Plant α -1,4- Glucan Phosphorylase

by
J. HOLLÓ D. Sc.

E. LÁSZLÓ C. Sc.

Dr. Á. HOSCHKE

AKADÉMIAI KIADÓ, BUDAPEST

J. HOLLÓ, E. LÁSZLÓ and
Á. HOSCHKE:

PLANT α-1,4-GLUCAN PHOSPHORYLASE

For more than a decade, the three authors have been engaged in the field of preparation, kinetic study and industrial application of enzymes of plant, animal and microbial origin. In the present work they report their own investigations and summarize the results of other researchers published in the literature on plant α-1,4-glucan phosphorylase. In addition to discussing plant phosphorylases, the monograph describes the important characteristics of phosphorylases of animal and other origin, and the similarities and differences in the properties of the enzymes are compared.

The occurrence of α-1,4-glucan phosphorylases is discussed, followed by an evaluation of the methods of preparation of these enzymes; the composition, structure, physical-chemical properties of the enzymes of plant and animal origin, the determination of the functional groups of the enzyme and the substrate are dealt with. The results reported in the literature on the mechanism of action and molecular mechanism of phosphorylases are summarized.

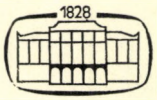
A large collection of analytical and preparative methods is found at the end of the monograph.

AKADÉMIAI KIADÓ
Publishing House of the
Hungarian Academy
of Sciences
BUDAPEST

RECENT DEVELOPMENTS IN THE CHEMISTRY OF NATURAL CARBON COMPOUNDS

RECENT DEVELOPMENTS IN THE CHEMISTRY OF NATURAL CARBON COMPOUNDS

VOLUME III



AKADÉMIAI KIADÓ

PUBLISHING HOUSE OF THE HUNGARIAN ACADEMY OF SCIENCES

BUDAPEST 1971

PLANT α–1,4–GLUCAN PHOSPHORYLASE

BY

J. HOLLÓ D. Sc.

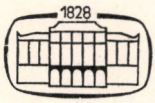
Corresponding Member of the Hungarian Academy of Sciences
Professor of Agricultural Chemical Technology
Technical University, Budapest

E. LÁSZLÓ C. Sc.

Associate Professor of Agricultural Chemical Technology
Technical University, Budapest

Dr. Á. HOSCHKE

Associate Professor of Agricultural Chemical Technology
Technical University, Budapest



AKADÉMIAI KIADÓ
PUBLISHING HOUSE OF THE HUNGARIAN ACADEMY OF SCIENCES
BUDAPEST 1971

Translation revised by

D. A. DURHAM Ph.D.

Printed in Hungary

CONTENTS

I. INTRODUCTION

Hardly more than a century has elapsed since urea, a compound present in living organisms, was first prepared in the laboratory by chemical synthesis by the German chemist Wöhler. In so doing he opened the field of study of the vast number of chemical compounds occurring in living organisms. Since Wöhler's work, the role, fate, formation and decomposition of a large number of these compounds have been successfully elucidated. In particular, it has been established that certain biocatalysts, the enzymes, which are capable of performing reactions under extremely mild conditions (such as nearly neutral solution, relatively low temperature) due to their very high transfer number actually play a central role in these processes. The effects of enzymes have been proved to be particularly specific.

Just these specific properties have made possible their widespread use in industry. Research scientists have endeavoured to utilize enzymes under industrial conditions on the basis of their role in living organisms. At present, many problems of industrial production and use have been solved for quite a number of enzymes. Of these, the amylolytic enzymes are quite prominent. Their application offers remarkable advantages in starch degradation processes. As a comparison of the degradation operations carried out with chemical agents such as acids, the hydrolysis of glucosidic bonds is performed by amylolytic enzymes at identical proton concentrations and temperatures 10^9–10^{12} times faster than by hydrochloric acid.

In addition to this extraordinary advantage, however, enzymes also have a number of unfavourable properties which limit their further use in industry. Of these, one of the most inportant is that enzymes are very complicated protein molecules and thus extremely sensitive to external effects such as hydrogen ion concentration, temperature, ionic and non-ionic contaminants, etc. Another problem of industrial importance concerns the repeated use of enzyme catalysts.

Recently, more or less successful attempts have been made to overcome these difficulties by producing insoluble enzyme preparations. Though the problem of the repeated use has been solved by the availability of these preparations, the sensitivity of protein molecules invariably remains high

even in those enzymes which are bound to solid carriers. Further, preparations of this type prove to have very short life times at the optimum temperatures of the catalyzed reactions.

Where is the solution of this problem to be found? In our opinion, the chemical nature of enzymatic catalysis must first be cleared up and then, in the light of this knowledge, the protein-nature of enzymes must be reduced. That this is a realistic conception may be illustrated by two quite extreme examples. One of these is the subject of the present monograph, α-1,4-glucan-phosphorylase of plant origin, with a molecular weight of 200 000 and an extremely complicated ternary and quaternary structure; the other is the α-amylase prepared from *Bacillus thermophylicus*, with a molecular weight of 15 000, with virtually no ternary–quaternary structure.

In the knowledge of the above-discussed and similar facts, attempts to approach this aim have been, and still are being carried out in our Institute. We have set ourselves the task of investigating the overall complexity of the problem rather than one single enzyme, by studying a group of enzymes which are in some way correlated to each other but which, at the same time, differ from each other in their effects. For this reason, we chose amylolytic and starch-synthesizing enzymes for our studies, in particular α- and β-amylases, amyloglucosidase and phosphorylase.

In the selection of the enzyme groups for our investigations, an important aspect was the fact that our Department has a very wide 15 year experience in research into the substrates of the above-mentioned enzymes (in starch research), and also has a research team of adequate training and orientation. The selected enzymes are of importance because they are widely used in industry.

However, the most important reason was the fact that starch, the substrate of the enzymes chosen for study, is a homogeneous polymer (in contrast to proteins and nucleic acids) which lends itself readily to the determination of the actual kinetic constants of enzyme-catalyzed reactions. This is possible since, on employing special labels or on determining the distribution ratios of the products in an extremely precise way, the probability of effective enzyme-substrate collisions can easily be measured for each single enzyme; this known probability is then suitable for the determination of the actual rate constants of the reactions of formation and dissociation of the enzyme-substrate complex studied.

In the present monograph, the findings of earlier publications dealing with α-1,4-glucan-phosphorylase are surveyed.

II. OCCURRENCE OF THE ENZYME

The effect of the enzyme phosphorylase* was first detected in 1925 by Bodnár [9] who observed that during the incubation of pulverized peas with phosphate buffer the inorganic phosphate content of the reaction mixture shows a steady decrease. Later, in 1935, it was proved experimentally by Tankó [94] that the decrease of the inorganic phosphate content is actually due to the formation of organic ester phosphate bonds with reducing sugars.

In 1937, Cori *et al.* first succeeded in isolating phosphorylase (the enzyme which synthesizes polysaccharides) from animal muscle tissues of various origin [13], and then again in 1938 from yeast [14].

Since then, the occurrence of phosphorylase in the leaves, roots and seeds of a great number of higher plants has been proved by many authors. In 1940 Hanes first demonstrated the presence in potato and sugar beet tubers of a plant phosphorylase which is capable of synthesizing amylose from glucose-1-phosphate [32]. Later, the presence of this enzyme in potato tubers was proved. The enzyme has been investigated at various stages of plant development and during storage, and enzyme preparations of various degrees of purity have been produced under in vitro conditions by a number of authors [6, 7, 22, 28, 32, 33, 36, 39, 41, 42, 44, 51, 57, 58, 61, 63, 67, 73, 79, 80, 85, 88, 90, 91, 99]. Phosphorylase has been detected in the tubers of sugar beet [11, 33, 50], in sweet potatoes [1, 12, 43, 66, 84, 86, 92] and in tapioca roots [65]. The presence of the enzyme has been investigated by Krech in the tubers of a number of higher plant types. Phosphorylase activity has been detected in the tubers of *Saxifraga crassifolia*, *Iris sibirica*, *Hyacinthus orientalis*, *Galanthus nivalis* and *Tulipa gesneriana*, but the tubers of various *Allium* varieties did not show phosphorylase activity [47].

The presence of phosphorylase has been observed by a number of authors in the leaves of higher plants, e.g. in the leaves of potatoes [59, 80], tapioca [89, 96], tobacco [26, 52], rice [29, 98], geranium and sugar beet [33, 50, 100] and in the leaves of other higher tuberose plants [47].

* In the following, the term phosphorylase will be used throughout instead of α-glucan phosphorylase (EC 2.4.1.1,α-1,4-glucan orthophosphate glucosyl transferase).

A number of papers deal with the determination of the enzymatic activity, and of the changes in the activity during the development of plants, of phosphorylase present in the plastides of the various plant cells [87], e.g. in rice plant [3, 49, 98], *Vicia faba* [78], cereals [83] and maize [21].

Phosphorylase activity has also been detected in the pollen grains of plants [16, 37, 60, 95].

Many authors have reported the detection and isolation of phosphorylase in fruits and seeds of higher plants such as barley [69, 75], waxy maize [8], sweet corn [72], maize [33, 40, 93], rice [2, 29–31, 101], various cereals [4, 48], peas [28, 32, 102, 103], broad beans [38, 48, 78], Lima beans [28, 68], jack beans [91], soybeans [100], green grain [77], squash [74], pumpkins [43, 66], *Trapa bispinosa* [19] and sea lettuce [45].

Phosphorylase has been detected in various types of algae *(Protophytes)* as well [5, 23–25, 56].

Phosphorylase systems prove to be present in microorganisms such as bacteria [10, 20, 34, 35, 70, 76], protozoa [53, 54], and yeasts [14, 46, 55, 62, 81, 82]. Further, it has been possible to detect phosphorylase in a great number of animal tissues and organs and to isolate it in a very simple way not only in a pure state free from contaminant enzymes but also in a crystalline form, e.g. in brain [14, 15, 17], fat tissue [64], heart [14, 17], liver [14, 17, 71, 97] and muscle tissue [13–15, 18, 27].

REFERENCES

1. Aimi, R., *Nippon Sakumotsu Gakkai Kiji 24*, 201 (1956); C. A. *53*, 16286f (1959).
2. Aimi, R., Fujimaki, K., *Nippon Sakumotsu Gakkai Kiji 29*, 11 (1960); *Chem. Abstr. 56*, 725c (1962).
3. Aimi, R., Murakami, T., *Nogyo Gijutsu Kenkyusho Hokoku 12*, 1 (1964); *Chem. Abstr. 63*, 10322g (1965).
4. Anisimov, A. A., Boikov, P. Y., *Agrokhimiya 5*, 37 (1965); *Chem. Abstr. 63*, 12268c (1965).
5. Bailey, J. M., Neish, A. C., *Can. J. Biochem. and Physiol. 32*, 452 (1954); *Chem. Abstr. 48*, 10364g (1954).
6. Barker, S. A., Bourne, E. J., Wilkinson, J. A., Peat, S., *J. Chem. Soc. 1949*, 1507 (1949).
7. Baum, H., Gilbert, G. A., *Nature 171*, 983 (1953).
8. Bliss, L., Naylor, N. M., *Cereal Chem. 23*, 177 (1946).
9. Bodnár, J., *Biochem. Z. 165*, 1 (1925).
10. Carlson, A. S., Hehre, E. J., *J. Biol. Chem. 177*, 281 (1949).
11. Cashen, N. A., Friloux, J. J., *J. Agr. Food Chem. 14*, 434 (1968).
12. Chithazanjan, N.; *Naturwissenschaften 50*, 667 (1963); *Chem. Abstr. 60*, 1955e (1964).
13. Cori, C. F., Colowick, S. P., Cori, G. T., *J. Biol. Chem. 121*, 465 (1937).
14. Cori, C. F., Colowick, S. P., Cori, G. T., *J. Biol. Chem. 123*, 375 (1938).
15. Cori, G. T., Cori, C. F., *J. Biol. Chem. 135*, 733 (1940).
16. Cori, G. T., Cori, C. F., *J. Biol. Chem. 151*, 57 (1943).
17. Cori, G. T., Cori, C. F., Schmidt, G. T., *J. Biol. Chem. 129*, 629 (1939).

