhibitor concentration, and hence, the smaller the rate of RNA synthesis. Furthermore, as soon as the concentration of any one amino acid falls to the very low level that obtains in an auxotroph starved for its growth factor, the effective concentration of the inhibitor homologous to that amino acid rises high enough to cut off RNA synthesis altogether.

The set of 20 or more transfer RNA species, each of which is known to enter into combination with only one cognate amino acid (Berg and Ofengand 1958) commended itself as a likely possibility for the postulated inhibitors of RNA synthesis (Stent and Brenner 1961, Kurland and Maaløe 1962). Thus, amino acid-free transfer RNA molecules would inhibit RNA synthesis, whereas enzymatic transfer of the cognate amino acid to the ribose moiety of the terminal adenine nucleotide of the homologous transfer RNA molecule would abolish the inhibitory effect. The hypothesis of the transfer RNA molecules as regulators of RNA synthesis subsequently de-

rived support from the finding (Tissières et al. 1963) that the *in vitro* action of the DNA-dependent RNA polymerase—the enzyme presumed to be responsible for the *in vivo* synthesis of bacterial messenger RNA—is inhibited by amino acid-free transfer RNA and that this inhibition can be relieved by charging the transfer RNA molecules with amino acids. Thus, if the DNA-dependent RNA polymerase is responsible not only for the synthesis of messenger but also for the synthesis of the total bacterial RNA, as would be the case if the bacterial DNA really provides the template for all species of RNA polynucleotides, then the amino acid control of RNA synthesis could be understood in terms of the inhibition of RNA polymerase function by amino acid-free transfer RNA molecules.

RELAXED CONTROL AND THE CATHOLIC INDUCER

There exists, however, an exception to this general finding of *stringent* amino acid control of bacterial RNA synthesis, namely the behavior of the methionine-requiring mutant 58–161 of *E. coli* K12 (Borek et al. 1955a, Borek et al. 1955b). Cultures of this mutant strain continue to synthesize RNA even after having been deprived of all exogenous methionine. During methionine starvation, the bacteria lose more than double their relative content of both ribosomal as well as transfer RNA (Mandel and Borek 1962, Neidhardt 1963) and, without having lost the ability to give rise to colonies on nutrient agar, undergo a progressive physiological deterioration that delays the resumption of normal anabolic activities after restoration of methionine to the growth medium. The reason for the methionine-independent RNA synthesis in this strain is not that methionine, unlike other amino acids, does not happen to participate in the control of RNA synthesis, since methionine-auxotrophs of *E. coli* other than strain 58–161 do cease net synthesis of RNA as soon as methionine is removed from their growth medium. Subsequent experiments with auxotrophic derivatives of this strain requiring various amino acids, such as leucine, isoleucine, valine, threonine, arginine, histidine, lysine or tryptophan showed that in this exceptional strain RNA synthesis can proceed in the absence of not only methionine but also of other amino acids necessary for growth. That is, in strain 58–161 the normally stringent amino acid control of RNA synthesis is *relaxed*.

Genetic crosses involving conjugation between Hfr donor bacteria derived from strain 58–161 and F^- recipient bacteria, derived from other lines of *E. coli* K12, that manifest the normal, stringent control of RNA synthesis, showed that the property of relaxed amino acid control of RNA synthesis can be transferred from donor to recipient cell, and that it segregates among the recombinant bacteria produced. Hence there appears to exist an RNA control, or *RC*, genetic locus in *E. coli* which is involved in the regulation by amino acids of RNA synthesis, and which can exist in two allelic states, *stringent*, or RC^{st}, and *relaxed*, or RC^{rel} (Stent and Brenner 1961). Amino acid auxotrophs carrying the RC^{st} allele stop synthesizing not only protein but also RNA as soon as they are deprived of an exogenous source of their amino acid growth requirement, whereas auxotrophs carrying the RC^{rel} allele continue to synthesize RNA at a nearly normal rate for a considerable period after removal of the required amino acids from the growth medium.

A more detailed study of the segregation pattern of complementary *RC* genotypes among the recombinant progeny of such crosses showed that the *RC* gene is situated on the *E. coli* chromosome between the loci responsible for resistance to streptomycin (str-r) and for synthesis of serine (*ser/gly*) (Alföldi et al. 1962).

Study of the physiology of growth of RC^{rel} bacteria showed that their synthesis of RNA appears to be under normal control under most conditions of balanced or unbalanced growth. Thus, it is true for RC^{rel} strains, as it is for the normal RC^{st} strains, that the richer the growth medium, and hence the faster the rate of bacterial growth, the greater the differential rate of RNA synthesis, and hence the greater the relative cellular content of RNA. Furthermore, it is true for RC^{rel} strains, as it is for RC^{st} strains, that upon downshift of growing cultures into media of nutritionally inferior carbon and energy or nitrogen sources, there ensues an immediate reduction in the differential rate of RNA synthesis (Neidhardt and Eidlic 1963). However, a striking difference in the behavior of RC^{rel} bacteria is evident upon downshift from amino acid-supplemented media to minimal ones. Under these conditions, RC^{rel} bacteria *continue* rather than cease their synthesis of RNA and enter a long lag, lasting for many hours, before resuming growth in the minimal medium. Apparently, the unbalanced, post-downshift accumulation of RNA drives the RC^{rel} bacteria into a physiological impasse in which the synthesis of previously repressed enzymes necessary for the resumption of growth has become very difficult. Indeed, colony assay on minimal glucose agar of RC^{rel} bacteria growing rapidly in amino acid-supplemented medium reveals that only about 20% of the cells thus subjected to downshift are ultimately able to escape from this impasse. *Leucine* greatly aggravates the deleterious effects of downshift transfer for RC^{rel} genotypes, since the presence of leucine in the minimal glucose agar reduces to 0.1% the fraction of cells capable of ever resuming growth, i.e. of forming a colony on the minimal agar (Alföldi et al. 1963). Thus the regulatory aberration of RC^{rel} bacteria becomes manifest only upon starvation of the bacteria for *one or a few required amino acids*. It is under this special metabolic restriction that the normal control of RNA synthesis shown by RC^{st} genotypes breaks down in RC^{rel} genotypes.

Since the behavior of RC^{rel} bacteria is quite normal under most conditions of balanced and unbalanced growth, it follows that their RNA synthesis is not to be considered amino acid-independent, or 'constitutive'. Rather, their behavior suggests that the regulatory aberration of RC^{rel} bacteria derives from a *breakdown of the specificity* of the neutralization reaction between the amino acids and the postulated inhibitors of RNA synthesis. That is, one may suppose that, in contrast to normal RC^{st} genotypes, in which each inhibitor species can be neutralized by only one of the standard amino acids, in RC^{rel} bacteria each inhibitor species can be neutralized not only by its cognate amino acid but also by a *catholic inducer*. This catholic inducer might be a metabolite that bears some structural or metabolic relation to amino acids, or might in fact, be *itself* an amino acid. In RC^{rel} bacteria, just as in RC^{st} bacteria, RNA synthesis would then proceed at a reduced rate as long as nutritional limitations of the growth medium render many amino acids in short intracellular supply. These same nutritional limitations would cause the postulated catholic inducer also to be in short

supply, and its intracellular concentration to be insufficient for the neutralization of an appreciable fraction of the amino acid-free inhibitor molecules. However, if RC^{rel} bacteria are starved for only one, or a very few required amino acids, then the intracellular concentration of the catholic inducer would suffice to neutralize enough inhibitor molecules cognate to the few missing amino acids to allow maintenance of RNA synthesis. Leucine would be thought to bear a metabolic relation to the catholic inducer, so that its toxic effect during downshift transfer from amino acid-supplemented medium to minimal one would be explained by an increase in the internal concentration of the inducer and hence by an overstimulation of the pernicious post-downshift RNA synthesis.

Three alternative roles can now be envisaged for the *RC* locus. (1) *RC* might govern the structure of the inhibitor molecules; here, the RC^{rel} allele would produce a structural modification of all inhibitor species, so that they would accept the catholic inducer. (2) *RC* might govern the synthesis of one or more enzymes that catalyze the combination of the amino acids with the cognate inhibitor; here, the RC^{rel} allele would produce a modified enzymatic ensemble that could combine the catholic inducer with all inhibitor species. (3) *RC* might govern the synthesis of the catholic inducer; here the RC^{rel} allele would produce an increase in the intracellular concentration of the catholic inducer. At present I favor the last of these three hypotheses.

Experiments have been carried out to test directly whether, provided that the transfer RNA molecules are really the inhibitors of RNA synthesis, one of the 20 standard amino acids is the postulated catholic inducer (Martin et al. 1963). For this purpose, transfer RNA molecules extracted from RC^{rel} bacteria were charged with one or more of the 20 standard amino acids by the use of amino acid-activating enzymes isolated from the same bacteria. The charged transfer RNA was oxidized with periodate, and then reisolated and retested for its residual capacity to accept an amino acid that was absent from the preliminary charging mixture. If preliminary charging transferred an amino acid to a non-cognate transfer RNA species belonging to an absent amino acid, then the acceptor capacity for the missing amino acid would survive periodate oxidation and reveal its presence on recharging with that amino acid after post-periodate reisolation of the transfer RNA (Berg et al. 1962). The results of these experiments showed that there does *not* appear to exist any major breakdown in amino acid transfer specificity in RC^{rel} bacteria: preliminary charging of the transfer RNA from RC^{rel} bacteria with 19 of the 20 standard amino acids does not afford protection against periodate oxidation for any appreciable fraction of the transfer RNA molecules cognate to the absent 20th amino acid. It is unlikely, therefore, that the catholic inducer is one of the 20 standard amino acids. If, as is of course possible despite the suggestive evidence, transfer RNA is not, after all, the inhibitor of RNA synthesis, and amino acids exert their regulatory role by combining with, and neutralizing, an entirely different class of inhibitor molecules, then the present experiments are not relevant to the nature of the catholic inducer. But if one wishes to retain both notions of the regulatory role of transfer RNA and of the presence of a catholic inducer in RC^{rel} strains, one is led to infer that the catholic inducer may be a nonstandard amino acid, or simply an organic acid, that combines aspecifically

with the terminal adenosine residues of amino acid-free transfer RNA molecules and to neutralize their inhibitory effect on the RNA polymerase. Accordingly, we are now attempting to ascertain whether, as predicted by this idea, transfer RNA molecules extracted from amino acid-starved RC^{rel} bacteria are, in fact, combined with a substance that is not their cognate amino acid.