18. Cori, G. T., Green, A. A., *J. Biol. Chem. 151*, 31 (1943).
19. Datta, J., *Ann. Biochem. Exptl. Med. 20*, 117 (1960); *Chem. Abstr. 61*, 12250f (1964).
20. Doudoroff, M., Hassid, W. Z., Putman, E. W., Potter, A. L., Lederberg, J., *J. Biol. Chem. 179*, 921 (1949).
21. Duvick, D. N., *Botan. Gaz. 115*, 82 (1953).
22. Fischer, E. H., Hilpert, H. M., *Experimentia (Basel) 9*, 176 (1953).
23. Fredrick, J. F., *Physiol. Plant. 4*, 621 (1951).
24. Fredrick, J. F., *Phyton 13*, 15 (1959).
25. Fredrick, J. F., *Phytochemistry 1*, 153 (1962).
26. Gerola, F. M., Vaccari, E., *Rend. ist. lombardo Sci. Classe Sci. mat. e nat. 88*, 575 (1955); *Chem. Abstr. 50*, 6590e (1956).
27. Green, A. A., Cori, G. T., Oncley, J. L., *J. Biol. Chem. 151*, 21 (1943).
28. Green, D. E., Stumpf, P. K., *J. Biol. Chem. 142*, 355 (1942).
29. Hamada, J., *Nippon Kogaku Zasshi 80*, 180 (1959); *Chem. Abstr. 59*, 14241g (1963).
30. Hamada, J., *Nippon Kogaku Zasshi 80*, 182 (1959); *Chem. Abstr. 59*, 14241h (1963).
31. Hamada, J., *Mie Kenritsu Daigaku Suisan Gakubu Kiyo 5*, 75 (1961); *Chem. Abstr. 61*, 4714d (1964).
32. Hanes, C. S., *Proc. Roy. Soc. London B 128*, 421 (1940).
33. Hanes, C. S., *Proc. Roy. Soc. London B 129*, 174 (1940).
34. Hehre, E. J., Carlson, A. S., Neill, J. M., *Science 106*, 523 (1947).
35. Hehre, E. J., Hamilton, D. M., *J. Bact. 55*, 197 (1948).
36. Hidy, P. H., Day, H. G., *J. Biol. Chem. 160*, 273 (1945).
37. Hixon, R. M., in *Chemistry and Industry of Starch* by R. W. Kerr, Acad. Press. New York 1944. p. 172.
38. Hobson, D. N., Whelan, W. J., Peat, S., *J. Chem. Soc. 1950*, 3566.
39. Holló, J., László, E., Hoschke, Á., *Die Stärke 16*, 243 (1964).
40. Hori, Sh., *Botan. Mag. (Tokyo) 67*, 228 (1954); *Chem. Abstr. 49*, 7657i (1955).
41. Husemann, E., Fritz, B., Lippert, R., Pfannemüller, B., *Makromol. Chem. 26*, 199 (1958).
42. Hyde, R. B., Morrison, J. W., *Am. Potato J. 41*, 163 (1964).
43. Inoue, Y., Onodera, K., *Repts. Inst. Chem. Research, Kyoto Univ. 15*, 70 (1946); *16*, 28 (1947).
44. Kamogawa, A., Fukui, T., Nikuni, Z., *J. Biochemistry 63*, 361 (1968).
45. Kashiwabara, Y., Suzuki, H., Nishizawa, K., *Plant Cell Physiol.* (Tokyo) *6*, 537 (1965); *Chem. Abstr. 64*, 5460a (1966).
46. Kiessling, W., *Naturwissenschaften 27*, 129 (1939).
47. Krech, E., *Beitr. Biol. Pflanz. 30*, 379 (1954).
48. Krenke, A. N., *Trud. Gl. Botan. Sada, Akad. Nauk SSSR 8*, 97 (1962); *Chem. Abstr. 56*, 14643 (1962).
49. Kurasawa, H., Igaue, J., Hayakawa, T., Ogami, H., *Nippon Nogei Kagaku Kaishi 33*, 393 (1959); *Chem. Abstr. 54*, 9005d (1960).
50. Kursanov, A., Pavlovina, O., *Biokhimiya 13*, 378 (1948).
51. Lee, Y. P., *Biochim. Biophys. Acta 43*, 18 (1960).
52. Lovrekovich, L., Klement, Z., Farkas, G. L., *Nature 197*, 917 (1963).
53. Lwoff, A., Ionesco, H., Gutmann, A., *Compt. rend. 228*, 342 (1949).
54. Lwoff, A., Ionesco, H., Gutmann, A., *Biochim. et Biophys. Acta 4*, 270 (1950).
55. Mager, J., *Biochem. J. 41*, 603 (1947).
56. Manners, D. J., Taylor, D. C., *Biochem. J. 94*, 17 (1965).
57. Marré, E., *Atti accad. ligure sci. let. (Pavia)* 6, 311 (1949); *Chem. Abstr.* 46, 2135a (1952)
58. Marré, E., Felici, L., *Atti accad. nazl. Lincei, Rend., Classe sci. fis. mat. e nat. 9*, 111 (1950); *Chem. Abstr. 46*, 11345a (1952).
59. Marré, E., Felici, L., *Atti. accad. nazl. Lincei., Rend. Classe sci. fis., mat. e nat. 9*, 188 (1950); *Chem. Abstr. 46*, 11346b (1952).
60. Marré, E., Teunber, F. G., Murneek, A. E., *Nuovo giorno bot. ital. 61*, 9 (1954); *Chem. Abstr. 50*, 7241c (1956).
61. Maruo, B., *J. Agr. Chem. Soc. (Japan) 23*, 271 (1950); *Chem. Abstr. 44*, 8391 (1950).
62. Meyer, K. H., Bernfeld, P., *Helv. Chim. Acta 25*, 399 (1942).

63. MEYER, K. H., TRAZ, C., *Helv. Chim. Acta 27*, 840 (1944).
64. MIRSKI, A., *Biochem. J. 36*, 232 (1942).
65. MURTHY, H. B. N., SWAMINATHAN, M., SUBRAHMANYAN, V., *J. Sci. Ind. Research* (India) *13B*, 223 (1954); *Chem. Abstr. 48*, 11573i (1954).
66. NAKAMURA, M., *J. Agr. Chem. Soc. (Japan) 25*, 413 (1952) *Chem. Abstr. 46*, 11266a.
67. NAKAMURA, M., *J. Agr. Chem. Soc. (Japan) 26*, 260 (1952) *Chem. Abstr. 48*, 757e (1954).
68. NAKAMURA, M., *J. Agr. Chem. Soc. (Japan) 27*, 70 (1953); (1952) *Chem. Abstr. 48*, 758e (1954).
69. NAKAMURA, M., YAMAZAKI, K., MARUO, B., *J. Agr. Chem. Soc.* Japan *24*, 197, 299, 302 (1951) *Chem. Abstr. 45*, 6668 (1951).
70. NASR, H., BAKER, F., *Nature 164*, 745 (1949).
71. OSTERN, P., HOLMES, E., *Nature 144*, 34 (1939).
72. PEAT, S., WHELAN, W. J., TURVEY, J. R., *J. Chem. Soc. 1956*, 2317.
73. PETROVA, A. N., BOLOTINA, T. T., KOBZEVA, A. A., *Biokhimiya 19*, 64 (1964); *Chem. Abstr. 47*, 7606f (1953).
74. PHILLIPS, T. G., AVERILL, W., *Plant Physiol. 28*, 287 (1953).
75. PORTER, H. K., *Biochem. J. 47*, 476 (1950).
76. POTTER, A. L., LEDERBERG, J., *J. Biol. Chem. 179*, 921 (1949)
77. RAM, J. S., GIRI, K. V., *Arch. Biochem. Biophys. 38*, 231 (1952).
78. RONGINE, M. A., FEKETE, DE, *Arch. Biochem. Biophys. 116*, 368 (1966).
79. ROZENFELD, E. L., SHUBINA, A. J., *Biokhimiya 19*, 289 (1954); *Chem. Abstr. 48*, 11559i (1954).
80. RUBIN, B. A., GLUSCHENKO, I. E., SAVELEVA, O. N., *Doklady Akad. Nauk SSSR 72*, 735 (1950). *Chem. Abstr. 44*, 9006f (1950).
81. SCHÄFFNER, A., *Naturwiss. 27*, 195 (1939).
82. SCHÄFFNER, A., SPECHT, H., *Naturwiss. 26*, 494 (1938).
83. SHAW, M., *Can. J. Botany 32*, 523 (1954).
84. SHINJO, S., UEZUMI, N., SUZUKI, T., *Eiyo to Shakuryo 10*, 68 (1957); *Chem. Abstr. 55*, 21274h (1961).
85. SHOICHIRO, H., *Botan. Mag. (Tokyo) 67*, 57 (1954); *Chem. Abstr. 49*, 7657h (1955).
86. SHOICHIRO, H., *Botan. Mag. (Tokyo) 68*, 347 (1955); *Chem. Abstr. 49*, 7657h (1955).
87. SISAKYAN, N. M., KOBYAKOVA, A. M., *Doklady Akad. Nauk SSSR 61*, 1065 (1948); *Chem. Abstr. 43*, 3050 (1949).
88. SOKOLOVA, V. E., *Biokhimiya Plodov i Ovoshchei Sbornik 1*, 45 (1949); *Chem. Abstr. 46*, 2632f (1952).
89. SRIVASTAVA, L. M., KRISHNAN, P. S., *J. Sci. Ind. Research (India) 20c*, 306 (1961); *Chem. Abstr. 56*, 6372g (1962).
90. STAERK, J., SCHLENK, H., *Biochim. Biophys. Acta 146*, 120 (1967).
91. SUMNER, J. B., CHOU, T. C., BEVER, A. T., *Arch. Biochem. 26*, 1 (1950).
92. SUZUKI, SH., TAMURA, T., HIROMATA, T., NEMOTO, Y., ARAI, K., *Nippon Nogei Kagaku Kaishi 31*, 859 (1957); *Chem. Abstr. 53*, 20301a (1959).
93. TANAKA, K., *Symposium on Enzyme Chemistry (Japan) 10*, 189 (1954); *Chem. Abstr. 49*, 16078h (1955).
94. TANKO, B., *Biochem. J. 30*, 692 (1936).
95. TEUNBER, F. G., MARRÉ, E., MURNEEK, A. E., *Nuovo giorn. bot. ital. 61*, 9 (1954); *Chem. Abstr. 50*, 7241e (1956).
96. VISWANATHAN, P. N., SRIVASTAVA, L. M., KRISHNAN, P. S., *Plant Physiol. 37*, 283 (1962); *Chem. Abstr. 57*, 5026f (1962).
97. WAJZER, J., *Arch. Sci. Physiol. 3*, 93 (1949); *Chem. Abstr. 44*, 2057 (1950).
98. WATABE, T., FUJIWARA, T., OSHIHI, R., *Nippon Sakumotsu Gakkai Kiji 29*, 219 (1961); *Chem. Abstr. 61*, 3417e (1964).
99. WEIBULL, G., TISELIUS, A., *Arkiv Kemi, Mineral. Geol. A 19*, 1 (1945).
100. YIN, H. C., SUN, C. N., *Science 105*, 650 (1947).
101. YOSHIDA, T., TAKAHASHI, J., *Nippon Dojo Hiryogaku Zasshi 29*, 341 (1958); *Chem. Abstr. 55*, 1816h (1961).
102. ZALESKI, J., *Przemysl Spozywczy 13*, 179 (1959); *Chem. Abstr. 54*, 1763h (1960).
103. ZALESKI, J., *Roczniki Technol. Chem. Tyunosei 4*, 75 (1960); *Chem. Abstr. 54*, 1763h, 4791b (1960).

III. PREPARATION

A crystalline enzyme or at least a homogeneous enzyme preparation free of protein contaminants is an indispensable prerequisite for the accurate study of the detailed mechanism of enzymatic synthesis and phosphorolysis, and for the elucidation of the enzyme structure. In the case of plant enzymes (apart from a few exceptional cases), this is a very difficult task which has only become possible during the last few years by the use of high-capacity ion exchangers.

Nearly all the methods of protein chemistry (salting-out based on the differences in the solubilities of proteins; precipitation by solvents; selective separation by heavy metal salts; various adsorption procedures; zone electrophoresis; ion-exchange; and molecular sieves) have been applied to obtain, purify and crystallize plant phosphorylases.

It has been pointed out in Chapter II that a great number of starting materials are available for the preparation of phosphorylase products.

Potato tubers are used by the majority of authors as starting material because enzyme proteins are present in a dissolved state in potato tubers and because relatively mild and simple methods are sufficient to destroy the cells and obtain potato juice rich in enzyme. The drawback of potatoes, however, is that the enzyme content is not stable and the enzymatic activity continually changes during the storage period [22, 47, 48].

The use of other starting materials can lead to problems: on the one hand the removal of plant fats is complex and high losses in activity are faced (e.g. in the case of peas, beans or waxy maize), and on the other hand high losses and poor yields occur during extraction and dissolution of proteins (cereals, rice) due to the presence of enzyme proteins present in the bound state.

On taking into account all these aspects, potato tubers appear to be the most suitable starting material. Hence the various methods of purification are described in detail for this material.

1. Pretreatment and Production of Potato Juice

In the papers dealing with the production of potato phosphorylase a more or less identical technique is suggested for the preparatory and juice-producing operations. Different methods, however, are applied for the inhibition of enzyme contaminants (phenoloxidases and amylases).

In the first step potatoes are peeled and sliced. However, prior to juice production, the effect of phenoloxidases must be inhibited because non-inhibited phenoloxidases induce appreciable changes in enzyme proteins.

No inhibition of phenoloxidases was applied by Hanes [13], Hidy and Day [14], Meyer and Traz [31], Maruo [30], Nakamura [33] or Husemann [19 – 21]. The action of phenoloxidases was inhibited by Green and Stumpf [11] (treatment for 2 hours at room temperature with a 0·005 M solution of potassium cyanide), and by Weibull and Tiselius [45] (treatment at 0°C with a solution of sodium hyposulphite of unknown concentration).

Sodium dithionite has been used by all other authors as inhibitor.

A 0·5% solution of sodium dithionite (20 minutes at room temperature) was applied by Barker *et al.* [2], and by Holló *et al.* [16].

Phenoloxidases were inhibited by Lee [28] and by Staerk and Schlenk [43] during a one-minute homogenization at 3°C by a buffer containing 0·5% sodium dithionite and 0·5% sodium citrate, using 100 ml of buffer for 1 kg of potatoes.

Baum and Gilbert [3] used a 0·7% solution of sodium dithionite for 10 minutes at room temperature.

Potato slices were treated for an hour by Kamogawa *et al.* [25] with a buffer containing 0·7% sodium dithionite and 0·7% sodium citrate.

Inactivation was brought about by Fischer and Hilpert [7] by a 30-minute treatment with 5% sodium dithionite at room temperature.

After the inactivation of phenoloxidases, the potato slices are thoroughly washed and made up to a pulp. Juice is generally produced by pressing [2, 7, 11, 14, 28, 30, 31, 43, 45], filtering through linen sacks [16] and by centrifugation [25]. Pure potato juice was obtained by cooling to a temperature of 0 to 3°C by Hanes [13], Baum and Gilbert [3], Lee [28], Staerk and Schlenk [43], Holló *et al.* [16] and Kamogawa *et al.* [25]; or simply at room temperature, by Green and Stumpf [11], Husemann and Pfannemüller [20], Fischer and Hilpert [7] and Barker *et al.* [2].

In the next step, the potato starch is removed by sedimentation or centrifugation. Subsequently, prior to the fractionation or to other purifying operations, heat treatment has been applied by a number of authors in order to inhibit the effect of amylases.

The removal of amylase contaminants by heat treatment has been investigated in detail by Husemann *et al.* [20] who observed the conditions of heat treatment by control tests. They proved in this way that it is possible to subject potato juice of pH 7·0 to 54–56°C for long periods without any essential deterioration of phosphorylase (Table III/1).

Table III/1

Heat Treatment of Potato Juice [20]

Temperature of 15-minute heat treatment (°C)	Phosphorylase activity* (Units/ml)	Hydrolyzing activity** (sec/60 min)
Untreated	22·5	13·3
37	22·8	12·8
45	22·5	8·4
50	23·2	3·2
54	21·9	2·1
56	18·0	—
60	2·9	—

* Activity measured by the Hidy and Day method [14].
** Hydrolyzing activity (expressed by the decrease in seconds of the time of outflow after inhibition for 60 minutes, referred to hydroxyethylamylose as control standard).

Amylase is thus inactivated as proved by the fact that a sample added to a solution of hydroxyethylamylose did not cause any decrease in viscosity (Fig. III/1).

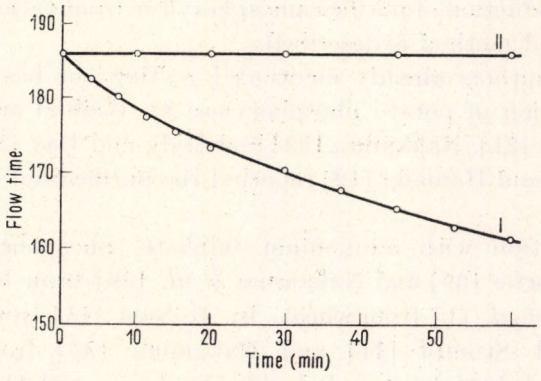


Fig. III/1. Investigation (by viscosimetry, using a solution of hydroxyethylamylose) of the amylase content of heat-treated (II) and untreated (I) potato juice [20]. The heat-treatment was carried out for 10 minutes at 54°C

A five-minute heat treatment at 50°C was applied by Green and Stumpf [11], Weibull and Tiselius [45] and Nakamura [33].

Husemann *et al.* [20] applied at first a treatment of 54–56°C for 10–15 minutes. According to their data, a temperature of 54°C is sufficient for the inactivation of amylases in freshly harvested potatoes. However, in stored potatoes (in late springtime) a temperature of 56°C must be applied. In their later paper [21] a treatment for 40 minutes at 55·5 ± 0·2°C (at pH 7·0) is described. Potato juice was heat-treated by Holló *et al.* [16] for 15 minutes at 54 ± 0·1°C (pH 7·0) while a 10-minute treatment at 55–56°C (pH 7·2) has been applied by Kamogawa *et al.* [25]. According to all the authors, the heat-treated potato juice was quickly cooled in ice-water, the precipitated proteins removed by centrifugation, and the pure supernatant solution was processed in the later operations of enzyme purification.

2. *Salting Out*

Fractionation by ammonium sulphate, the simplest method of enzyme purification known and applied since the earliest time, has been employed by a great number of authors, and it is still used in the most recent and up-to-date techniques as a method of prefractionation.

Green and Stumpf were the first [11] to employ (in 1942) purification with ammonium sulphate for the production of phosphorylase from potatoes and lima-beans. Since then, a large number of papers have been published in which the production of purified phosphorylase from various plants by the above-mentioned method is described.

Besides the authors already mentioned, salting out has been employed for the production of potato phosphorylase by Weibull and Tiselius [45], Meyer and Traz [31], Nakamura [33] and Hidy and Day [14], while Kurasawa *et al.* [26] and Hamada [12] reported the purification of rice phosphorylase.

By fractionation with ammonium sulphate, phosphorylase has been produced by Porter [39] and Nakamura *et al.* [36] from barley; by Shaw [40] and Aimi *et al.* [1] from wheat; by Hobson [15] from broad beans; by Green and Stumpf [11] and Nakamura [35] from lima beans; by Sumner *et al.* [44] from jack beans; by Inoue and Onodera [23, 24], Nakamura [34] and Shoichiro [41] from sweet potato; by Kursanov and Pavlovina [27] from sugar beet; by Inoue and Onodera [23, 24] from squash; by Phillips and Averill [38] from pumpkins; by Srivastava and

Krishnan [42] from tapioca; by Bliss and Naylor [4] from waxy maize; by Peat *et al.* [37] from sweet corn; and by Fredrick from *Oscillatoria princeps* [9].

Of the great number of above-mentioned methods based on ammonium sulphate, that suggested by Hidy and Day [14] is the best and the most detailed technique for purifying potato phosphorylase.

The specific gravity of potato juice obtained by the method described in Section III. 1 was adjusted at room temperature to 1·085 with ammonium sulphate. The precipitated material was removed by centrifugation, then the specific gravity of the supernatant solution was adjusted with ammonium sulphate to 1·152, and the precipitate (containing the phosphorylase) was dissolved in water (employing 250 ml of distilled water to 1000 ml of potato juice). The fractionation was repeated between the specific gravity limits 1·095 and 1·145, followed by two further fractionations between the limits 1·100 and 1·140, and 1·100 and 1·135, respectively. The enzyme fraction was dissolved in distilled water and stored at a temperature of 1–2°C.

It follows from the description of the method that neither the phenoloxidases nor the amylases were inhibited by Hidy and Day. For this reason, Husemann *et al.* combined the Hidy and Day method with a heat treatment. According to this modification, the potato juice adjusted with citrate buffer to pH 7·0 was heat-treated for 40 minutes at 55·5 ± 0·2°C, and subjected to a single fractionation with ammonium sulphate between the specific gravity limits 1·1 and 1·15. The phosphorylase obtained in this way was ten times purer and free of amylase.

On investigating the methods based on ammonium sulphate, a critical study of the technique appeared to be vital since the majority of papers (mainly the earlier ones) did not contain any data (enzymatic activity, specific activity, percentage yield, enzyme contaminants) to serve as a basis of evaluation of the enzyme purification techniques based on salting out. Hamada [12], however, has reported that his rice phosphorylase is of 25-fold purity and that it is contaminated by amylase, phosphatase and phosphoglucomutase.

The technique of Hidy and Day has been repeated by Holló *et al.* with the modification that the phenoloxidases were inhibited by a 20-minute treatment with a 0·5% solution of sodium dithionite [17]. In these repeated experiments, the enzymatic activity (by the method described in Chapter VII) and the protein content (according to Lee [28]) were determined throughout the operations, and the specific activities and yields were established from the measured values (Table III/2).

Table III/2

Repetition of the Method of Hidy and Day [17]

Fractionation		Volume (ml)	Enzyme activity (Enz. unit)	Protein content (mg)	Specific activity (Enz.unit/mg)	Yield (%)
Potato juice		410	76 700	18 700	4·1	100
First	purification with ammonium sulphate	250	19 600	5 310	3·8	25·6
Second		200	9 800	835	11·7	12·8
Third		100	6 400	325	19·8	8·4
Fourth		10	3 080	89	34·2	4·2

Initial amount: 1 kg of peeled potatoes.
Activity and protein content measured by the Lee method [28].

It appears from the results that with this method 4% of the phosphorylase activity can be extracted, and the enzyme purification is 8·4-fold.

3. Precipitation with Organic Solvents

The available data regarding the use of organic solvents are rather contradictory.

According to the investigations of Meyer and Traz [31], even minute amounts of organic solvents, mainly ethanol and acetone, cause an irreversible inhibition.

However, in the methods of Baum and Gilbert [3] and of Kamogawa *et al.* [25] to be described later, precipitation by ethanol is utilized for the production of the enzyme. According to these authors, a crystalline enzyme can be produced by any of the suggested techniques. In the strongly cooled medium (−5 to −10°C) employed in these experiments, phosphorylase was not inhibited by an ethanol concentration of 11–16% in the presence of a substrate.

No inactivation of phosphorylase was observed by Fujimura *et al.* [10] during the investigation of homogenized acetone-extracted rice leaves.

In the experiments of Maruo [30], the enzyme precipitate prefractionated with ammonium sulphate was dissolved in citrate buffer and the solution cooled to 0°C; the enzyme protein was precipitated with 0°C dioxan (employing 2 ml of dioxan for 1 ml of enzyme solution) and washed with ether. The dry enzyme preparation proved to be very stable; its activity had not disappeared after a 2-year storage at room temperature.

4. Selective Precipitation with Heavy Metal Salts

A novel method has been evolved by Barker *et al.* [2] for the purification of potato phosphorylase. The essence of this technique is that the phosphorylase and the Q-enzyme can be precipitated from potato juice with lead acetate and subsequently selectively redissolved. After the removal of starch, the pH of potato juice was adjusted to 7·2, with the use of 0·02 N sodium hydroxide. After the addition of 30 ml of a solution of lead acetate (prepared by dissolving 63·3 g lead acetate in 1 000 ml of water to 100 ml of potato juice, the lead-protein precipitate formed was centrifuged and suspended in 0·2 N sodium carbonate solution (10 ml to 100 ml of original potato juice). After stirring for 5 minutes, carbon dioxide gas was allowed to bubble through the suspension for a further 2·5 minutes, and then the precipitate was removed by centrifugation. The supernatant solution which contained the redissolved phosphorylase and the Q-enzyme was further fractionated with ammonium sulphate.

At first, the ammonium sulphate concentration of the supernatant solution was adjusted to 18 g per 100 ml, with a saturated solution of ammonium sulphate (prepared by dissolving 50 g of ammonium sulphate in 100 ml of distilled water of pH 7·0). The Q-enzyme precipitate formed was removed by centrifugation, and the concentration of the supernatant solution increased to 35 g per 100 ml by adding saturated ammonium sulphate solution. The phosphorylase precipitate obtained was removed by centrifugation, dissolved in distilled water and repeatedly fractionated, similarly to the previous treatment. The last phosphorylase fraction was dissolved in a 0·2 M sodium citrate buffer of pH 7·0 and stored at a temperature of 0–2°C. No data are given by Barker *et al.* as regards enzyme activity, enzyme purification or yields.

Phosphorylase enzyme has also been produced from tapioca by Murthy *et al.* [32] using the Barker method.

By a repetition of this method, potato phosphorylase was produced by Whelan *et al.* [46] who subsequently subjected the enzyme solution to freeze drying. For this product an enzyme activity value of 7·5 units per g of dry substance was given, measured by the Green and Stumpf [11] method. However, no conclusions can be drawn from this value because the protein content is unknown, and the major part of the product consists of sodium citrate.

With respect to the critical investigation of the method, the selective precipitation by heavy metal salts has been repeated by Holló *et al.* Enzyme activities and protein contents according to Lee [28] were established

throughout the steps, and the specific activity and yield were determined in the solutions (Table III/3).

Table III/3

Repetition of the Method of Barker *et al.* [17]

Fractionation	Volume (ml)	Enzyme activity (Enz.unit)	Protein content (mg)	Specific activity (Enz.unit/mg)	Yield (%)
Potato juice	440	65 600	18 700	3·5	100
Lead protein	55	25 200	2 400	10·4	38·4
First precipitation by	50	9 450	470	20·1	14·4
Second ammonium sulphate	9	3 350	101	32·2	5·1

Initial amount: 1 kg of peeled potatoes.
Activity and protein content measured by the Lee method [28].