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It should be evident from this discussion that none of the main hypotheses presented here concerning the mechanism of amino acid control of RNA synthesis, plausible as they may appear, can as yet lay claim to very much *direct* experimental support. In that respect, however, they may not be at a very serious disadvantage *vis à vis* some other theories designed to render account of various regulatory phenomena. Furthermore, these hypotheses would seem to have at least the virtue of suggesting further experiments that should be capable of testing their validity, experiments that we are presently endeavoring to carry out.

REFERENCES

Alföldi, L., Stent, G. S. and Clowes, R. C. (1962) *J. molec. Biol. 5*, 348
Alföldi, L., Stent, G. S., Hoogs, M. and Hill, R. (1963) *Z. Vererbungsl.* (in press)
Aronson, A. I. and Spiegelman, S. (1958) *Biochim. biophys. Acta (Amst.) 29*, 214
Berg, P., Lagerkvist, V. and Dieckmann, M. (1962) *J. molec. Biol. 5*, 159
Berg, P. and Ofengand, E. J. (1958) *Proc. nat. Acad. Sci. (Wash.) 44*, 78
Borek, E., Rockenbach, J. and Ryan, A. (1955a) *J. Bact. 71*, 318
Borek, E., Ryan, A. and Rockenbach, J (1955b) *J. Bact. 69*, 460
Gros, F. and Gros, F. Mrs (1956) *Biochim. biophys. Acta 22*, 200
Gros, F. and Gros, F.Mrs (1958) *Exptl. Cell Res. 14*, 104
Kjeldgaard, N. O., Maaløe, O. and Schaechter, M. O. (1958) *J. gen. Microbiol. 19*, 607
Kurland, C. G. and Maaløe, O. (1962) *J. molec. Biol. 4*, 193
Jacob, F. and Monod, J. (1961) *J. molec. Biol. 3*, 318
Maaløe, O. (1960) *Symp. Soc. gen. Microbiol. 10*, 272
Mandel, L. R. and Borek, E. (1962) *Biochem. biophys. Res. Commun. 9*, 11
Martin, E. M., Yegian, C. and Stent, G. S. (1963) *Z. Vererbungsl.* (in press)
Neidhardt, F. C. (1963) *Biochim. biophys. Acta 68*, 365.
Neidhardt, F. C. and Eidlic, L. (1963) *Biochim. biophys. Acta 68*, 380
Neidhardt, F. C. and Magasanik, B. (1960) *Biochim. biophys. Acta 42*, 99
Pardee, A. B. and Prestidge, L. (1956) *J. Bact. 71*, 677
Schaechter, M. O., Maaløe, O. and Kjeldgaard, N. O. (1958) *J. gen. Microbiol. 19*, 529
Stent, G. S. and Brenner, S. (1961) *Proc. nat. Acad. Sci. (Wash.) 47*, 2005
Tissières, A., Bourgeois, S. and Gros, F. (1963) *J. molec. Biol. 7*, 100

DISCUSSION

SZYBALSKI: Do you plan to propose this as an alternative hypothesis or it is an accessory hypothesis? I ask this because there are some experiments which show that the control might be on the first level of the synthesis of messenger RNA.

STENT: Yes, of course, there are experiments, those of Attardi, which show that the rate of messenger synthesis is in some way connected with the rate of enzyme synthesis. Probably, it is necessary to imagine that the removal of the messenger is connected with its reading. When the modulating sRNAs are not available, the messenger cannot be removed from the DNA. Therefore, the rate of messenger synthesis would depend on whether, or not, the protein is made. I am prepared to defend this hypothesis and to show you that it is in fact no more complicated, than the assumptions of Jacob and Monod, as for each you can find the explanation of the whole equipment. That is, this hypothesis is only completing in essence the ideas of Jacob and Monod. The repressor, which hypothetically combines with the DNA, can be an enzyme, which does a specific distracting action on the modulating sRNA. That is about the equipment of the repressor. The equipment of the operator would be a separated assumption. The operator is possibly some piece of DNA for which the repressor has affinity. And for that we substitute the idea that the messenger does not come off, that the DNA does not let being read, which, I think, is no worse an assumption.

STAHL: Do you think there are enough triplets to the modulation?

STENT: No, there are no triplets, the modulating sRNA has no triplets.

STAHL: Oh, I beg your pardon, there are no triplets on the operon.

STENT: No, the modulation must not play on triplets, but must play probably on sextuplets or something else. Again, of course, that is also no worse an assumption, because there is the code of the beginning and there is the code for the end, and so forth. We would say that the code of beginning is in fact at the first series. You have a sequence of six nucleotides at the beginning, two of which represent the whole equipment, followed by four which give the variety for modulation.

STAHL: Considering the polarity mutation, what is the answer for proper objection?

STENT: I think there is an answer for proper objection. You would ask now, how we explain polarity mutants? They have, and I think that is the question, triplets, probably sextuplets, which would be the same. You have two triplets now in the middle of the molecule. If you make a change next just in a mononucleotide, you would change this to the modulating type of sextuplets and you would now concern the sRNA at this place, making polypeptide in fact not more different but probably by one amino acid, and so forth. I would be willing to state that the obvious objection can be answered. Theoretically, it is no more complicated than the one proposed before. I think it has the advantage that the repressor so far has proved elusive.

WOLLMAN: You must introduce then a new enzyme.

STENT: The enzyme is known, but what is not known is whether it acts specifically on different sRNAs. The enzymes are known, but what is not known is to what extent they persist, i.e. the degree of specificity which we wish to attribute to them. We can, however, search for it. We are not identifying unknown enzymes. We only attribute allosteric control to them.

SZYBALSKI: It is very nice, however, now to test, because you could look for these enzymes in temperature sensitive mutants. Because, if you have

temperature sensitive enzymes you should have to look for temperature sensitive nuclease, you should have substrate to work with.

STENT: I think the advantage of this series is that predictions are possible; for instance, one can predict that there must be several *I* loci, i.e. more than one locus, which control the so-called repressor-system, because one within them corresponds to the synthesis of the phosphorylase enzyme, and there would be a second which would correspond to the synthesis of modulating sRNA. There would be then two *I* loci, both of which were trans, and one would be dominant and one would be recessive.

CLOWES: It should perhaps also be pointed out that the genetic evidence presented for a specific operator region, is now less specific with the finding of polarity mutants, of which the 0^0 mutants are presumed to be a type occurring within the leading locus, and of constitutive mutants, which are now found in many parts of the complex locus (operon) and are not restricted to 0^0 mutants.

STENT: That is right. Of course, in an 0^0 mutant there is one which is now dominant constitutive, which will make the enzyme in the absence of the inducer; one in which the mutation takes place, which calls now for methionin, and one is its non-modulating representation in ordinary mutants, for which the methionin is available, whether the inducer is present or not. This is an 0^0 mutant. In 0^0 mutant there is one, which would be either nonsense or would call for methionin in molecular representation, which exists as a stimulator in the system. Concerning its function it should be an operator mutation. The operator, however, must first be demonstrated and not played with philosophically.

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RECOMBINATION IN BACTERIOPHAGE T4. HETEROZYGOSITY AND CIRCULARITY

By

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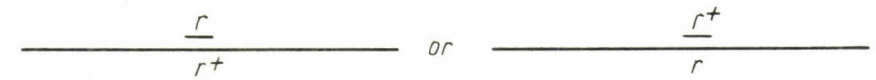
INTRODUCTION

The phenomena of bacterial transformation and of bacteriophage recombination have an interesting aspect in common; both phenomena imply an interaction between DNA molecules leading to the formation of genetically recombinant molecules. In this lecture I shall describe recent ideas and experiments in the genetic analysis of the recombination process in the coliphage T4. I shall leave it to this audience of transformers to judge the degree of applicability to their own work of what will be described here. The recent, unpublished experiments which I shall describe are primarily from the laboratory of George Streisinger at Eugene; a few are from my own lab. These experiments will be displayed in a theoretical framework proposed semi-publically several years ago by Streisinger. Let us turn now to a review of those experiments in phage genetics which have led Streisinger to his proposal.

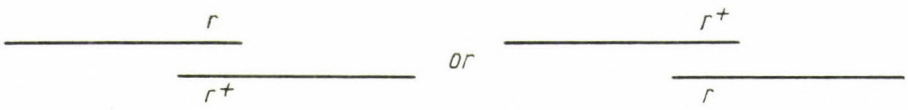
EARLY OBSERVATIONS ON HETEROZYGOSIS (1951—1960)

If a bacterium is infected by two hereditary types of phage, say T4*r* and T4r^+, the cell will produce both types of particles. About 1% of the particles produced are extraordinary. Upon replication these extraordinary particles produce offspring of both *r* and r^+ genotype and, in addition, an occasional extraordinary particle. Such particles were termed 'phage heterozygotes' (HETs) by Hershey and Chase (1951). They demonstrated that any one of many loci in the T-even phages can become heterozygous as a result of genetically mixed infection; as a rule, however, a particle heterozygous at one locus is also heterozygous at a second only if the second locus is closely linked to the first. It appears as if regions of heterozygosity are not only hereditarily unstable but are also rather short and that the number per phage particle is not very much greater than one. This viewpoint suggested two simple *formal* models for a HET. Model I is the sort of thing which a blackboard geneticist would draw if a fragment of DNA were matured along with a whole phage chromosome chosen 'willy-nilly' from the vegetative pool. (For the instance of heterozygosity for an *r* marker see Model I.)

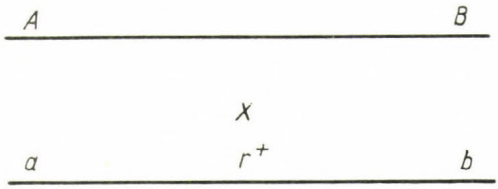
In Model II we suppose that heterozygosity results when large parts of chromosomes derived from two different individuals are combined into one mature particle. The models were distinguished by Levinthal (1954) from the results of a three-factor cross of the type (Model III).