According to this investigation, a 9·6-fold enzyme purification can be attained, and 5·1% of the enzyme activity can be extracted [17].

5. Adsorption

The methods described thus far in the present paper were unsuitable for the production of a homogeneous, possibly crystalline enzyme of adequate purity. Thus, it was necessary to develop new techniques, one of which consists in employing various adsorbents.

The first attempt was carried out by Hanes [13] who treated potato juice with kaolin.

Two methods for the production of potato phosphorylase were published simultaneously in 1953. For the adsorption of the enzyme, retrogradated amylose from marrowfat peas was employed by Baum and Gilbert [3], while Fischer and Hilpert [7] used granular starch for the same purpose. Adsorption by starch grains was used in 1960 by Datta [5] for the purification of phosphorylase from *Trapa bispinosa*, and in 1966 by Fekete [6] for *Vicia faba* phosphorylase. Neither enzyme was crystalline.

The potato juice produced in the way described in Section III. 1 was subjected to freeze drying by Baum and Gilbert [3], and this potato extract served as starting material for the production of the enzyme.

The potato extract was dissolved in water and cooled to 0°C; the ethanol concentration was adjusted to 11% with a 50% ethanol-citrate buffer (i.e. a 0·1 M citrate buffer of pH 6·0, containing 50 w/v % of ethanol). The precip-

itate formed was removed by centrifugation in the cold, and the supernatant solution treated with a 20% suspension of marrowfat pea amylose in water (using 2 ml of amylose suspension to 1 ml of solution). Subsequently, the ethanol concentration of the suspension was again adjusted to 11% by the addition of 50% ethanol-citrate buffer with vigorous stirring. After continuing stirring for a further 10 minutes at 0°C the precipitate was collected by centrifugation, and washed with 11% ethanol-citrate buffer. The precipitate was twice extracted for 15 minutes with three times the volume of 0·05 M citrate buffer of pH 6·0, the salt concentration of the extract adjusted to 30% with saturated ammonium sulphate solution (pH = = 7·0), and the mixture was allowed to stand for one hour at 0°C. The precipitate formed was again dissolved in citrate buffer, and the solution adjusted to 19% ammonium sulphate concentration. The resulting precipitate (consisting predominantly of Q-enzyme) was removed by centrifugation. The last step was an increase of the concentration of ammonium sulphate in the supernatant solution to 25% at 0°C when phosphorylase could be obtained in the crystalline state. However, no data referring to yield, purity etc. are given by the authors who mention only that the enzyme crystallized in such a small amount that it was impossible to determine its activity and contaminants.

Fischer and Hilpert [7] adjusted the saturation of the crude potato juice to 62%, with ammonium sulphate (pH 6·2). The precipitate formed was collected by centrifugation, and fractionation with ammonium sulphate was repeated in the ranges 32–62 and 40–62%, respectively. The precipitated phosphorylase was treated with a 10% sodium chloride solution and twice fractionated with ammonium sulphate; the enzyme solution was subjected to chromatography on a column packed with granular starch, using a 10% solution of ammonium sulphate containing 0·006% Brij. On treating the eluate with ammonium sulphate the phosphorylase precipitated in crystalline form. This product had a 300-fold purity and was free of phosphatase and maltase, though it did contain some α-amylase. The percentage yield, the method of determination of activity and the basis to which the 300-fold purity referred, were not given by the authors.

Of the adsorption methods, that of Baum and Gilbert [3] was repeated by Holló *et al.* [17]; the operations were carried out throughout at 0°C, and the activity and the protein content were measured according to Lee [28] (Table III/4).

The experiment did not result in a crystalline product. 1·8% of the initial enzyme activity was recovered, and 13-fold enzyme purification was attained.

Table III/4

Repetition of the Method of Baum and Gilbert [17]

Fractionation	Total volume (ml)	Enzyme activity (Enz.unit)	Protein content (mg)	Specific activity (Enz.unit/mg)	Yield (%)
Potato juice	200	12 200	7 856	1·55	100·0
After centrifugation with 11% ethanol	255	11 600	7 800	1·50	95·0
Supernatant liquid obtained after second adsorption	1 461	6 800	6 920	0·99	58 5
Enzyme eluate	110	312	72	4·30	2·6
Fractionation with ammonium sulphate	10	224	12	20·30	1·8

Initial amount: 0·5 kg of peeled potatoes.
Activity and protein content measured by the Lee method [28].

The conditions of adsorption have been studied in detail with regard to the extremely poor yield. The experimental data proved that 58% adsorption occurs in the case of dissolved amylose (the applied amylose concentration range was 4–5 mg per ml).

Elution was essentially much less effective. In general, only 17% of the adsorbed enzyme could be eluted with citrate buffer.

6. Zone Electrophoresis

In the Lee method [28], fractionation by ammonium sulphate was combined with zone electrophoresis. Pure potato juice was fractionated with ammonium sulphate (using 20 g of ammonium sulphate per 100 ml of potato juice), the system was allowed to stand overnight, and the precipitate formed was removed by centrifugation and filtration through Celite. Finely powdered amylose (1 g of amylose to 100 ml of potato juice) was added to the clear filtrate with stirring which was continued for an hour. The ammonium sulphate concentration of the mixture was adjusted to 18%, stirring was maintained for a further hour, and then the suspension was centrifuged, and the precipitate washed with 5 × 50 ml of 1·8 M ammonium sulphate solution (of pH 6·5). The residue was extracted with small volumes of 0·1 M citrate buffer (of pH 6·5) (total volume 50 ml) while the extract still showed phosphorylase activity. After combining the phosphorylase-containing extracts, the fractionation with ammonium sulphate was repeated; then the precipitated phosphorylase was dissolved in 0·002 M potassium phosphate

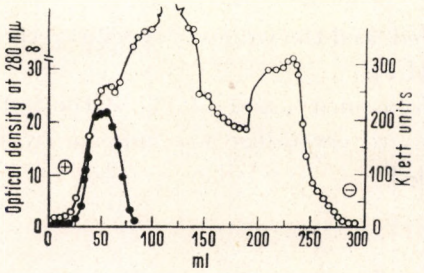


Fig. III/2. Electropherogram of the first zone electrophoretic separation of potato phosphorylase according to Lee [28]. ○ – ○: Protein concentration (O. D. at 280 mμ), ●–●: phosphorylase activity expressed in Klett units. 700 mg of the 2nd ammonium sulphate fraction, 38 mA for 22 h

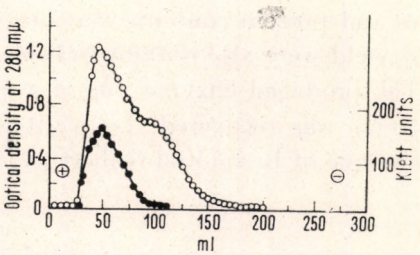


Fig. III/3. Electropherogram of the second zone electrophoretic separation [28]. ○ – ○: Protein concentration (O. D. at 280 mμ); ●–●: phosphorylase activity expressed in Klett units 65 mg of the first zone electrophoretic fraction, 38 m Å for 22 h

buffer (of pH 6·8) and the salts removed by dialysis. The precipitate formed was removed by centrifugation and the enzyme solution (containing 6·5% of protein) subjected to electrophoresis in a Porath apparatus packed with Munktell cellulose powder (Fig. III/2) (current intensity 38 mA; length of electrophoresis 21–22 hours; applied enzyme 700 mg/70 g cellulose powder). The column was eluted with 0·002 M potassium phosphate buffer (of pH 6·8) at a flow-rate of 50 ml buffer per hour. The combined enzyme fractions were treated with ammonium sulphate (28 g per 100 ml of solution); after 1 hour the suspension was centrifuged, and the supernatant solution again treated with ammonium sulphate (18 g per 100 ml). Subsequent to centrifugation, the precipitate was dissolved in 0·002 M potassium phosphate buffer, repeatedly subjected to electrophoresis, and eluted in a way similar to that employed previously (Fig. III/3). In all the operations, enzyme activ-

Table III/5

Purification of Potato Phosphorylase by the Lee Method [28]

Fraction	Total protein (mg)	Total enzyme (Enz.unit)	Specific activity (Enz.unit/mg)	Yield (%)
Crude potato juice	29 880	848 000	28·5	100
Precipitation–adsorption	4 160	705 000	170	74·5
Second amm. sulphate precipitation	2 560	487 000	191	57·5
First column electrophoresis	280	193 000	687	22·3
Second column electrophoresis	80	125 000	1 560	14·7

Initial amount: 5 kg of Idaho potatoes.

ities and protein contents were measured, and the values of specific activity and yield were also obtained (Table III/5).

The produced enzyme was of a 55-fold purity, and 14·7% of the initial activity was recovered. Lee's attempts to crystallize the enzyme by the technique of Baum and Gilbert [3] failed.

7. *Molecular Sieves*

Quite recently, the production of gel-type molecular sieves (Sephadex, Biogel etc.) and of high-capacity ion exchangers made possible the preparation of enzyme fractions of a purity exceeding all previous values; the amount of enzyme produced could also be raised.

Obviously, these substances and techniques have also been employed in the production of phosphorylase.

Molecular sieves Sephadex G-25 and Biogel P-2 were used by Holló *et al.* [18] for the purification of phosphorylase. Potato juice was prepared by the method described under Section III. 1. After purification by fractionation with ammonium sulphate according to the method of Hidy and Day [14], it was dissolved in 0·01 M citrate buffer (of pH 7·0), and the sulphate removed in a similar buffer. Amylase was inactivated by heat treatment (20 minutes at 56°C), then the system quickly cooled in a bath of ice-water, and the precipitate formed removed by centrifugation. The prefractionated enzyme showed a specific activity of 37·5 units per mg of protein and a 10·7-fold purity. The prefractionated, amylase-free phosphorylase was transferred onto gel filter columns packed with Sephadex G-25 and Biogel P-2. The zone precipitation technique was employed to enrich the enzyme.

In the preliminary experiments the optimum salt concentration proved to be 20% for both types of molecular sieve. The use of higher or lower concentrations of ammonium sulphate than the optimum value resulted in the case of both types of molecular sieve in a poorer separation and lower yields [18]. Accordingly, the columns were equilibrated with 0·01 M citrate buffer (of pH 7·0) containing 20% of ammonium sulphate. Equilibration and changes in salt concentration occurring during the experiments were followed by conductance measurements.

The 2 ml sample of prefractionated enzyme transferred onto the column (its characteristics are given in Table III/6) was eluted with 0·01 M citrate buffer of pH 7·0 (the dimensions of the column were 40 cm × 1 cm, elution rate 16 ml per hour, temperature 3–5°C). With molecular sieve Sephadex

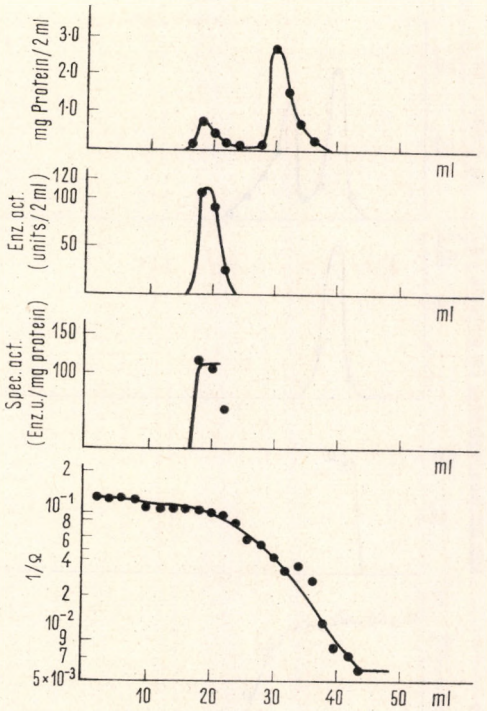


Fig. III/4. Purification, by zone precipitation on molecular sieve Sephadex G-25, of potato phosphorylase fractionated with ammonium sulphate [18]

G-25 (Fig. III/4), 38-fold purification was attained, with practically no loss in activity. This means a 3·6-fold enrichment referred to the prefractionation (Table III/6).

Table III/6

Purification of Potato Phosphorylase by Zone Precipitation with Molecular Sieves Sephadex G-25 and Biogel P-2 [18]

Fraction	Activity (Enz.unit/ml)	Protein content (mg/ml)	Specific activity (Enz.unit/mg)	Yield (%)	Enrichmet
Potato juice	14·1	4·0	3·5	100	
Heat-treated, dialyzed enzyme	124	3·3	37·5	14	10·7
Zone precipitation on Sephadex G-25	124	0·9	134·0	14	38·3
Zone precipitation on Biogel P-2	122	1·4	87·0	13·7	25·0

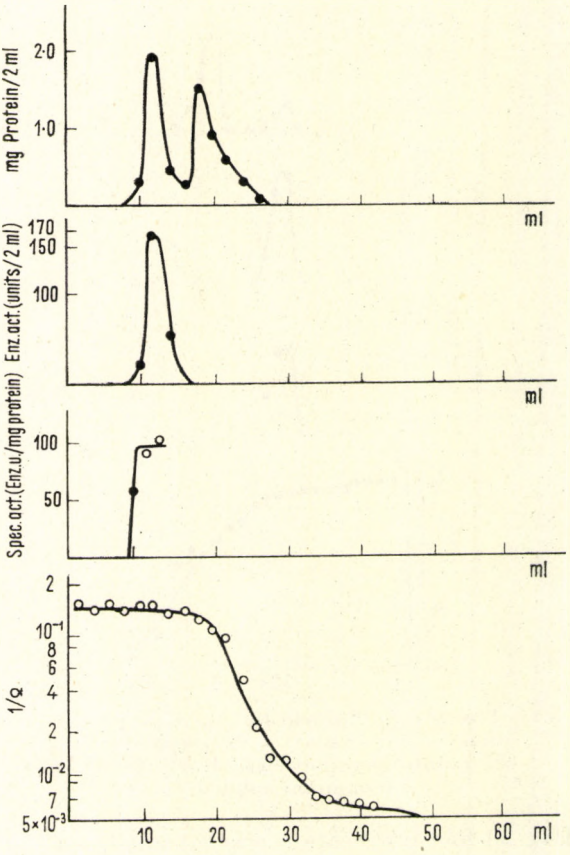


Fig. III/5. Purification, by zone precipitation on molecular sieve Biogel P-2, of potato phosphorylase fractionated with ammonium sulphate [18]

With molecular sieve Biogel P-2 (Fig. III/5), 25-fold purification was obtained, with practically no enzyme loss. In this case an approximately 2·3-fold enrichment was attained referred to the prefractionation (cf. Table III/6).

In the method of Staerk and Schlenk [43], the prefractionation technique of Lee was combined with ion exchange using DEAE-Sephadex A-50, and column chromatography with molecular sieve Sephadex G-200. The phosphorylase enzyme was produced from potato tubers. As the first step of purification, the Lee method [28] was applied with the modification that after the second precipitation by ammonium sulphate, instead of electro-

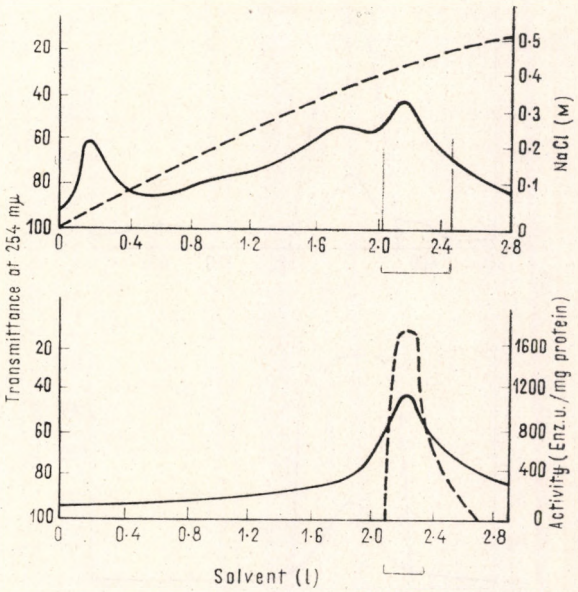


Fig. III/6. Purification of potato phosphorylase on DEAE-Sephadex A-50 by gradient elution with sodium chloride solution according to Staerk and Schlenk [43]. Chromatography was repeated with the most active fraction (lower part of the Figure). ———: The recorded transmittance at 254 mμ (LKB Uvicord); – – –: in the upper part indicates the salt concentration, and in the lower part the specific activity (units/mg protein)

phoresis, gradient elution with sodium chloride was employed on the ion exchanger DEAE-Sephadex A-50 (Fig. III/6). The phosphorylase-containing fractions were combined and the enzyme protein was precipitated with ammonium sulphate (40 g to 100 ml); the precipitate dissolved in 20 ml of tris buffer [tris(hydroxymethyl)aminomethane] of pH 6·9, residual ammonium sulphate removed by dialysis (the buffer was changed 3 times during 36 h), and the product repeatedly subjected to chromatography on ion exchanger DEAE-Sephadex A-50. (Conditions: The packing was equilibrated with buffer (pH 6·9) of 0·02 M tris, 0·02 M NaCl and 0·15 mM EDTA, the solution being one-fourth saturated with thymol. Active material from the fractionation was dialyzed against the buffer and about 700 mg was applied to a column, 3·8 cm × 86 cm (980 ml bed volume). The salt gradient was produced by pumping buffer containing 1 M NaCl, 45 ml/h, into a mixing reservoir with 4·2 l original buffer and from there to the column which

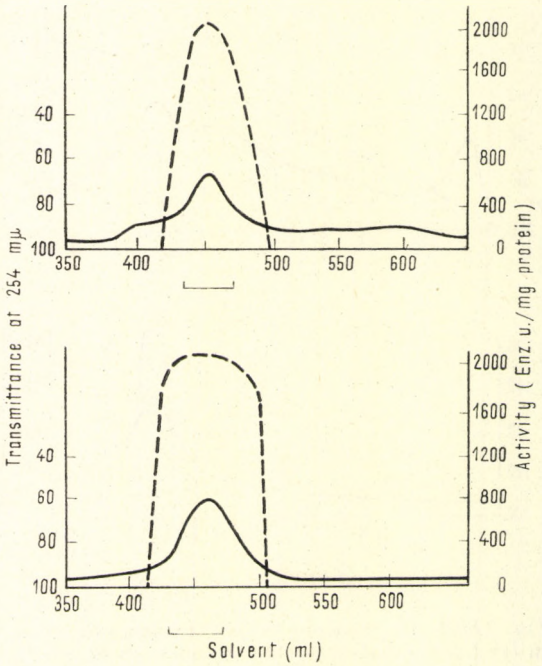


Fig. III/7. Final purification of potato phosphorylase by chromatography on Sephadex G-200 [43]. ———: The recorded transmittance at 254 mμ (LKB Uvicord); — — —: the specific activity (units/mg protein)

was kept at 8°C. The eluant was collected under cooling. Chromatography was repeated with the most active portion (lower part of the Figure). The full lines give the recorded transmittance at 254 mμ (LKB Uvikord). The dotted line in the upper part indicates the salt concentration. This line is omitted in the lower part where the broken line gives units/mg protein.)

The active enzyme fractions were combined and phosphorylase was subjected to column chromatography on molecular sieve Sephadex G-200 (Fig. III/7) (Conditions: The packing was equilibrated for 4 days with tris buffer and then filled into a column, 2·7 cm × 175 cm (1000 ml bed volume) at 8°C. The flow rate was reduced to 12 ml/h with a Teflon needle-valve stop-cock and remained then constant for many runs. The most active fraction was rechromatographed (lower part of the Figure). The full lines show the recording at 254 mμ; the broken line show specific activity, units/mg protein.) The molecular sieve process was repeated twice. By this technique

the authors attained about 60-fold purification and recovered 20% of the initial enzyme (Table III/7).

Table III/7

Purification of Potato Phosphorylase by the Method of Staerk and Schlenk [43]

Fractionation	Total enzyme units	Specific activity (Enz.unit/mg)	Yield (%)
Potato juice	694 000	34	100
First precipitation with ammonium sulphate	640 000	202	92
Second	446 000	322	64
First chromatography on DEAE-Seph. A-50	287 000	885	42
Second	233 000	1 560	34
First gel filtration on Seph. G-200	142 000	1 950	22
Second	138 000	2 050	20

Initial amount: 3·8 kg of potatoes.
Enzyme activity was determined by the Lee method and protein content by the method of Lowry *et al.* [29].

8. *Ion Exchange Techniques*

Ion exchange column chromatography was first employed for the purification of potato phosphorylase by Holló *et al.* [16]. According to their combined method, a prefractionation with ammonium sulphate was first applied, then amylase was inactivated by heat treatment, and finally column chromatography on a column of DEAE-cellulose was carried out stepwise, using potassium chloride buffers of various concentration. The production of potato juice and the inhibition of phenoloxidases and amylases were performed by the technique described in Section III.1.

The heat-treated potato juice was purified by fractionation with ammonium sulphate according to the method of Hidy and Day, with the modifications that the operations were carried out at temperatures between 0 and 2°C, and that the pH value of the solution during the fractionations was maintained at 7·0 by adding ammonium hydroxide. The last enzyme fraction was dissolved at 0°C in 0·01 M citrate buffer of pH 7·0, and the residual ammonium sulphate removed by dialysis at 0°C with the

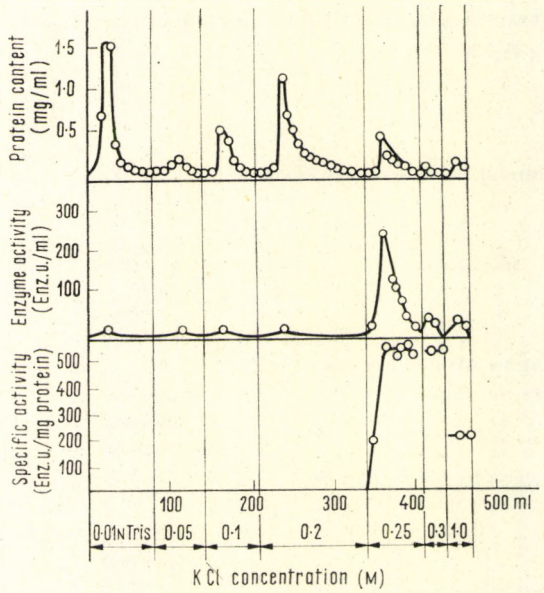


Fig. III/8. Purification of potato phosphorylase by DEAE-cellulose column chromatography according to Holló *et al.* [16]

same buffer (the buffer was changed five times during 15 h). The prefractionated enzyme was transferred onto a column equipped with a cooling jacket (100 mg protein per 65 ml), and eluted from section to section with a solution of potassium chloride (Fig. III/8). The elution temperature ranged from 0 to 2°C (column: 3 cm×40 cm). The column was equilibrated with 0·01 M tris-citrate buffer of pH 7·0 (the flow rate of the column was 40–50 ml per hour at an excess-pressure of 50–80 torr; concentration of buffer mixture applied for elution: 0·01 M tris, 0·05 M, 0·1 M, 0·2 M and 0·3 M potassium chloride). The enzyme fraction could be eluted with a buffer of 0·25–0·3 M potassium chloride. The combined enzyme fractions were subjected to dialysis in order to remove the salts. By this method, homogeneous phosphorylase free of amylase was produced. In the course of the operations, enzyme activity and protein contents according to Lee were measured [28], and specific activities and yields were determined from the measured values (Table III/8). It appears from these data that with the use of DEAE-cellulose column chromatography, the enzyme can be purified almost without loss. Referred to the initial values, 13·6% of the total activity can be recovered and 148-fold purification can be attained.

Table III/8

Purification of Potato Phosphorylase with DEAE-cellulose Column Chromatography by the Method of Holló *et al.* [16]

Fraction	Total volume (ml)	Enzyme amount (Enz.unit)	Protein content (mg)	Specific activity (Enz.unit/mg)	Yield (%)
Crude potato juice	470	58 600	16 800	3·5	100
First ammonium sulphate fraction	250	58 200	9 300	6·3	99·5
Second ammonium sulphate fraction	150	30 600	2 150	10·4	52·1
Third ammonium sulphate fraction	50	23 400	640	36·4	40·0
Fourth ammonium sulphate fraction	10	9 040	172	52·6	15·5
After dialysis	10	7 900	155	51·0	13·0
Amount transferred onto DEAE-cellulose column	6·5	5 100	100	51·0	100
Eluted enzyme amount	96	4 900	9·55	520	97

Initial amount: 1 kg of peeled potatoes.
Activity and protein content were determined by the Lee method [28].