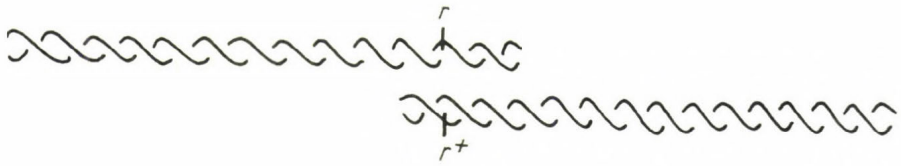
Model I



Model II



Model III



Model IV

Model V

The r/r^+ HETs resulting from the mixed infection were observed to be predominantly of genotypes Ab and aB arguing that a correct formal representation for most, if not all, HETs is given by Model II.

When Levinthal presented the results of his crosses he suggested two *molecular* models which might correspond to the formal Model II. Both of these models represented minor modifications of the then-recently-announced Watson-Crick structural model for DNA. In molecular HET-model A (see Model IV) heterozygosity is supposed to arise as a result of a region (or regions) of local 'diploidy' whose position is variable on the chromosome. An r/r^+ HET chromosome would 'look like' Model IV, i.e., two DNA molecules derived from genetically different phages are matured into the same particle. For reasons which will become clear later, we shall not inquire now into the nature of the forces which unite the two molecules to form a single chromosome.

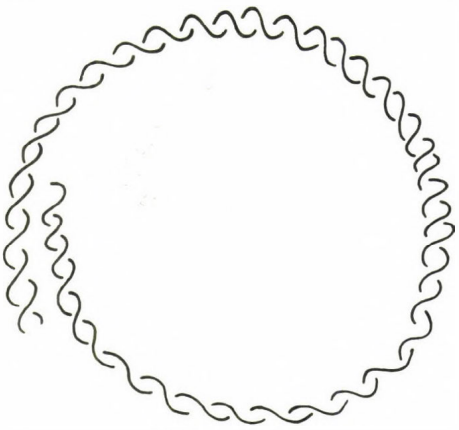
In molecular HET-model B (see Model V), a heterozygous chromosome has no region of structural redundancy, and its only genetic redundancy is that implied by the duplicity of the Watson and Crick DNA model. (The parts of the molecule derived from different parental phage chromosomes are differently shaded.)

Other molecular models for HETs have been proposed, but the two described above are outstanding with respect to both priority and simplicity. In addition, they are sufficiently distinct from each other to encourage experiments designed to choose between them. Experiments by Doermann and his collaborators (1960) succeeded in detecting HETs which have a behavior which might well be expected of those with a structure like Model A. However, those same experiments demonstrated HETs which behave as one would expect of those with a structure like Model B. The essence of Doermann's observation is the following. Two phage stocks differing by 6 or 8 closely linked markers were crossed and the multifactor HETs resulting were examined for the frequencies with which each of the markers appeared among the progeny of the HET. Some of the HETs gave rise to populations containing approximately equal numbers of each of the markers. Since DNA duplicates semi-conservatively, HETs with the structure of Model B should segregate in that fashion. Others of the HETs, however, showed strikingly different segregation patterns. For one of the outermost heterozygous loci, the frequencies of the two alleles among the offspring were usually grossly unequal. For the outermost one at the other end of the marked sequence of loci a similar inequality was often seen. However, in this case the allele in excess was derived from the parent which was poorly represented at the first heterozygous locus. Loci in between gave inequalities of intermediate magnitude with values varying monotonically from one end to the other. Such segregation behavior argues for the presence of structural singularities disposed *trans* to each other at each end of the heterozygous region. Model A has exactly that structural feature.

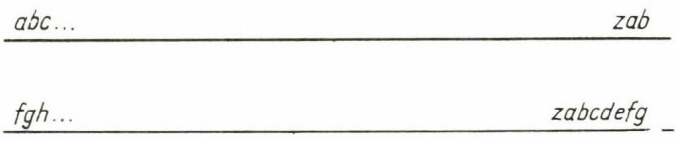
At the same Phage Meeting (Cold Spring Harbor, 1960) at which Doermann's associates presented genetic evidence for the existence of phage chromosomes with structures like that of Model A, Berns and Thomas (1961) presented physical evidence for their non-existence. They testified that their studies of whole T4 chromosomes showed a reduction in molecular weight by just a factor of two upon strand separation brought about by heating the DNA in formaldehyde. If their chromosome population contained many individuals with structures like that of Model A, the reduction in molecular weight could reasonably be expected to be greater than twofold. At this point Streisinger presented his model as a possible solution to this apparent paradox.

STREISINGER'S MODEL

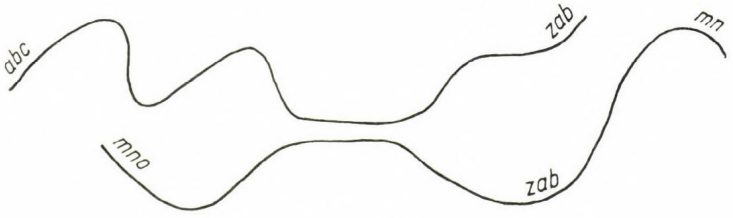
(a) *Genetic circularity*. Streisinger suggested that the apparent conflict between genetic and physical data regarding the existence of Model-A-like structures was resolved if one pictured a phage chromosome like Model VI. Since Model A HET structures can (presumably) occur at any genetic locus, we are led to the following picture of the chromosomes of a (mature) phage population. The genetic sequences from one chromosome to another are



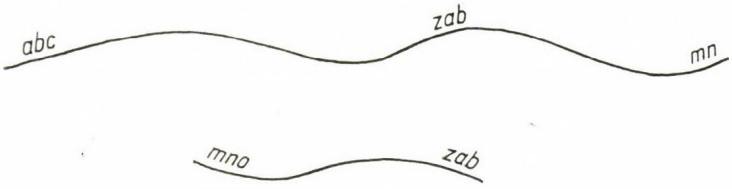
Model VI



Model VII



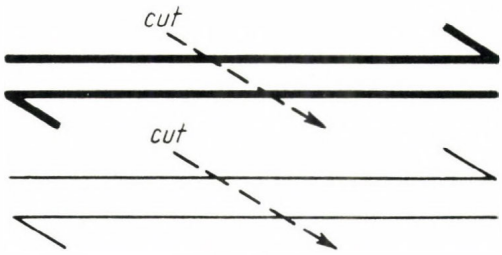
Model VIII



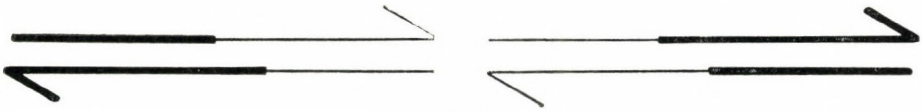
Model IX

circular permutations of each other. Each chromosome has a terminal repetition of its initial loci (an example is given in Model VII). The most obvious prediction of Model VII was tested promptly (Streisinger et al. 1961); upon close scrutiny the linkage map of T4 was indeed found to be circular!

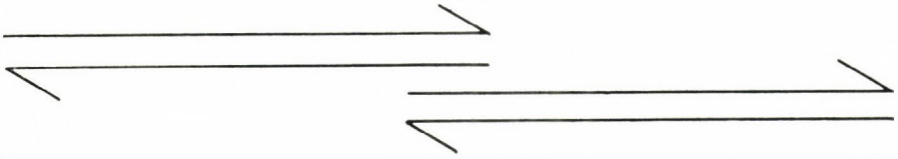
(b) *The mode of origin of* HETs. Emboldened by the initial success of the model, Streisinger resolutely examined its logical consequences. A 'mating'



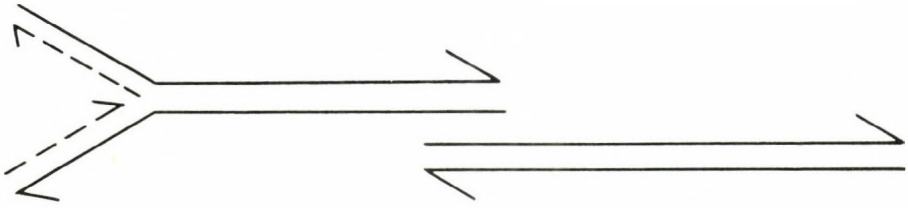
Model X



Model XI



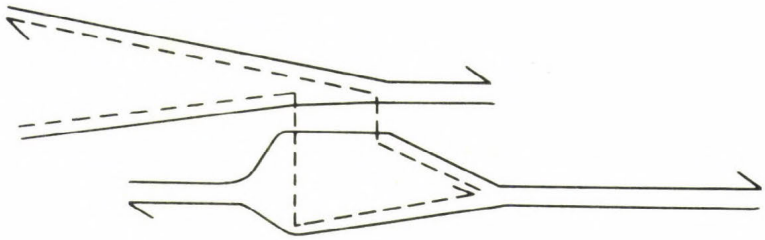
Model XII



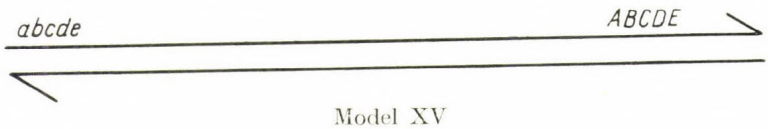
Model XIII

between two chromosomes which are circular permutations of each other may be pictured (on the blackboard) like Model VIII. Crossing-over in the synapsed region can lead to the formations in Model IX. Whatever the precise steps in the crossover process may be, the crossover product arises as if the two parental molecules were each cut on the bias and their parts rejoined (cf. Model X), giving rise to products shown in Model XI. *If the two mating chromosomes are genetically distinct in the synapsed region, then the recombinant products may be* HETs *of Type B.*

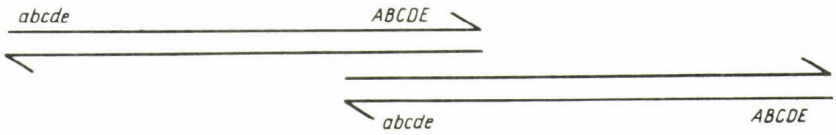
As is implied by the diagram of a 'mating' above, reiterated matings lead to the formation of giant 'chromosomes' in which the T4 genome is serially repeated. At some stage T4-sized lengths of DNA are removed from the giant polymers. It seems most reasonable that this step should occur hand-in-hand with phage maturation. At maturation, then, and perhaps



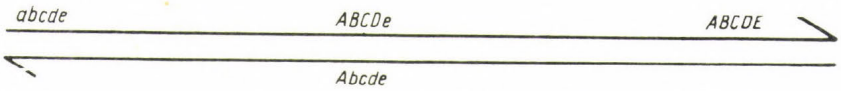
Model XIV



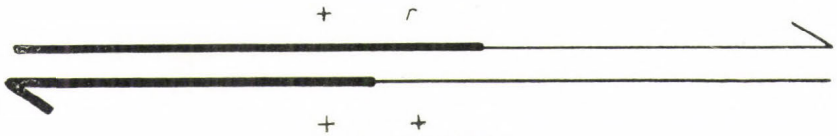
Model XV



Model XVI



Model XVII



Model XVIII

before, chromosomes with new permutations are created. *A matured chromosome may be a* HET *of Type A if it is cut from the polymer such that it is redundant for a marked locus.*

(c) *Segregation from* HETs. Polarized segregation from Type A HETs was originally explained by Doermann and his collaborators in terms of an 'internal copy-choice' scheme. The scheme is most conveniently presented by means of a diagram, which shows that a Type A structure (Model XII) duplicates from one end (Model XIII). The newly forming chains 'switch' in the redundant region, typically before reaching the end of the partial chromosome upon which they started. The two chains need not switch at exactly the same place (Model XIV). Upon completion of the duplication act, the chains segregate conservatively so that the two daughters are the

original Type A HET structure and an all-new chromosome, without chain interruptions, which may be a HET of Type B. Subsequent duplication acts of the Type A HET proceed in the same fashion except that the points of switching are variable from one act to another.