A novel method suitable for the production of crystalline potato phosphorylase was recently published by Kamogawa *et al.* [25]. In this method, adsorption, heat treatment and ion exchange are utilized for the purification of the enzyme. Ethanol (−15°C, up to 11% by volume) was added at 0°C within 10 minutes to the heat-treated potato juice (2 320 ml), the mixture stirred for a further 10 minutes at −3°C, and the precipitate obtained separated by centrifugation at −3°C (10 minutes; 10 000 g). 5 litres of a 2% cold solution of waxy rice starch was poured into the supernatant liquid cooled to −5°C, the mixture stirred 5 minutes at −2 to −3°C, then 620 ml of cold ethanol (−15°C) added in 16 minutes to the suspension (−2 to −5°C) with continuous stirring. Stirring was continued for a further 10 minutes, the precipitate formed was collected by decantation and washed once with 500 ml of 11% cold (−5°C) ethanol. Subsequently, the precipitate was quickly dissolved in 2 litres of 0·005 M citrate buffer (pH 6·3) and the solution allowed to stand overnight at 0°C. The slightly viscous solution was transferred in 10 hours onto a DEAE-cellulose column (6·5 cm × 13 cm) at room temperature. The column was equilibrated with 0·005 M citrate buffer (pH 6·3). After the transfer of the enzyme the column was washed with 2 litres of 0·005 M tris-hydrochloric acid buffer (pH 7·5), then the protein eluted by linear gradient elution (1 litre of basic buffer containing 1 M sodium chloride was added to 1 litre of basic buffer). The elution rate was 2 ml per minute. Fractions of 20 ml were collected, the active fractions (fractions 35–50) combined and dialyzed overnight at 5°C with 6 litres of

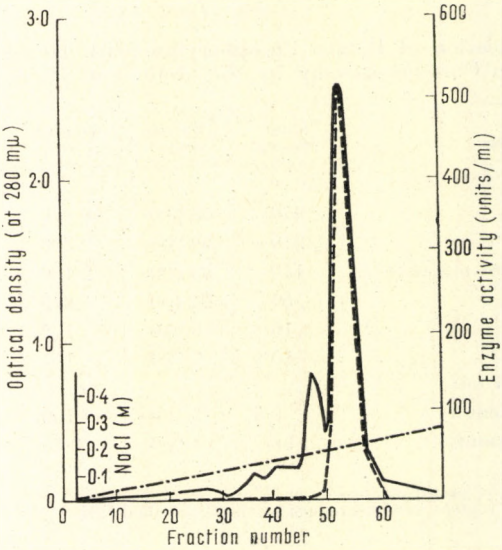


Fig. III/9. Purification of potato phosphorylase on DEAE-Sephadex *A*-50 by gradient elution with sodium chloride solution according to Kamogowa *et al.* [25]. ———: Optical density at 280 mμ; — — —: enzyme activity; —.—.—: concentration of NaCl

0·005 M tris-buffer (pH 7·5). The dialyzed enzyme solution was transferred in 2 hours onto a DEAE-Sephadex A-50 ion exchanger column (2·5 cm × 45 cm) and equilibrated with 0·005 M tris-hydrochloric acid buffer (pH 7·5). The enzyme proteins were eluted by linear gradient elution (adding to 1 litre of basic buffer, another litre of basic buffer containing 1 M sodium chloride, carrying out the elution at a rate of 2 ml per minute). The elution curve is shown in Fig. III/9. The active fractions (fractions 50–57) were combined and dialyzed at +5°C overnight against 2 litres of a saturated solution of ammonium sulphate (pH 7·5). The precipitate formed was collected by centrifugation (10 minutes; 15 000 g) and dissolved in 6·4 ml 0·005 M tris-hydrochloric acid buffer (pH 7·5). A solution of ammonium sulphate (pH 7·0) saturated at 0°C was added dropwise until a definite turbidity was observed. Then the temperature of the suspension was raised to 17°C and maintained for 3 days. Gradually needle-shaped crystals developed. Then the suspension was cooled to 4°C when the crystallization process was completed within some days.

In the opinion of the authors, the above-described conditions, and in particular the temperature of 17°C, are very important. The crystalline

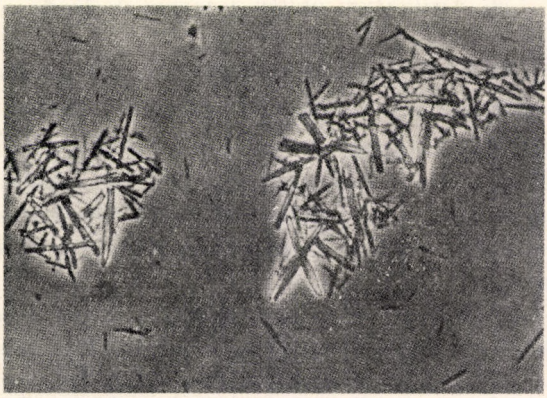


Fig. III/10. The microscopic appearance of once-crystallized potato phosphorylase according to Kamogowa *et al.* [25]. × 600 phase contrast

enzyme was removed by centrifugation (10 minutes, 10 000 g), washed with 5 ml of 50% ammonium sulphate solution, dissolved in 6·4 ml of 0·005 M tris buffer (the insoluble residue was separated and removed) and recrystallized as described above (Fig. III/10). When stored in a saturated ammonium sulphate solution the enzyme proved to be stable for months. Investigations by electrophoresis and ultracentrifugation proved that the enzyme is a homogeneous product. The values of activity and specific activity, and of yields in the individual steps are summarized in Table III/9. It appears from these data that 135-fold purification and 45% yield were attained.

Table III/9

Purification and Crystallization of Potato Phosphorylase by the Method of Kamogawa *et al.* [25]

Fraction	Total volume (ml)	Total activity (Enz.unit)	Total protein (mg)	Specific activity (Enz.unit/mg)	Yield (%)
Crude extract	2 420	5 700	32 700	0·174	100
Heat treated	2 320	5 600	26 700	0·210	98
Adsorption on starch	2 380	3 860	1 000	3·86	68
DEAE-cellulose eluate	315	4 160	202	20·6	73
DEAE-Sephadex eluate	117	3 370	140	24·0	59
First crystallization	6·4	3 190	134	23·8	56
Second crystallization	7·4	2 580	109	23·7	45

Initial amount: 5 kg of peeled potatoes.
Activity was determined under conditions chosen by the authors, and protein content by the method of Lowry *et al.* [29].

9. Evaluation of the Various Methods

In contrast to that of plant phosphorylases, the production of animal phosphorylases is relatively simple. According to Fischer and Krebs [8], crystalline rabbit-muscle phosphorylase *b* can be produced by a single precipitation with ammonium sulphate, followed by crystallization at 0°C with magnesium acetate in the presence of AMP. On repeating the crystalization with magnesium acetate three times, 23-fold purification and 33% yield were attained.

On the basis of the above-discussed methods, it can be stated that high purity or possibly crystalline plant phosphorylases free of enzyme contaminants can only be produced by combined methods. The production cannot be carried out by fractionation with ammonium sulphate or by selective precipitation with heavy metal salts because these techniques have proved unsuitable for attaining adequate enrichment degrees or for preparing homogeneous enzyme products. The products were contaminated mainly by amylase and phosphatase.

Table III/10

Comparison of Methods

Method	Enzyme yield (%)	Purification	Remarks
Ammonium sulphate method [17]	4·0	8·5	contaminated by amylase
Lead acetate method [17]	5·1	9·5	contaminated by amylase
Adsorption by amylose [3]	—	—	
Adsorption by amylose [17]	2	13	contaminated by amylase
Chromatography on granulated starch [7]	—	300	cryst. contaminated by α-amylase
Zone electrophoresis [28]	14·7	54·6	homogeneous
Zone precipitation [18]			
Sephadex G-25	14.0	38	free of amylase
Biogel P-2	13·5	25	free of amylase
Ion exchange gel filtration [43]	20	60	homogeneous
Ion exchange on DEAE-cellulose [16]	13·6	148	homogeneous, free of amylase
Ion exchange on Sephadex [25]	45	135	crystalline
Muscle phosphorylase *b* [8]	33	23	crystalline

A comparison and critical evaluation of the methods is almost impossible since the earlier papers contain no numerical data at all or they are not evaluable. In addition, nearly all the authors employed different conditions in the determination of enzyme activity. Thus, only the degrees of enzyme purification calculated from the ratio of specific activities, and the percentage yields calculated from the ratio of the enzyme activities published by the authors can be taken into account (Table III/10). For the sake of comparison, the similar data of rabbit-muscle phosphorylase *b* are also listed in this Table.

The answer to the problem of preparing a crystalline enzyme product may probably be found in the adsorption of the enzyme by a polysaccharide, but Holló *et al.*, though producing an enzyme solution of a specific activity and purity similar to those of the solution obtained by Kamogawa *et al.*, failed to isolate the crystalline enzyme.

The importance of the adsorption by polysaccharides appears to be proved by the fact that all three publications reporting the production of crystalline α-glucan phosphorylase [3, 7, 25] described some form of adsorption by a polysaccharide (marrowfat pea amylose, granulated starch and waxy rice starch). Of the adsorbents employed in the adsorption methods, the solid adsorbents (granulated starch, retrogradated amylose) are suitable for producing only small amounts of enzyme. Of the dissolved adsorbents which later precipitate or undergo retrogradation (marrowfat pea amylose, potatoes and waxy rice starch), only those are suitable for the purification of phosphorylase which are readily redissolved (i.e. the poor-yield elution can be avoided), and which can be separated from the enzyme by some technique, e.g. by ion exchange.

REFERENCES

1. Aimi, R., *Nippon Sakumotsu Gakkai Kiji 24*, 201 (1956); *Chem. Abstr. 53*, 16286f (1959).
2. Barker, S. A., Bourne, E. J., Wilkinson, J. A., Peat, S., *J. Chem. Soc. 1949*, 1507.
3. Baum, H. and Gilbert, G. A., *Nature 171*, 983 (1953).
4. Bliss, L. and Naylor, N. M., *Cereal Chem. 23*, 177 (1946).
5. Datta, J., *Ann. Biochem. Exptl. Med. 20*, 117 (1960); *Chem. Abstr. 61*, 12250f (1964).
6. Fekete, De, M. A. R., *Arch. Biochem. Biophys. 116*, 368 (1966).
7. Fischer, E. H., Hilpert, H. M., *Experimentia (Basel) 9*, 176 (1953).
8. Fischer, E. H., Krebs, E. G., *J. Biol. Chem. 231*, 65 (1958).
9. Fredrick, J. F., *Physiol. Plant. 4*, 621 (1951).
10. Fujimura, K., Kawabata, M., Ikeda, Sh., *Mem. Research Inst. Food Sci. Kyoto Univ. 15*, 23 (1958); *Chem. Abstr. 53*, 506b (1959).
11. Green, D. E., Stumpf, P. K., *J. Biol. Chem. 142*, 355 (1942).
12. Hamada, J., *Nippon Kogaku Zasshi 80*, 182 (1959); *Chem. Abstr. 59*, 14241h (1963).
13. Hanes, C. S., *Proc. Roy. Soc. London B 129*, 174 (1940).

14. Hidy, P. H., Day, H. G., *J. Biol. Chem. 160*, 273 (1945).
15. Hobson, P. N., Whelan, W. J., Peat, S., *J. Chem. Soc. 1950*, 3566.
16. Holló, J., László, E., Hoschke, Á., *Stärke 16*, 243 (1964).
17. Holló, J., László, E., Hoschke, Á., *Die Nahrung 9*, 317 (1965).
18. Holló, J., László, E., Hoschke, Á., Bánky, Zs., *Period. Polytech. (Chem.) 14*, 91 (1970).
19. Husemann, E., Fritz, B., Lippert, R., Pfannemüller, B., *Makromol. Chem. 26*, 199 (1958).
20. Husemann, E., Fritz, B., Lippert, R., Pfannemüller, B., Schupp, E., *Makromol. Chem. 20*, 181 (1958).
21. Husemann, E., Pfannemüller, B., *Makromol. Chem. 49*, 214 (1961).
22. Hyde, R. B., Morrison, J. W., *Am. Potato J. 41*, 163 (1964); *Chem. Abstr. 64*, 4169 (1966).
23. Inoue, Y., Onodera, K., *Repts. Inst. Chem. Research Kyoto Univ. 15*, 70 (1946); *Chem. Abstr. 45*, 4787 (1951).
24. Inoue, Y., Onodera, K., *Repts. Inst. Chem. Research Kyoto Univ. 16*, 28 (1947).
25. Kamogawa, A., Fukui, T., Nikuni, Z., *J. Biochem. 63*, 361 (1968).
26. Kurasawa, H., Igaue, J., Hayakawa, T., Ogami, H., *Nippon Nogei Kagaku Kaishi 33*, 393 (1959); *Chem. Abstr. 54*, 9005d (1960).
27. Kursanov, A., Pavlovina, O., *Biokhimiya 13*, 378 (1948); *Chem. Abstr. 42*, 8842 (1948).
28. Lee, Y. P., *Biochim. Biophys. Acta 43*, 18 (1960).
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem. 193*, 265 (1951).
30. Maruo, B., *J. Agr. Chem. Soc. (Japan) 23*, 271 (1950; *Chem. Abstr. 44*, 8391a. (1950).
31. Meyer, K. H., Traz, C., *Helv. Chim. Acta 27*, 840 (1944).
32. Murthy, H. B. N., Swaminathan, M., Subrahmanyan, V., *J. Sci. Ind. Research* (India) *13B*, 223 (1954); *Chem. Abstr. 48*, 11573i (1954).
33. Nakamura, M., *J. Agr. Chem. Soc. (Japan) 26*, 260 (1952); *Chem. Abstr. 46*, 11266a (1952).
34. Nakamura, M., *J. Agr. Chem. Soc. (Japan) 25*, 413 (1952).
35. Nakamura, M., *J. Agr. Chem. Soc. (Japan) 27*, 70 (1953); *Chem. Abstr. 48*, 758c (1954).
36. Nakamura, M., Yamazaki, K., Maruo, B., *J. Agr. Chem. Soc. (Japan) 24*, 197, 299, 302 (1951).
37. Peat, S., Whelan, W. J., Turvey, J. R., *J. Chem. Soc. 1956*, 2317.
38. Phillips, T. G., Averill, W., *Plant Physiol. 28*, 287 (1953).
39. Porter, H. K., *Biochem. J. 47*, 476 (1950).
40. Shaw, M., *Can. J. Botany 32*, 523 (1954).
41. Shoichiro, H., *Botan. Mag. (Tokyo) 68*, 347 (1955); *Chem. Abstr. 50*, 11443f (1956).
42. Srivastava, L. M., Krishnan, P. S., *J. Sci. Ind. Research (India) 20c*, 306 (1961); *Chem. Abstr. 56*, 6372g (1962).
43. Staerk, J., Schlenk, H., *Biochim. Biophys. Acta 146*, 120 (1967).
44. Sumner, J. B., Chou, T. C., Bever, A. T., *Arch. Biochem. 26*, 1 (1950).
45. Weibull, G., Tiselius, A., *Arkiv Kemi, Mineral. Geol. A 19*, 1 (1945).
46. Whelan, W. J., Bailey, J. M., *Biochem. J. 58*, 560 (1954).
47. Zaleski, J., *Roczniki Panstwowego, Zakladu Hig. 14*, 49 (1963); *Chem. Abstr. 59*, 120926 (1963).
48. Zaleski, J., *Roczniki Panstwowego, Zakladu Hig. 15*, 137 (1964); *Chem. Abstr. 61*, 7616 (1964).