This scheme explains the polarized segregation from Type A HETS and the observation (Doermann and collaborators 1961) that Type B HETS arise rather frequently in cells infected by Type A HETS. While it provides an apparently simple explanation for the behavior of Type A HETS, the scheme does have two obviously unpleasant features. (1) It proposes a copy-choice mechanism of recombination at a time when other experiments, more critically addressed to mechanism, clearly indicate break-reunion (Meselson and Weigle 1961). (2) It proposes a conservative mode of DNA duplication although there is no evidence that DNA duplication ever proceeds in other than a semi-conservative fashion (Meselson and Weigle 1961, Kozinski and Kozinski 1963).

In Streisinger's model, polarized segregation from Type A HETS results from 'head-to-tail' matings between daughter particles. For instance, a multi-factor HET (Model XV) duplicates, then synapses like in Model XVI. Crossing-over in the synapsed region leads to plus fragments as shown, for instance, in Model XVII. Duplication of this dimer may be followed by a second head-to-tail mating, etc. The points of crossing-over in these additional rounds will not generally be at precisely the same places as those in the first. Polarized segregation will be the result. Two other features of incestuous head-to-tail matings may be noted here. (1) Type B HETS arise from Type A HETS by the same cross-over mechanism with which they form in 'ordinary' matings. (2) Since polymers are formed, it is possible that monomers with new permutations arise in singly infected cells. (We may add that crossing-over between the head and the tail of a single chromosome would give rise to a topologically circular chromosome.) Such events may happen (it is difficult to see what might prevent them, but they are not a feature of the model in its present form. At this point we must make a short aside to introduce the genetic material which has been used to test predictions of Streisinger's model.

POINT-MUTANT HETS AND DELETION HETS

Mutants in the *r*II region of T4 can be assigned to either of two categories having the properties according to Table I.

Nomura and Benzer (1961) determined the frequency of HETS from crosses of point mutants by wild type and deletions by wild type. Crosses of the first type all gave 1.4% mottled plaques HETS, while crosses of the second type gave 0.4% HETS. This latter value was independent of the extent of the region deleted in the mutant employed. On the basis of this observation Nomura and Benzer proposed the existence of two classes of HET-structures. One class which occurs with a frequency of 2%* can be heterozygous for a wild type and a point mutation but not for a deletion and wild type.

* At genetic equilibrium, the frequency of HET structures is twice the frequency of HETS themselves, i.e. homozygous HET structures are implied but undetected.

TABLE I

Category	Point-mutants	Deletions
Definitive properties	{ reversion rate $\geqslant 10^{-8}$ { map at a point	No detectable reversion Fail to recombine with each of two or more mutants which are recombinable with each other

Both categories of mutants, on the other hand, can form HETs (with wild type) having structures of the second class, which occurs with a frequency of 0.8%. It occurred to Streisinger that these two classes of HET structures might correspond to Type B and Type A structures. The nucleotide sequences of a wild type and a point mutant differ by only one base pair. The construction of a Type B HET from two such chains represents a minimal, and likely tolerable, violation of the base-pairing rules of Watson and Crick. The construction of such a 'heteroduplex' HET from wild type and a deletion mutant, however, may well represent a violation intolerable to all concerned. On the other hand, there seems to be no reason why both categories of mutants should not *equally* well form HETs of Type A ('terminal redundancy HETs').

EXPERIMENTAL TESTS OF STREISINGER'S EXPLANATION OF HETS

Hershey and Chase (1951) and Levinthal and Visconti (1953) reported that the frequency of HETs among phages maturing at different times is essentially constant. Sechaud et al. (1962) have observed that this statement is true separately for each of the two classes of HETs, i.e., deletion HETs have a constant frequency of 0.4%, while point-mutant HETs occur with a frequency of 1.4% independent of the time after infection at which mature phages are examined. Since at the time of infection neither type of HET exists (although HET *structures* exist in a homozygous state), the values observed among particles sampled from the mating pool may be assumed to result from equilibria between reactions that form and reactions that destroy HETs. Let us examine those reactions which, in Streisinger's model, determine the equilibrium values of Type A and B HETs. The frequency of Type A HETs will reach equilibrium when sufficient recombination has ensued that any chromosome-polymer is essentially randomized with respect to the allele present at each representation of the marked locus. Two representatives of a given locus are 'maximally unliked', and the number of matings at the time when mature phages first appear is several (Visconti and Delbrück 1953). Thus, the observed value of 0.4% equals ½ times the probability that a matured monomer will be terminally redundant for the marked locus. The equilibrium value for Type B HETs will depend upon the rate at which they are formed as a result of crossing-over in their immediate neighborhood and the rate at which they are destroyed as a result of (semi-conservative) duplication. Thus, the reaction which destroys Type A HETs (recombination) is different from the one which destroys Type B HETs (duplication). A prediction of this notion is that under condi-

tions which permit a normal rate of genetic recombination while depressing the rate of DNA duplication, the frequency of Type B HETs should rise while the frequency of Type A HETs will be unaffected. Deletions can be used to score the frequency of Type A HETs; Type B HETs can be studied using point mutants which form predominantly ($^2/_3$ under normal conditions) HETs of this type.

Flouro-deoxyruidine (FUdR) inhibits DNA synthesis by interfering with the formation of thymidilic acid. Genetic recombination in T4 is not inhibited by the analogue (Simon 1961, Frye, Sister Celeste and Melechen 1961). In complete agreement with prediction, Sechaud et al. (1962) found that they could increase the frequency of point-mutant HETs from 1.4% to about 8% by the treatment of infected cells with FUdR. This increase paralleled the increase in recombinant frequency under the same conditions. The frequency of deletion HETs, on the other hand, was not changed by FUdR.

Further substantiation for the differing roles of heteroduplex HETs and terminal redundancy HETs in recombination comes from studies of crosses between closely linked *r*II markers (Shalitin and Stahl 1963). Wild-type recombinants arising in crosses between *r*II mutants in the same cistron can be selected by adsorbing the progeny phage to *E. coli* strain K. Among those individuals which grow in K the 'pure wild-type' particles can be distinguished from the 'recombinant HETs' (Edgar 1958) by plating the infected K cells on strain B. Recombinant HETs give mottled plaques. In a cross between two point-mutant *r*'s near opposite ends of the B cistron (about 3% recombination), 16% of the wild-type recombinants were HETs. The value rose to 63% when the cross was performed in FUdR. This result is in fine accord with the notion that the primary product of recombination between close markers is usually a recombinant HET which looks like Model XVIII. Segregation of this HET by duplication to give 'pure wild-type' particles is blocked by FUdR. The same experiment performed with two small deletions near opposite ends of the B cistron gave, as expected, a different result. Only 6% of the wild-type recombinants are HETs, and this value does not change when the cross is performed in FUdR. The value of 6%, however, struck us as being immoderately high; it indicated a rather strong correlation between crossing-over and terminal redundancy and implied either that ends of T4 chromosomes engaged in crossing-over at a higher rate than did the other regions or that crossing-over determined the location of the end. On second thought, the idea of crossing-over near ends is seen to be consistent with the high rate of head-to-tail matings invoked earlier to account for polarized segregation of terminal redundancy HETs.

Streisinger's model proposes that a 'machine' (maturation?) which is blind to nucleotide sequence measures monomers of T4 out of the intracellular chromosome polymers. These monomers are of such a size that any one of them contains the complete T4 genome plus a small terminal repetition. Particles which are terminally repetitious for a marked locus can be spotted as deletion HETs. If a matured monomer is somewhere genetically deleted over a length about equal to or larger than that of a typical terminal redundancy, it should itself carry an abnormally long redundancy. Operationally, phages which carry large deletions in one region of their genome should show a higher-than-ordinary frequency of deletion heterozygosis in another region. To perform an experiment which tested this

prediction, Streisinger and his friends apparently needed *two* regions in which deletion mutants were available. The appropriate material was provided by their demonstration that the h^+_2 and h^+_4 alleles behave *as if* one of the two were a deletion; i.e., the frequency of h^+_2/h^+_4 HETs is low (somewhat less than 0.5%) and this frequency is not increased by FUdR. The prediction was tested then by two crosses of the type *r*-deletion $h^+_4 \times r^+ h^+_2$ (Streisinger et al. 1963). In one cross the *r*-deletion was very short, in the other very long. Particles HET for h^+_2 and h^+_4 were examined for the allele carried at the *r* locus. In the case of the cross involving the short *r*-deletion the h^+_2/h^+_4 HETs were equally *r* or r^+. In the case of the long deletion, however, the h^+_2/h^+_4 particles were more frequently *r* than r^+.* These results confirmed the results of another set of experiments in which the frequency of h^+_2/h^+_4 HETs arising in mixed infection was compared with that of another. In the first case both phages carried the same short *r*-deletion, in the second, the same long *r*-deletion. The frequency of h^+_2/h^+_4 HETs was observed to be higher in the second case than in the first one.

SUMMARY

Streisinger proposed a model for the T4 chromosome which resolved apparently contradictory genetic and physical evidence. This model, which is still being tested at Eugene as well as elsewhere, assumed that the chromosomes in a mature T4 population were circular permutations of each other and that each was terminally redundant. The model successfully predicted a circular map for T4 and, when elaborated in the most obvious manner, successfully predicted several outrageous properties of phage heterozygotes.

*

To Professor Streisinger and his friends, without whom I would have had no reason to address this audience, I am both indebted and grateful. Support from the National Science Foundation (U.S.A.) (Res. Grant GB-294) helped to make possible both attendance at this meeting and the preparation of this manuscript.

REFERENCES

Berns, K. I. and Thomas, C. A. jr. (1961) *J. molec. Biol. 3*, 289
Doermann, A. H. et al. (1960) personal communication
Doermann, A. H. et al. (1961) personal communication
Edgar, R. S. (1958) *Genetics 43*, 235
Frye, Sister Celeste and Melechen, N. E. (1961) personal communication
Hershey, A. D. and Chase, M. (1951) *Cold Spr. Harb. Symp. quant. Biol. 16*, 471
Kozinski, A. W. and Kozinski, P. B. (1963) *Virology 20*, 213

* In these as in some of the other experiments reported here preparative centrifugation in a sucrose gradient was used to remove a third class of HETs. These HETs are simultaneously heterozygous for distantly linked markers. The fact that they can be sedimented away from the primary population of particles suggests that they may be clumps or siamese particles.

Levinthal, C. (1954) *Genetics 39*, 169
Levinthal, C. and Visconti, N. (1953) *Genetics 38*, 500
Meselson, M. and Weigle, J. J. (1961) *Proc. nat. Acad. Sci. (Wash.) 47*, 857
Nomura, M. and Benzer, S. (1961) *J. molec. Biol. 3*, 684
Sechaud, J., Streisinger, G., Lanford, H., Reinhold, H. and Stahl, M. M. (1962) personal communication
Shalitin, C. and Stahl, F. W. (1963) personal communication
Simon, E. (1961) personal communication
Streisinger et al. (1963) personal communication
Streisinger, G., Edgar, R. S. and Harrar Denhardt, G. (1961) personal communication
Visconti, N. and Delbrück, M. (1953) *Genetics 38*, 5

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INDUCTION OF MUTATIONS IN THE PHAGE KAPPA

By

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The temperate phage kappa grows on several strains of the bacterium Serratia. The plaques are turbid and surrounded by violet halos on the orange red layer of the indicator strain *HY*. There are three main groups of plaque mutants which can be easily distinguished and scored on a plate: (1) clear centre (*c*-type), (2) narrow halo (*e*-type), (3) pale or no colour halo (*b*-type). Different other mutant types are much rarer. Each class of mutants certainly arises by mutation in several genes. This is shown by the different degree of phenotypic deviation of the mutants within one class and also by crossing experiments.

The phage is exceptional in so far as it can mutate by UV- or X-irradiation of the free particles without irradiation of the host cells. UV does not produce perfect mutations in the extracellular particles but first some kind of premutational lesions occur. They can be partially reverted by the post-irradiation conditions. Heat or visible light on the UV-irradiated free phage decreases the frequency of mutations. Further on, if another indicator strain, *CN*, is used for plating the UV-irradiated phage, no mutations are observed. Nevertheless, mutant strains of the phage show the mutant plaque type also on this strain. So we conclude that strain *CN* has, in contrary to strain *HY*, a strong intracellular apparatus for destroying UV-induced permutations.

The easy scoring of 4 different classes of mutations allowed to study the mutation spectrum in dependence on the mutagenic agent, and so to distinguish differences in mutation mechanisms. The mutation spectrum is the relative frequency of the 4 types of plaque mutations (*c*, *e*, *b* and rare types) produced by a mutagen. Since each class represents the mutations in several genes, the differences in the type spectrum reflects differences in the sensitivity of these gene groups to the mutagens. Very probably, these differences are due to differences in the average chemical composition of the DNA of the 3 or 4 gene groups, if the mutagen is applied to the extracellular phage. One can assume that hot spots of mutability may vary between the gene groups and that a given hot spot owes its high mutability not to one of the 4 DNA-bases alone but to a special group of bases, e.g. duplets or triplets.

We applied several mutagens to the extracellular phage: UV, X-rays (in broth), H_2O_2, nitrite, hydroxylamine, TEM, EMS, heat (45°C at p_H 4.6). The spectrum of the spontaneous mutations was also obtained. Special experiments exluded the contribution of a selection of spontaneous mutants to the induced mutation spectra obtained. The main results are the following. (1) UV and X-rays give very different spectra, UV preferentially *c*-muta-

tions and only $^1/_4$ of other types, x-rays give not very different amounts of *c*, *e* and *b*. So UV has a high electivity for *c*-genes. (2) Hydrogen peroxide produced nearly only *c*-mutations. So x-ray mutations cannot be due mainly to H_2O_2 which is produced by x-rays from water decomposition. The mutagenic effect of H_2O_2 is more similar to that of UV; but the relative killing effect of H_2O_2 is much greater than that of UV, so UV does not act mainly

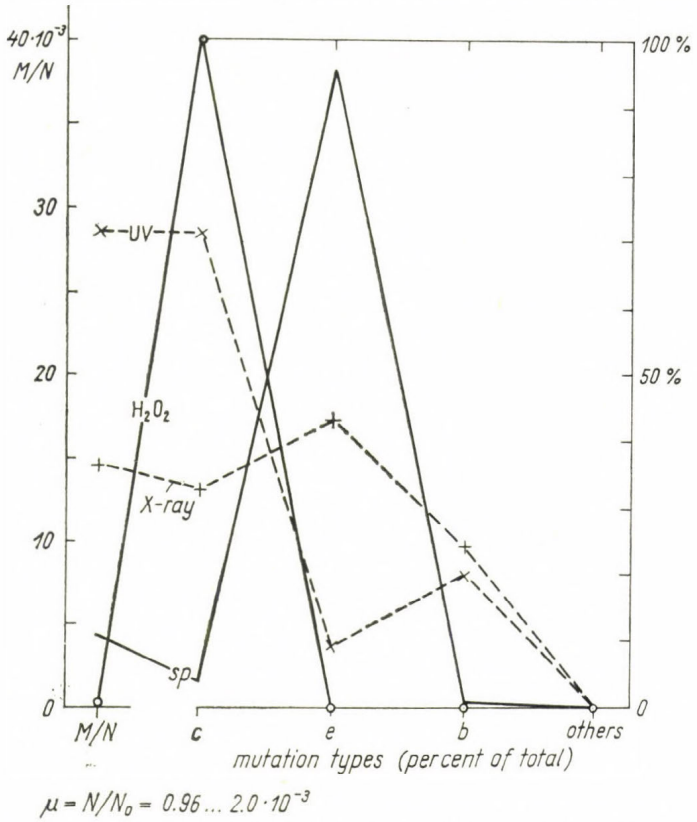

FIG. 1.—Extracellular induced mutation spectra of the phage *kappa* (after H. Beckmann and W. Rüger)

by the production of H_2O_2 from water (Fig. 1). (3) All mutagens used gave significantly different mutation spectra, their mutation mechanisms must be different. EMS and heat only produced the same spectra, and both agents have about the same relative killing power. This similarity indicates the same mutation mechanism. This mechanism may be preferential removing the guanine from DNA, perhaps, at the same hot spots (Fig. 2). (4) The spontaneous mutations are mostly only *c*-mutations. This may indicate a rather special mechanism for production of spontaneous mutations, not a mixture of different ones. It is probably not the thermic vibration in DNA, since the heat spectrum contains only very few *c*-mutations and much more other types.

Recently we have tried to induce mutations in vegetative phage by excess adenine and other DNA-bases and nucleotides, but without success. Unnatural purines, as 5-aminopurine and coffein, did not give an increase in mutation yield, despite their growth inhibitory effect. So we found no indication that spontaneous mutations in our phage are due to excess DNA-bases in normal metabolism. It would certainly be interesting to find a metabolic or other normal intracellular factor which gives the same mutation spectrum as spontaneous mutations do.

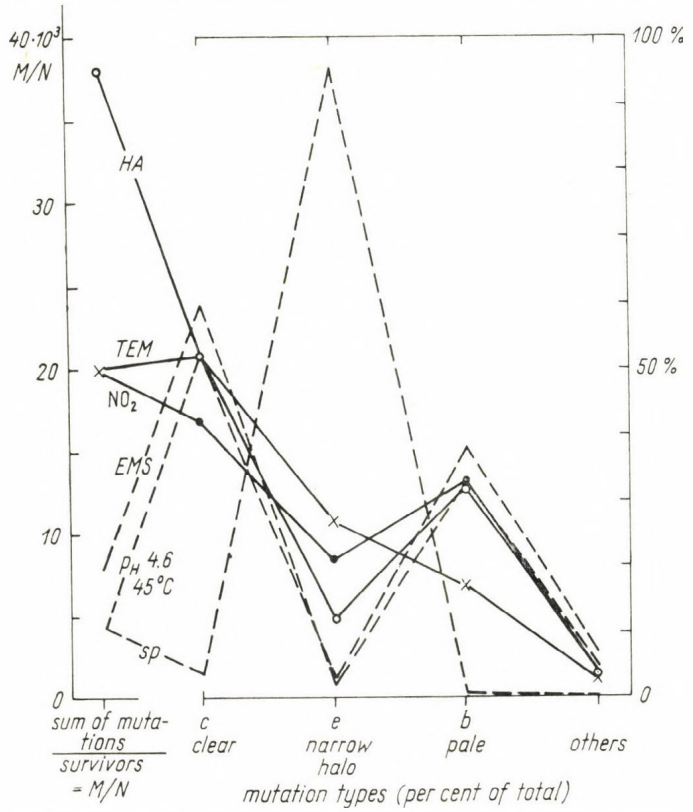

FIG. 2.—Extracellular induced mutation spectra of the phage *kappa* (after H. Beckmann)

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The following members of the Institute of Microbiology of Frankfurt a. M. cooperated in the work reported: Helga Beckmann, W. Rüger, H. Steiger and U. Winkler.

DISCUSSION

STAHL: Is proflavine mutagenic in your phage system?

KAPLAN: Investigations are planned; perhaps, there will be some results when I am back.