IV. STRUCTURE AND PROPERTIES OF PHOSPHORYLASE

All the enzymes known at present are of the nature of proteins. Thus, certain properties of enzymes are particular protein properties. Accordingly, the structure of the protein molecule, the degree of dissociation of the functional groups and consequently their catalytic activity also are affected by the hydrogen ion concentration, the temperature and by the various activators and inhibitors. The isoelectric point relating to the aminoacid composition, the presence of various functional groups and the general texture of the enzyme molecule are similarly characteristic properties of the enzyme protein.

Many difficulties are encountered in a study of the structure of enzymes, merely as a result of their protein nature. Up to the present, the structures of plant phosphorylases (and with a few exceptions those of other enzymes in general), are unknown. The investigations are limited to the analysis for aminoacids, to indirect reaction kinetic or inhibitive proofs of functional groups in the active centres which are possibly required for the catalytic activity, and to some analytical tests.

In marked contrast to animal phosphorylase, very few literature data are available of the investigations of the structure of plant phosphorylases. Thus, it appeared necessary to include here the description of literature data referring to animal phosphorylase as well.

1. Molecular Weight

In raw potato juice, two sedimentation constants have been determined ($S_{20} = 2{\cdot}41$ and $5{\cdot}89$) by Weibull and Tiselius [107] and by Green and Stumpf [38].

The first homogeneity investigations of purified potato phosphorylase were also carried out by Weibull and Tiselius [107] using an ultracentrifuge. From an examination of potato phosphorylase purified with ammonium sulphate, three sedimentation constants were found ($S_{20} = 2{\cdot}5$, $5{\cdot}9$ and $9{\cdot}2$). When however, the activities of the fractions were determined by the Tise-

lius method [98] in parallel measurements, catalytic activity was detected only in the fraction of highest molecular weight ($S_{20} = 9{\cdot}2$). The results of electrophoretic experiments showed that only the component with the highest mobility (corresponding to the ultracentrifugal component of $S_{20} =$ $= 9{\cdot}2$) was active. The active fraction amounted to about 5–7% of the total protein content, it was electrophoretically homogeneous, and according to ultracentrifugation tests monodisperse [107].

For the investigation of homogeneity, Tiselius electrophoresis and ultracentrifugation experiments were carried out by Lee [66], who performed electrophoresis at three different pH values (6·9, 7·2 and 7·5) at ionic strength 0·1 and 1°C. In the experiments at pH 6·9 and 7·2, the electrophoresis detected two components while only one component appeared at pH 7·5.

The sedimentation constant was determined by ultracentrifugation (enzyme concentrations: 6·5, 5·0 and 3·5 mg/ml; pH 6·7; maximum speed of rotor: 52 640 rpm). The determined sedimentation constant was $7{\cdot}94 \times$ $\times 10^{-3}$ sec and only one symmetrical peak was obtained. On the basis of these experimental results this potato phosphorylase proved to be homogeneous and showed a molecular weight of 207 000. The diffusion constant required for the determination of molecular weight was established at 4°C (protein concentration 6·5 mg/ml; pH 6·7). The value of D_{20} was $3{\cdot}76 \times$ $\times 10^{-7}$ cm^2sec^{-1} [66].

The homogeneity of the crystalline potato phosphorylase prepared by Kamogawa *et al.* [51] was investigated by these authors by electrophoretic tests (enzyme concentration 11·8 mg/ml; 0·01 M potassium phosphate buffer of pH 6·9; 0·1 M NaCl; 5°C; 99 V and 11 mA). After 170 minutes of electrophoresis the enzyme moved to a symmetric arrangement and proved to consist of only one component. In ultracentrifuge experiments, a symmetrical limiting surface was obtained (enzyme concentration 10·4 mg/ml; 0·05 M tris-phosphate-HCl buffer of pH 7·5; maximum rotor speed 46 600 rpm) and the measured sedimentation constant was $S_{20} = 6{\cdot}5$. In a study of the effect of *p*-chloromercuribenzoate (*p*CMB) it was found that, in contrast to muscle phosphorylase [71], potato phosphorylase does not dissociate to subunits though its catalytic activity is suspended (experimental conditions: enzyme concentration 12·8 mg/ml; buffer as above; *p*CMB concentration 10^{-3} M; duration of treatment in 0·1 M NaCl at 15·9°C, 18 hours). On subsequent centrifugation (maximum rotor speed 46 700 rpm) and addition of cysteine in excess, the original ultracentrifugal distribution and activity could not be recovered, in contrast to animal phosphorylase. In the sedimentation test in 4 M urea, only one peak was obtained. However, the S_{20} value decreased to 5·5 [51].

The above experimental results proved that potato phosphorylase does not undergo any allosteric changes and does not dissociate into sub-units.

These statements are in complete contrast to the literature data referring to animal phosphorylase since, e.g. according to the investigations of Keller [53–55], rabbit muscle phosphorylase *a* can be decomposed to sub-units. The molecular weight of one of these sub-units, denoted as phosphorylase *b*, amounts to half that of phosphorylase *a*. On treating phosphorylase *b* and phosphorylase *a* with *p*CMB, both can be decomposed under identical conditions to the same monomers [69, 71]. A comparison of the various types of phosphorylase is given in Table IV/1.

Table IV/1

Comparison of Various Animal and Plant Phosphorylases

Type of phosphorylase	$S_{20} \times 10^{13}$ (Svedberg units)	$D_{20} \times 10^{7}$ ($cm^2 \cdot sec^{-1}$)	V_{20} ($ml \cdot g^{-1}$)	f/f_0	M.W.$\times 10^5$	Ref.
Rabbit muscle *a* (tetramer)	13·2	2·6	0·75	1·55	4·95	[6, 54, 55, 60, 101]
Rabbit muscle *b* (dimer) (from *a* with PR enzyme)	8·2	3·3	0·75	1·55	2·42	[17, 36, 48, 54, 55. 57, 60]
Rabbit muscle *b'* (after decomposition by trypsin)	8·2	3·1	—	1·65	2·57*	[17, 54, 60]
Rabbit muscle *c* (monomer) (after *p*CMB treatment from *a* and *b)*	5·6	4·0	0·75	—	1·35	[71, 72]
Dog liver	8·4				2·42	[96, 112, 113]
Human muscle *a*	13·5					[114]
Human muscle *b*	8·9					[114]
Potato	7·9	3·8		1·43	2·07*	[65, 66]
	6·5					[51]
	9·2					[108]

* In the determination, V_{20} was presumed to be 0·75.

Thus, phosphorylase *a* must be considered a tetramer and phosphorylase *b* a dimer. This is proved also by the fact that two moles of phosphorylase *b* can be converted into one mole of phosphorylase *a* [19–21].

The presence of the subunit of phosphorylase (molecular weight 120 000–125 000) has also been confirmed by light scattering experiments, and the

molecular dimensions of the subunit proved to be exactly half those of phosphorylase *b* and a quarter of those of phosphorylase *a* [72].

Though phosphorylase does not dissociate to fragments smaller than the subunits, in 'aged' phosphorylase solutions, in the absence of cysteine or other SH compounds, the formation of minute amounts of aggregates with polymerization degrees over four has been detected by ultracentrifuge investigations. Similarly, a polydisperse aggregate is also formed from phosphorylases treated with *p*CMB and denatured slightly [71]. With the use of a specific phosphatase (denoted as PR enzyme) which splits off phosphates from the phosphoserine groups of the enzyme, muscle phosphorylase *a* can be reversibly converted into form *b* [15, 55, 58]. Phosphorylase *b*, in turn, is convertible into form *a* by means of a specific kinase which transfers the phosphate of ATP to serine [24, 27, 58, 60]. Phosphorylase *a* can be converted with trypsin into a polypeptide denoted as phosphorylase *b'* and into a hexapeptide [15, 54]. This form *b'* cannot be activated by kinase since the phosphorylable serine group is located in the hexapeptide.

2. *Primary Structure of Phosphorylase*

The amino acid composition of the plant phosphorylases has been given only in the case of potato phosphorylase. Phosphorylase which had been repeatedly crystallized, desalted, lyophilized and dried in vacuum to constant weight was dissolved by Kamogawa *et al.* [51] in 6 N hydrochloric acid and subjected to hydrolysis in a sealed tube at 110°C for 24 and 48 hours, respectively. The results of the amino acid analysis, together with the data referring to rabbit muscle phosphorylase and human muscle phosphorylase are given in Table IV/2.

It can be seen in that Table that the amino acid composition of potato phosphorylase, with the exception of the data for arginine, shows a fair agreement with the values obtained for muscle phosphorylase.

From a knowledge of the molecular weights, the character and number of the side chains with amino acid functions located on the protein chain can be established from the amino acid composition (Table IV/3).

Both in the plant and in the animal phosphorylases the positively charged groups predominate, i.e. the protein molecule is of a basic character. According to Velick and Wicks [101], the isoelectric point of phosphorylase *a* calculated from the amino acid composition is about 10. It is of interest to note that a value lower than 5·8 has been given by Green [36] for the isoelectric point of phosphorylases *a* and *b* in a potassium phosphate buffer. Similar

Table IV/2

Amino acid Composition of Various Phosphorylases

Aminoacid	Grams of aminoacid in 100 g of protein				
	Potato phosphorylase hydrolyzed for		Phosphorylase		
			of rabbit muscle		of human muscle
	24 hours	48 hours			
	(1)	(1)	(2)	(3)	(3)
Lysine	8·42	9·14	7·2	7·64	7·41
Histidine	2·17	2·37	3·3	3·72	3·91
Ammonia	0·69	0·81	1·2	1·31	1·26
Arginine	6·11	6·40	11·6	11·78	11·94
Tryptophan	3·14	—	2·0	2·54	2·46
Aspargic acid	10·34	10·64	10·5	12·91	13·53
Threonine	4·45	3·57	4·4	4·05	4·09
Serine	3·94	3·52	3·4	2·66	2·80
Glutamic acid	14·11	14·66	13·6	14·10	13·99
Proline	3·57	3·75	4·7	4·13	4·08
Glycine	3·39	3·73	3·9	3·60	3·71
Alanine	5·29	5·47	4·8	5·63	5·91
Semi-cystine	—	—	0·4	0·45	0·57
Valine	5·50	5·54	7·3	7·24	7·13
Methionine	1·74	1·84	2·7	3·23	3·47
Isoleucine	6·09	6·46	6·5	6·51	6·55
Leucine	7·60	7·89	10·5	10·61	10·61
Tyrosine	4·94	5·02	5·9	6·59	6·36
Phenylalanine	5·60	5·76	6·2	6·43	7·05
Total	100·39	99·71		115·13	116·83

(1): Data of Kamogawa *et al.* [51].
(2): Data of Velick and Wicks [101].
(3): Data of Appleman *et al.* [2].

values were also published by Keller (5·6) for the isoelectric point of phosphorylase *b* in sodium glycerophosphate buffer [53]. The difference between the calculated and observed isoelectric points may be attributed to an intrinsic binding of buffer ions to proteins of cationic character [54, 101]. Since proteins may possess different mobilities under various conditions some conclusions may be drawn as regards their primary structure. Anionic bonds may possibly be responsible for the deviations in the electrophoretic properties of phosphorylases *a* and *b*. It is of interest that the differences in the primary structure of phosphorylases *a* and *b* appear only in the four phosphoserine residues (form *a*), and that the number of negative charges increases during the conversion of form *a* into form *b* (in a pH range where proteins are soluble and stable).