Symp. Biol. Hung. 6, 147–52 (1965)

REGULATION MECHANISMS CONTROLLING BACTERIAL SPORULATION

By

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The morphogenetic processes underlying bacterial sporulation undoubtedly result from biochemical reactions, controlled by the genetic material of the cell. They will therefore be best analyzed by a combination of cytological, physiological, biochemical and genetic studies carried out concurrently on a sporogenous (Sp^+) wild type strain and its sporulation mutants (Schaeffer and Ionesco 1960). These mutants have been known for a long time (Roux 1890, Eisenberg 1912) but the systematic comparison of their properties with those of the wild type began only recently (Schaeffer and Ionesco 1960, Ryter et al. 1961, Szulmajster and Schaeffer 1961, Aubert and Millet 1961, Aubert et al. 1961, Schaeffer et al. 1963, Aubert and Millet 1963). Study of genetic transfer so far can only be carried out with those strains of bacilli which, like the Marburg strain of *B. subtilis*, can be transformed (Spizizen 1958, 1959, Schaeffer and Ionesco 1959, Marmur et al. 1963), or transduced (Thorne 1962, Takahashi 1961).

Several reviews on sporulation have recently appeared (Foster 1958, Murrel 1961, Halvorson 1962); in this article, only those facts are brought together which seem meaningful in our attempt to identify the regulation mechanisms controlling the sporulation process.

MORPHOLOGICAL STUDIES

As many as six intermediary stages can be recognized cytologically during the process of sporulation (Young and Fitz-James 1959, Ryter et al. 1961, Schaeffer et al. 1963), each of which is found to predominate in the population at a well-defined time after the end of exponential growth. Sporulation mutants are found which, whether Sp^- or *Osp* (see below), may be blocked at any morphological stage (Ryter et al. 1961, Schaeffer et al. 1963).

GENETIC STUDIES

Because of their lesser opacity, mutant colonies with an impaired sporulation can easily be recognized, even in species with non-pigmented spores. A method has been described that selects at least those mutant forms which are blocked early in the process (Schaeffer et al. 1963). Upon further testing, the sporulation mutants can be subdivided into: (1) those which will never sporulate, under any cultural conditions, except of course when an exception-

al reverse mutation has taken place and (2) those which under 'optimal' standard conditions, will sporulate with a low frequency. The first category is called asporogenous (Sp^-), the second is oligosporogenous (*Osp*) (Schaeffer and Ionesco 1960). The low probability of an *Osp* mutant to sporulate under standard conditions is a stable, inbuilt property of the strain, but it will vary with the cultural conditions (Aubert and Millet 1961). Some *Osp* mutants at least obviously have all the specific information required to sporulate but, some regulatory mechanism being presumably disturbed, this information becomes only exceptionally expressed.

Normal sporulation can be restored, both in Sp^- and in *Osp* mutants, by transformation (Schaeffer and Ionesco 1960) or by transduction (Takahashi 1963). The transformation frequencies to the Sp^+ condition usually are of the same order of magnitude as for the transformations involving a nutritional character. Lastly, the restoration of the ability to sporulate occurs, as a rule, with the same frequency, whether the transforming DNA is extracted from the wild type or from another sporulation mutant. The combined results lead to the conclusion that the genes are many (several tens at least), at which a mutation can affect sporulation (Schaeffer and Ionesco 1960, Schaeffer et al. 1963). Transduction studies are in agreement with this conclusion (Takahashi 1963).

A method is now available permitting chromosomal mapping from transformation data, and a still rudimentary map of *B. subtilis* has been built, using the method of Yoshikawa and Sueoka (1963). It would be interesting to know the location of the sporulation genes on this map.

PHYSIOLOGICAL STUDIES

STUDIES WITH WILD TYPE BACILLI

(a) *Sporulation en masse.*— Conventionally, sporulation is studied in starved cultures, where it proceeds in the majority of the cells. Morphological and biochemical studies are best performed on such (more or less) synchronously sporulating populations. Carbon starvation and nitrogen starvation can both be used to induce the sporulation process (Grelet 1951). The complexity of this process is well illustrated by the fact that it requires some 14 generation times for its completion.

When bacteria sporulate in their exhausted growth medium, the sporulation medium can no longer be considered chemically defined. The procedure consisting in suspending washed growing cells in a defined medium not supporting growth has therefore much in its favor (Hardwick and Foster 1952). We have recently performed a number of experiments of this type, with cultures of *B. subtilis* (Marburg strain) growing in broth; re-suspension of the washed cells in a nitrogen-free, mineral base-medium leads to extensive lysis. The result remains the same in the presence of ammonium salts; but when glucose is added to the base, lysis does not occur and the culture sporulates. Some thirteen hours are required for the formation of heat-resistant spores under those conditions. When both glucose and ammonium salts are added to the base, growth resumes (after a lag of several hours) in the re-suspended culture, which eventually sporulates after growth has stopped; but 13 hours after transfer, no resistant spores are present. Since

both induction of lysis by carbon starvation (Monod 1942) and sporulation in a nitrogen-limited culture (Grelet 1951) are well-known phenomena, nothing really new was learned from these experiments. They do show, however, that (1) sporulation is not induced in the simultaneous presence of utilizable carbon and nitrogen sources, even during the long lag that follows transfer of actively growing cells from a rich to a minimal medium (Kjeldgaard et al. 1958), (2) in *B. subtilis* at least (in contrast with the results observed with *B. mycoïdes* by Hardwick and Foster 1952), *sporulation is not inhibited by glucose alone*, in the absence of nitrogen. Far from being inhibitory, glucose is actually required for sporulation only because it prevents the occurrence of cell lysis.

(b) *Sporulation during growth.*—No heat resistant spores are found in a culture growing rapidly in a rich medium. This is no longer true, however, when growth occurs in minimal medium: spores are constantly being formed and their number increases in parallel with the viable count (Aubert et al. 1961a). Another important observation was made by the same authors with cultures of *B. megaterium* grown in the chemostat. A mineral medium was used, with glucose as the rate-limiting factor. The growth rate could only be lowered from 0.7 down to 0.5 divisions per hour, since mass-sporulation was induced (and the chemostat was washed out), when the flow rate was lowered further. Rates as low as 0.2 divisions per hour could be maintained for several days, however, when a sporulation mutant was used instead (Aubert and Millet 1961).

In unpublished experiments with *B. subtilis*, both heat resistant spores and total viable cells have been counted during exponential growth in various defined media. With NH_4Cl, the nitrogen source, present in excess, the value of ϱ (the ratio of these two counts) was found to decrease regularly from 5×10^{-4} to 2×10^{-7}, as the glucose concentration was raised from 0.3 to 50 grams per liter (these changes in glucose concentration do not affect the growth rate). A similar concentration dependence was observed with either glycerol or citrate as the carbon source; but glycerol was much less inhibitory to spore formation than glucose, and citrate much less so than glycerol. With a substrate concentration of 2 grams per liter, the values of ϱ, the sporulated fraction, were 1.4×10^{-4}, 1.7×10^{-3} and 2.0×10^{-2} on the three substrates, while the corresponding growth rates were 0.90, 0.75 and 0.20 divisions per hour. In the same mineral-glucose medium, but with histidine as the only nitrogen source, the growth rate was 0.4 divisions per hour, and the value of ϱ was 7.7×10^{-2}.

STUDIES WITH SPORULATION MUTANTS

As a rule, no auxotrophy is found associated with the mutant condition. In both minimal and complete media, the mutants, whether Sp^- or *Osp*, generally show the same rate of growth and the same yield as the Sp^+ strain from which they were derived. In other words, *they start behaving as mutants only when growth stops, or at least slows down*; recognition of the existence of such genes may be of importance also in the study of differentiation in eucaryotic cells.

Since sporulation so far could be restored in the mutants neither by syntrophy with the wild type, nor by supplementation of the cultures with

various extracts (Schaeffer et al. 1963), attention was focussed on those bacterial products which are known to appear only after the end of growth. An antibiotic, detected routinely on staphylococcus, was found to be produced by the Marburg strain, which is therefore Sp^+ Ab^+, but not by some of its sporulation mutants. When various Sp^- Ab^- mutants were exposed to wild type DNA, allowed to sporulate and challenged by heat treatment, *all* the selected Sp^+ transformants (several thousands of them) were found to be also Ab^+. From this and other results it was concluded that the production of the antibiotic in question was due to a sporulation-specific reaction (Balassa et al. 1963, Schaeffer et al. 1963). It is interesting to speculate at this time on the physiological function of this antibiotic production. Edein, an antibiotic produced by *B. brevis* (Kurylo-Borovska 1962) inhibits electively DNA synthesis (its production has admittedly not been shown to be necessarily associated with sporulation). On the other hand, sporulation is known to proceed in the absence of DNA synthesis (Young and Fitz-James 1959, Szulmajster and Canfield 1963), while RNA is turning over (Young and Fitz-James 1959, Spotts and Szulmajster 1962, Del Valle and Aronson 1962, Szulmajster and Canfield 1963, Balassa 1963a,b). It would seem to be a clever thing to do for a sporulating bacillus to produce an antibiotic that would block DNA synthesis electively; many other interpretations of the scanty information available are of course possible (see Bernlohr and Novelli 1960).

SUMMARY

The facts reported in this article, together with the current notions on the repression of enzyme synthesis (Jacob and Monod 1961, Magasanik 1961) seem to make the following picture of the sporulation process a reasonable working hypothesis. Sporulation results from a long chain of largely unidentified specific biochemical reactions. Although, like all genes, the sporulation genes controlling these reactions are present at all times, the chain is not started, and is therefore believed to be repressed, under conditions where rapid growth is possible. The chain can also be blocked at anyone of its steps by mutation.

Catabolites seem to be responsible for the repression of the initial triggering of the chain. All the essential features of the repression by catabolites (Magasanik 1961) can be observed when sporulation, rather than synthesis of a given enzyme, is studied: repression requires that both carbon and nitrogen sources be actively metabolized; the relative efficiency with which catabolites endowed with repressive activity are formed from the various carbon and nitrogen sources are the same (glucose > glycerol > citrate; NH_4^+ > histidine), whether enzyme synthesis or sporulation is being considered. The inverse correlation observed between the probability of a cell to sporulate and the rate of its growth and multiplication is to be expected if the two are inverse functions of one and the same variable, the intracellular concentration of some nitrogen containing catabolite(s).

When sporulation is repressed during growth, its specific products do not seem to be formed at all; this is certainly true of dipicolinic acid (bibliography in Halvorson 1962) and of N-succinyl-glutamic acid (Aubert et al. 1961b), of which no trace is present in rapidly growing bacteria; it seems also

to be true of the sporulation antibiotic mentioned earlier. The whole chain therefore seems to be repressed, not only its initial steps, and the question must be raised whether the sporulation genome constitutes one single operon. The absence of genetic linkage between the sporulation genes investigated by transformation speaks against this possibility; it seems rather to suggest the existence of several operons, scattered all over the bacterial chromosome. If this is so, it is perhaps surprising that their repression is so well coordinated; the case of arginin synthesis in *E. coli* (Gorini et al. 1961, Maas 1961) may be invoked, however, which shows that even distinct operons may be repressed by one and the same metabolite.

Another fact must be introduced in the discussion at this point: when the sporulation process has progressed beyond a certain stage, some three hours after its onset, it will go to completion even if conditions supporting growth are restored: an irreversible committment has taken place (Grelet 1951, Hardwick and Foster 1952), as if the genes which become expressed late in the process were insensitive to catabolite repression. Our working hypothesis is, therefore, that sporulation is controlled by several operons, which become expressed in a definite order; the first one at least would be repressible by catabolites, the others, expressed later, being induced in a sequential manner (Stanier 1950). Much more work is needed, however, before this model can be accepted.

REFERENCES

Aubert, J. P. and Millet, J. (1961) *C. R. Acad. Sci. (Paris) 253*, 1880

Aubert, J. P. and Millet, J. (1963) in *Colloqu. Intern. C. N. R. S. (Marseille) 124*

Aubert, J. P., Millet, J. and Castoriadis-May, C. (1961a) *C. R. Acad. Sci. (Paris) 253*, 1731

Aubert, J. P., Millet, J., Pineau, E. and Milhaud, G. (1961b) *Biochim. biophys. Acta 51*, 529

Balassa, G. (1963a) *Biochim. biophys. Acta 72*, 497

Balassa, G. (1963b) in *Colloqu. Intern. C. N. R. S. (Marseille) 124*

Balassa, G., Ionesco, H. and Schaeffer, P. (1963) *C. R. Acad. Sci. (Paris) 257*, 986

Bernlohr, R. W. and Novelli, G. D. (1960) *Biochim. biophys. Acta (Amst.) 41*, 541

Del Valle, M. R. and Aronson, A. I. (1962) *Biochem. biophys. Res. Commun. 9*, 421

Eisenberg, P. (1912) *Zbl. Bakt., I. Abt. Orig. 63*, 305

Foster, J. W. (1958) *Quart. Rev. Biol. 31*, 102

Gorini, L., Gundersen, W. and Burger, M. (1961) *Cold Spr. Harb. Symp. quant. Biol. 26*, 173

Grelet, N. (1951) *Ann. Inst. Pasteur 81*, 430

Halvorson, H. (1962) in *The Bacteria*, ed. by I. C. Gunsalus and R. Y. Stanier, Academic Press, *IV*, 223

Hardwick, W. A. and Foster, J. W. (1952) *J. gen. Physiol. 35*, 907

Jacob, F. and Monod, J. (1961) *Cold Spr. Harb. Symp. quant. Biol. 26*, 193

Kjeldgaard, N. O., Maaløe, O. and Schaechter, M. O. (1958) *J. gen. Microbiol. 19*, 607

Kurylo-Borovska, Z. (1962) *Biochim. biophys. Acta 61*, 897

Maas, W. K. (1961) *Cold Spr. Harbor Symposia quant. Biol. 26*, 183

Magasanik, B. (1961) *Cold Spr. Harb. Symp. quant. Biol. 26*, 249

Marmur, J., Seaman, E. and Levine, J. (1963) *J. Bact. 85*, 461

Monod, J. (1942) in 'Recherches sur la Croissance des cultures bactériennes', *Actualités Scientifiques et Industrielles 911*, Hermann, Paris
Murrel, W. G. (1961) *Sympos. Soc. gen. Microbiol. 11*, 100
Roux, E. (1890) *Ann. Inst. Pasteur 4*, 25
Ryter, A., Ionesco, H. and Schaeffer, P. (1961) *C. R. Acad. Sci. (Paris) 252*, 3675
Schaeffer, P. and Ionesco, H. (1959) *C. R. Acad. Sci. (Paris) 249*, 481
Schaeffer, P. and Ionesco, H. (1960) *C. R. Acad. Sci. (Paris) 251*, 3125
Schaeffer, P., Ionesco, H., Ryter, A. and Balassa, G. (1963) in *Colloqu. Intern. C. N. R. S. (Marseille) 124*
Spizizen, J. (1958) *Proc. nat. Acad. Sci. (Wash.) 44*, 1072
Spizizen, J. (1959) *Fed. Proc. 18*, 957
Spotts, C. R. and Szulmajster, J. (1962) *Biochim. biophys. Acta (Amst.) 61*, 635
Stanier, R. Y. (1950) *Bact. Rev. 14*, 179
Szulmajster, J. and Canfield, R. E. (1963) in *Colloqu. Intern. C. N. R. S. (Marseille) 124*
Szulmajster, J. and Schaeffer, P. (1961) *C. R. Acad. Sci. (Paris) 252*, 220
Takahashi, I. (1961) *Biochem. biophys. Res. Commun. 5*, 171
Takahashi, I. (1963) *XI Intern. Congress of Genetics, The Hague* (in press)
Thorne, C. T. (1962) *J. Bact. 83*, 106
Yoshikawa, H. and Sueoka, N. (1963) *Proc. nat. Acad. Sci. (Wash.) 49*, 559
Young, I. E. and Fitz-James, P. C. (1959) *J. biophys. biochem. Cytol. 6*, 467, 483

DISCUSSION

WATANABE: I remember you have previously reported that the sporulation factor might be an episome in the cytoplasm. I should like to ask you about your present opinion on this point.

SCHAEFFER: The possibility has been considered that sporulation, a dispensable function, might be controlled by an episome which would become expressed or stay repressed, depending on whether it is in the autonomous or the integrated state. The ability to form spores would then be lost together with the episome itself. The various treatments capable of curing a cell from its carried episomes (exposure to submaximal temperature, acridine dyes, UV light or streptomycine) have all been tried on the sporogenous (Sp^+) Marburg strain. None of them carried out the predicted mass conversion of the Sp^+ into Sp^- cells.

The hypothesis suggests that a 'cured' Sp^- mutant might become Sp^+ again by reinfection; this also has been looked for unsuccessfully.

This negative evidence is insufficient to discard the hypothesis, but the following observation seems more convincing. With most if not all the Sp^- mutants tested, the ability to form spores could be restored by transformation with purified wild-type DNA; this would not happen with mutants having lost a sporulation episome. The mutants could, of course, carry an episome inactivated by mutation, and therefore restorable. The final proof is hard to get, but the weight of the evidence is certainly not in favour of the hypothesis.

Symp. Biol. Hung. 6, 153–4 (1965)

TEMPERATURE SENSITIVITY OF MUTANTS OF *BACILLUS ANTHRACIS* CAUSED BY A BLOCK IN THYMINE-NUCLEOTIDE SYNTHESIS

By

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Mutants were isolated from an acapsulogenic strain of *Bacillus anthracis*, strain Vollum, which were unable to grow at a temperature above 34° in the absence of certain pyrimidines. At elevated temperatures one of the mutants, VC^-TdR^- was found to be dependent on thymidine while the other, VC^-T^- required thymine. Both mutants, however, grew normally in the absence of pyrimidines at near room temperature. A relatively high concentration of thymine was needed to overcome the thymidine requirement of mutant VC^-TdR^- at 37°, whereas a combination of a low concentration of thymine with different deoxyribosides (deoxyadenosine, deoxyguanosine, deoxycytidine) gave good growth of the mutant. This observation is suggestive of the presence of a particular enzyme, trans-*N*-deoxyribosylase in the mutant VC^-TdR^-, an enzyme which appears to be of limited distribution in nature. The second mutant, VC^-T^-, utilized added thymine readily at 37° and the base could not be substituted by its nucleoside, thymidine. In fact, thymidine and deoxyribonucleosides inhibited the growth of mutant VC^-T^- in the presence of thymine.

Both mutants also grew well at 37° in the presence of thymidine-5-phosphate, which indicated that the *de novo* pathway of pyrimidine synthesis is blocked above 34° somewhere in the pathway between deoxycytidine-phosphate and thymidine-5-phosphate. This block in the pyrimidine synthesis occurring at elevated temperatures caused an unbalanced synthesis of macromolecules accompanied by an abnormal cell wall formation. At 37° germinated spores showed an abnormal elongation of the initial cell concomitant with a gradual loss of viability. At this temperature cell wall formation was also abnormal at a limiting concentration of pyrimidines, and minor deficiencies in cell wall structure of the mutants were still apparent even in the presence of a large excess of pyrimidine. This, however, did not involve any change in virulence of mutant VC^+TdR^- in the homoiothermic mouse.

It is assumed that the mutants produce either an altered enzyme protein corresponding to a block in *de novo* synthesis of pyrimidine, or an inhibitor is produced at high temperatures which diminishes and finally prevents the action of the normal enzyme.

DISCUSSION

Szybalski: How do you explain these thymidine mutants? Would you not think that the other possibility might be that in your mutants, which cannot utilize thymidine, there is a block for permeability of thymidine?

Iványovics: Yes, I have speculated about that. But the funny story is that the thymidine monophosphate is utilized by both enzymes. Therefore, this is the case also when nucleotides are able to penetrate into the cells as in the case of *Hemophilus influenzae*.

Szybalski: But I do not know about any well-proved case yet that nucleotide could be taken up thus, which could be very easily explained by splitting TMP, should thymine be absorbed so directly.

Iványovics: Yes, most of this investigation has been carried out with *Enterobacteriaceae*, and it makes some difference in the case of other microorganisms. I have just referred to *Hemophilus influenzae*. Factor B is actually a nucleotide, a nicotinic acid nucleotide; unless it penetrates, it is not able to grow.

Symp. Biol. Hung. 6, 155–6 (1965)

RELAXATION AND AMINO ACID SENSITIVITY OF *E. COLI* K12 MUTANTS

By

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E. coli carrying the 'relaxed' allele of the RNA control, or *RC*, gene show a much longer growth lag upon downshift transfer from an amino acid supplemented into a minimal medium than normal strains carrying the 'stringent' allele. The prolonged growth lag seems to be the consequence of an abnormal continuation of RNA synthesis by RC^{rel} genotypes following the downshift transfer. This unbalanced RNA synthesis appears to lock RC^{rel} cells into a physiological impasse in which the derepressed synthesis of new enzymes necessary for the resumption of growth has become more difficult. Colony assay of broth-grown bacteria on minimal-glucose salts agar reveals that only about 20% of RC^{rel} cells are able to escape from this impasse.

Certain amino acids seriously aggravate the effects of downshift transfer of RC^{rel} bacteria, since in the presence of methionine, leucine, isoleucine, valine, phenylalanine, cysteine, or serine only 10^{-2} to 10^{-3} portion of the population can form macroscopic colonies on the minimal-glucose salts agar. The sensitivity of the RC^{rel} bacteria to the amino acids above-mentioned changes with the physiological state of the bacteria. The most sensitive are 2 h tryptone broth-grown bacteria.

The deleterious effect of toxic amino acids on RC^{rel} bacteria can be counteracted if the minimal medium contains specific combinations of leucine, valine, isoleucine, and threonine. It is suggested, therefore, that all the amino acid sensitivities exhibited by the RC^{rel} bacteria in downshift transfer have their origin in feedback control anomalies of the joint biosynthetic pathways of leucine, valine, isoleucine and threonine.

Finally, the interrelationship between relaxation phenomenon and amino acid sensitivity have been discussed. Details of these experiments will be published elsewhere.

DISCUSSION

SZYBALSKI: I should like to ask for the interpretation of this phenomenon. What does it mean from the point of view of enzyme synthesis?

STENT: I think it is possible that the mutation to relaxation affects the feedback control of some biosynthetic enzymes, too, and that the postulated catholic inducer is an effective intermediate in this pathway. So it may be that the amino acid sensitivity in some way reflects the stimulation of the production of this catholic inducer, since even a greater amount of RNA will also be synthetized in the presence of this toxic amino acid.

Symp. Biol. Hung. 6, 157–9 (1965)

A STRAIN-SPECIFIC PROTEIN FOR THE REPLICATION OF BACTERIOPHAGES T2 AND T4*

By

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The two closely-related bacteriophage strains T2 and T4 induce in their *E. coli* host cells the appearance of several new enzymatic activities, termed 'early proteins', necessary for the replication of the phage DNA. Experiments carried out by Fermi and Stent (1961), involving primary infection of bacteria with one T4 genotype and delayed superinfection with a second T4 genotype, have shown that the early proteins induced by the primary phage genome can serve also for the replication of the DNA of the homologous secondary phage. I should like to report the results of an experiment, similar to that of Fermi and Stent, designed to determine whether the early proteins induced by T2 infection can serve for the replication of T4 DNA.

E. coli cells are infected with two T2 bacteriophages per cell. Intracellular phage development is allowed to proceed at 37° six minutes, at which time the enzymes necessary for DNA synthesis have been formed (Melechen 1955, Kornberg et al. 1959). Further protein synthesis is then inhibited by an addition of 300 mmg/ml of the antibiotic chloramphenicol to the culture, and the bacteria are superinfected with ten T4 phages per cell. Addition of chloramphenicol six minutes after infection certainly allows replication of the DNA of the primary T2, and the DNA formed in the presence of chloramphenicol is incorporated into the viable progeny phages ultimately formed after removal of the antibiotic (Hershey and Melechen 1957). If the DNA of the superinfecting T4 replicates along with that of the primary T2 in the presence of chloramphenicol, then it may be concluded that the enzymes induced by the T2 DNA can serve to replicate T4 DNA.

Replication of the DNA of the superinfecting T4 is determined in the following way. The T2 primary phage carries a 'deletion' in the *r*II region of its genome, and is thus unable to give rise to r^+ wild type revertants (Benzer 1957). The superinfecting T4 phage carries an *r*II *point mutation*, which is highly revertible by the mutagen 5-bromodeoxyuridine (BUdR), and which does not recombine with, or is covered by, the *r*II deletion carried by the primary T2 phage. After addition of chloramphenicol and superinfection by T4, the culture is divided into two aliquots, and 200 mmg/ml BUdR mutagen is added to one part. Both aliquots are incubated for one hour, at which time chloramphenicol and BUdR are removed, in order to allow resumption of protein synthesis and to stop further mutagenesis. Finally, both aliquots

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** Predoctoral Fellow of the National Science Foundation.

are incubated further, and infective progeny phages are allowed to form. Since BUdR acts only on replicating DNA, the phage progeny of the aliquot incubated in the presence of BUdR should contain a higher proportion of r^+ wild type revertants than the progeny of the aliquot incubated in the absence of BUdR, if and only if the DNA of the superinfecting T4 genome containing the BUdR-revertible *r*II point mutation replicates in the presence of chloramphenicol. Control experiments are performed in which bacteria primarily infected with a T2 *r*II deletion mutant are superinfected with a T2 BUdR-revertible *r*II point mutant, and bacteria primarily infected with a T4 *r*II deletion mutant are superinfected with a T4 BUdR-revertible *r*II point mutant. In these controls, according to the finding of Fermi and Stent, the superinfecting genomes should replicate, using their homologous early proteins. Results of a typical experiment are shown in Table I.

TABLE I

Primary infection	Superinfection	Ratio of revertants and total progeny		Revertant frequency +BUdR −BUdR
		+BUdR	−BUdR	
T2*r*II del.	T2*r*II pt.	2.2×10^{-8}	8×10^{-10}	27
T4*r*II del.	T4*r*II pt.	3.7×10^{-8}	1.8×10^{-9}	20
T2*r*II del.	T4*r*II pt.	1.7×10^{-9}	8.4×10^{-10}	2

It is clear that when primary infection with T2 is followed by superinfection with T4, the ratio of r^+ revertants to total progeny in the aliquot incubated in the presence of BUdR is not appreciably higher than the ratio in the aliquot incubated in the absence of BUdR. The slight stimulation observed might be accounted for by residual protein synthesis during the period after superinfection before the inhibition by chloramphenicol becomes fully effective, allowing some formation of early protein by a few superinfecting phages. The ratios of r^+ revertants to total progeny in both T2–T2 and T4–T4 controls showed approximately twentyfold stimulation by BUdR. It can be concluded, therefore, that the early protein induced by T2 infection does not allow the replication and production of stable T4 DNA.

These results appear to show that there is some strain-specific component of the early protein required for the replication and production of stable T4 DNA that *cannot* be induced by the *T2* genome, but *can* be induced by the *T4* genome. We are presently trying to identify this specific component, and to map the position of the gene controlling its synthesis on the T4 genome.

REFERENCES

Benzer, S. (1957), in *Chemical Basis of Heredity*, ed. by McElroy and Glass, Johns Hopkins Press, Baltimore, p. 70

Fermi, G. and Stent, G. S. (1961) *J. molec. Biol. 4*, 179—92

Hershey, A. D. and Melechen, N. E. (1957) *Virology 3*, 207—36

Kornberg, A., Zimmerman, S. B., Kornberg, S. and Josse, J. (1959) *Proc. nat. Acad. Sci. 45*, 772—85

Melechen, N. E. (1955) *Genetics 40*, 584

STAHL: Upon what strain of *E. coli* do you assay for the particular virotype T2r^+?

ECKHART: It is K12S. It is of a high efficiency, in particular.

STAHL: What does S mean in this case?

ECKHART: This is re-lysogenized.

SZYBALSKI: I think that you did already run, just for curiosity, together with T4, which is preferred to T2 in the reciprocal experiment.

ECKHART: No, the experiment is difficult to perform because, as it is known, the exclusion is of high per cent. The infection of T4 and T2 results in an approximately 100fold yield of T4 to T2, so that it is difficult to get T2 participating in the vegetative pool.

CLOSING REMARKS

By

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Now we are near the end of the Symposium and standing here again, I am afraid there is little useful that I can add in my closing remarks.

In these three days we have become acquainted with some highlights of current researches still running in laboratories; we have heard a lot of exciting problems and how they were attacked and brilliantly solved; we have enjoyed lively discussions.

In these days we obtained an idea about the successful progress of studies on several problems so intensively pursued at the molecular and subcellular level all over the world. Among others we have also heard some of the newest achievements by means of transformation of competent cells, a promising and competent way to the intentionally directed genetic alteration of the lower organisms. The spectacular successes achieved in molecular genetics from one week to the other make some people nearly intoxicated, and it is no wonder if they spread the rumour: the Huxleyan Brave New World is already before the gate.

However, it does not seem to a student of genetics that an intentional directing of the genetic constitution of higher organisms, or something like that, as e.g. human engineering, is to be easy, or even at all. A student of genetics, who knows the regulatory mechanisms at the subcellular-molecular level controlling the development, as well as the principles of population dynamics operating in the long-run evolution, should also know that similar regulatory mechanisms are operating both in the short-term development and in the long-term of the science of genetics, which warrants the balance in the increasingly extending population system of our discipline.

We have experienced the unbalance, nearly the breakdown of genetics caused by some suppressor taboo-mutations, interfering with the homeostasis in the development of sciences. We were already eyewitness to some extravagant and fashionable trends flourishing vigorously, due to some genetic drifts, and simulating an evolution by the way of natural selection. This experience made me feel my personal gratification mentioned in my opening remarks.

Never concealing our adherence to the scientific truth, also in genetics, we survived many ups and downs. The time has come for organizing this Symposium which, and I think you will agree with me, also belongs to the general scheme of genetics. Genetics has a privileged position among the biological and medical sciences owing to its perspectives in the future of mankind. This gratifies us to a great extent. Now I sincerely hope that this Symposium will either directly or by feedback mechanism contribute to the

better understanding of the goals and of the importance of genetics as well as to the full recognition and the further development of our discipline also in Hungary.

We, Hungarian geneticists, feeling the responsibility, are very thankful to all you who co-operated in one way or another to the success of our meetings. The Symposium is now nearly over, each of you can judge its defects and merits for yourself. So I ask you to forgive us if we have erred in some things. I hope they will be soon forgotten and I thank you for so many kind words and so much encouragement. May I express also my desire that you will leave Hungary with the feeling that the time and thought so generously given have been spent well.

Thank you!

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