Table IV/3

Classification of Side Chains in Phosphorylases

Type of group	Amino acid	Number of molecules in the side chain per enzyme molecule		
		in plant	in rabbit muscle *b*	in human muscle *b*
		phosphorylases		
Ionizable				
guanido	arginine	73	164	166
amino	lysine	120	126	123
imidazole	histidine	28	58	61
carboxyl	Asp + Glu	275	281	295
Hydrophilic				
amide	Asp (NH_2) + Glu(NH_2)	85	186	179
hydroxyl	serine			
	threonine	78	82	83
	tyrosine			
Hydrophobic				
hydrogen	glycine	95	116	120
alkyl	alanine	123	153	161
	valine	98	148	147
	leucine	157	196	196
	isoleucine	96	120	121
pyrrolidine	proline	65	87	86
aromatic	phenylalanine	70	94	103
	tryptophan	32	30	29
S-containing	methionine	24	52	56
	semi-cystine	3	9	11
All groups		1 562	1 869	1 909
All groups of cationic nature		221	348	350
All groups of anionic nature		275	281	297
All carboxyl and amide groups		360	467	476
All hydrophilic groups		212	231	232
All hydrophobic groups		762	997	1 019

On studying the interaction of protein and phosphorylase, Madsen and Cori [70] obtained an indirect proof of the linkage of buffer anions to the protein of cationic character. Phosphorylase *a* was readily precipitable from its solution by means of protamine. A 'semi-maximum' precipitate formation was observed at pH 5·7, a value agreeing with the isoelectric point of phosphorylase *a* measured in glycerophosphate buffer [53].

The investigation of the sequence of aminoacids in phosphorylase presents almost unsurmountable difficulties, due to the large dimensions of its mole-

cule. Though it is possible to decompose phosphorylase *a* to four subunits [48, 71], the determination of the amino acid sequence of the enzyme protein is still extremely complicated. The first experiments proved that no NH_2-terminal amino acid residues are present in phosphorylase *b* which can be decomposed to two subunits [49]. In further experiments, no end groups could be detected in the enzyme by the fluorodinitrobenzene, hydrazine, amino- and carboxypeptidase methods [59]. From these results it was concluded that the molecule may have a cyclic structure.

The paper of Fischer *et al.* [24] represented a marked advance in this field. These authors reported that, on treating phosphorylase *a* with trypsin, a phosphorylase *b* (which proved to possess full activity in the presence of 5′-AMP) and a hexapeptide containing phosphoserine are liberated. Four molecules of hexapeptide are liberated from one molecule of phosphorylase *a*. The hexapeptide showed the amino acid sequence:

$$\begin{array}{c} \text{Lys-Glu-Ileu-Ser-Val-Arg} \\ \quad | \qquad\qquad\quad | \\ \quad NH_2 \qquad\quad P \end{array}$$

This phosphoserine residue in phosphorylase *a* is the substrate of the PR enzyme [58].

The high selectivity of the trypsin reaction and the fact that the serine residue is always attacked by this enzyme, prove beyond doubt that the four hexapeptide units identified by these authors occupy an important site in the phosphorylase molecule. On the basis of the results of the decomposition experiments, Fischer concluded that the hexapeptide must be located in the polypeptide in a non-amino-terminal position [24].

3. *Functional Groups*

It was pointed out in Section IV.2 that an enzyme molecule contains a great number of ionizing amino groups of hydrophilic and hydrophobic nature. Of these groups, those which play a role in developing the optimum form of the molecule and the catalytic activity are denoted as functional groups.

Functional groups can be determined by aminoacid analysis, by group analysis as applied in organic chemistry, or by inhibition and reaction kinetic methods of investigation.

(i) SH Groups

The number of SH groups in the molecule of potato phosphorylase has been investigated by Lee [66]. The enzyme solution was dialyzed (0·1 M tris-phosphate buffer of pH 7·5; 10 mg of enzyme protein) and subjected to amperometric titration with 10^{-3} M $AgNO_3$ or 10^{-3} M $HgCl_2$. Titration with $AgNO_3$ indicated 5·9–6·0 moles of SH group while the spectrophotometric Boyer method [12] showed 6·8 moles of SH group per enzyme molecule. In contrast to muscle phosphorylase, the potato phosphorylase inactivated by *p*CMB did not recover its original activity on the addition of excess cysteine (Fig. IV/1).

The number of papers dealing with the determination and catalytic role of the SH groups of muscle phosphorylase is much higher than those referring to plant phosphorylases. In rabbit muscle phosphorylase, 18 SH groups were determined by Velick and Wichs [101], while the spectrophotometric titration of Madsen and Cori [71] indicated 18·5 moles of SH group per enzyme molecule. On inactivating the enzyme with *p*CMB, monomeric units were formed, and the inactivation was complete when 17 mole equivalents of *p*CMB were added. After separating the monomer from the tetra-

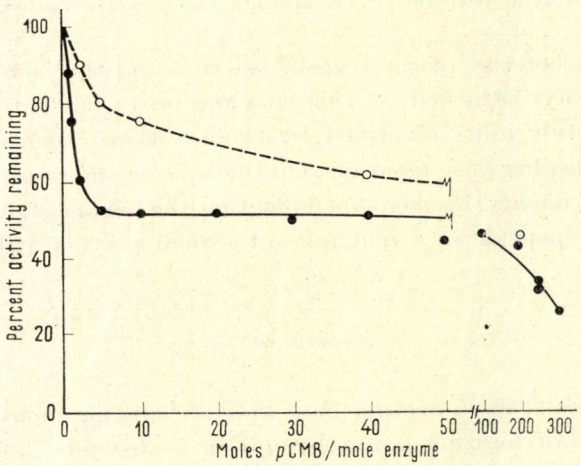


Fig. IV/1 Effect of *p*-chloromercuribenzoate (*p*CMB) on the enzyme activity of potato phosphorylase [67]. The enzyme at a concentration of 1×10^{-6}M, was incubated for 10 h at 2 ± 1°C with *p*CMB in 0·04 M glycerophosphate buffer (pH 6·3). The enzyme activity which remained was determined ●—●: without further treatment; ○—○: after treatment with 5×10^{-3}M cysteine for 3 h

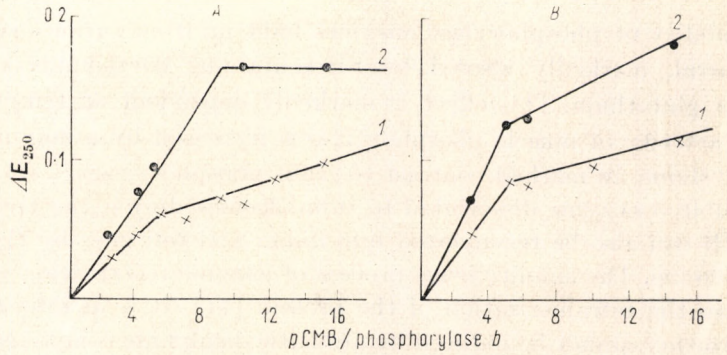


Fig. IV/2. Quantitative determination of the SH groups of phosphorylase *b* by titration with *p*CMB [102]. *A:* In α-glycerophosphate buffer; *B:* in imidazole solution; *1:* reaction period 10 minutes; *2:* reaction period 1·5 hours

mer by ultracentrifugation of the partly inhibited phosphorylase *a* solution, it was found that all the thiol groups of the monomer combined with *p*CMB while the tetramer contained only a small amount of *p*CMB. In the opinion of Madsen, this confirms the 'all-or-none' assumption regarding the SH groups of the enzyme [69, 72].

In contrast, Kudo and Shukuya found, although they too detected 18 moles of SH group in muscle phosphorylase, that half of the SH groups react quickly with *p*CMB while the other half have lower reactivity, and the degree of inhibition is not parallel to the reaction of SH groups [61].

The reactivity of the SH groups of muscle phosphorylase *b* has been studied by a number of authors [9, 35, 84, 102]. According to Vulfson and Skolisheva [102], two types of SH groups are present in the enzyme (Fig. IV/2). Of the 9 SH groups measured, 5 were readily titratable (10 minutes) with *p*CMB in glycerophosphate buffer while the other 4 groups could be titrated only after a long incubation period (1·5 hours). Five SH groups could also be readily titrated with N-ethylmaleic imide (NEMI) but the residual 4 groups which could be titrated with difficulty with *p*CMB, showed no reaction at all with NEMI. On the basis of activity measurements, the 5 readily reacting SH groups do not play any role in catalytic activity, neither in the enzyme structure. The residual 4 groups which reacted with *p*CMB only slightly, and did not react at all with NEMI, play an important role in the stabilization of enzyme conformation and undoubtedly in catalytic activity too. Imidazole proved to stabilize the enzyme activity and to exert a protecting effect during the treatment with *p*CMB and NEMI (Fig. IV/2). Thus, imidazole is of great importance in preserving the structure and the catalytic properties of enzymes [102].

The solubility of phosphorylase enzymes built up from various subunits is, in general, markedly altered by SH-containing compounds such as cysteine or glutathion. This effect is markedly dependent on temperature [37]. The activity of muscle phosphorylase is increased by a maximum of 30% by cysteine, in marked contrast to plant phosphorylases.

The inhibited enzyme dissociated to submolecules during the treatment with *p*CMB can also be reconverted into dimer and tetramer by the addition of cysteine. The simultaneous process of enzyme reactivation is much slower than the tetramerization of the protein [71]. It is of interest that monomerization cannot be induced by aromatic disulphide compounds such as 2,2-dithiodibenzoic acid, which possibly act via the reaction SH → disulphide. A marked protection against their effect is offered by AMP, and the reaction is reversed by an excess of cysteine [2].

(ii) Phosphate Groups

Significant amounts of phosphorus have been detected both in animal and in plant phosphorylases (Table IV/4). The total phosphorus content of the dialyzed enzyme solution was determined by Lee [67] using the method of Chen *et al.* [14]. According to his experimental data, 2 moles of phosphorus

Table IV/4

Number of Phosphate and SH Groups Present in Various Animal and Plant Phosphorylases

Type of phosphorylase	Number of groups/protein molecule			Ref.
	Phospho-serine	Pyridoxal-5-phosphate	SH group	
Rabbit muscle *a* (tetramer)	4·0	4·0	18	[6, 17, 57, 61]
Rabbit muscle *b* (dimer) (from *a* with PR enzyme)	0	2·0	9	[9, 17, 35, 57, 84, 102]
Rabbit muscle *b′* (after decomposition with trypsin)	0	2·0		[17, 24, 60]
Rabbit muscle *c* (monomer) from *a* and *b* after treatment with *p*CMB		+		[71, 72]
Dog liver		2·0		[96, 112, 113]
Human muscle *a*		4·3		[114]
Human muscle *b*		2·0		[114]
Potato		2·0	6	[65, 67]

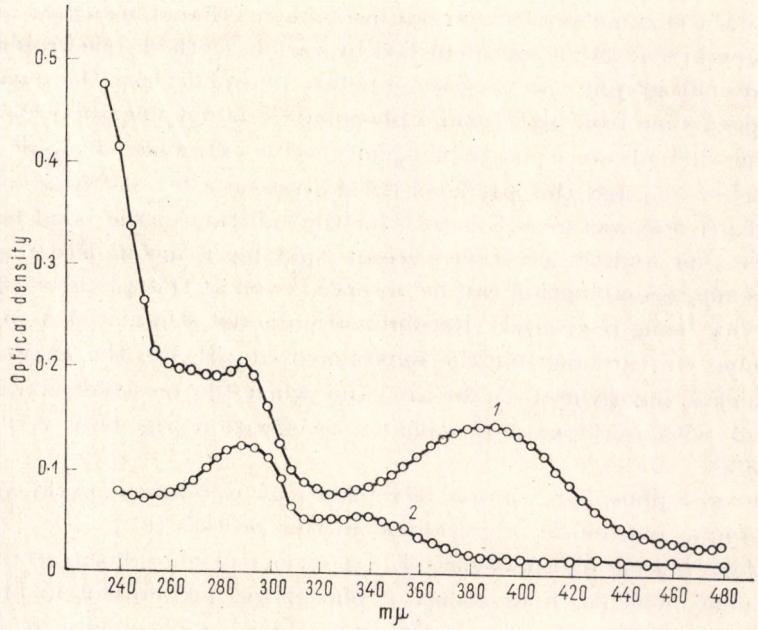


Fig. IV/3 Determination of pyridoxal-5-phosphate content of potato phosphorylase by spectrophotometric method [67]. Absorption spectra of the extract of 2·29 mg of potato phorphorylase; *1:* in 0·1 N NaOH; *2:* in 6% perchloric acid

are present in each mole of potato phosphorylase, and organic phosphate has been detected only in the form of pyridoxal-5-phosphate. No significant phosphorus content was present in the enzyme protein residue after precipitation by perchloric acid.

In contrast to muscle phosphorylase, no serine phosphate is present in potato phosphorylase; the latter proved to possess its full activity even in the absence of 5′-AMP [67]. The lack of serine phosphate is confirmed also by the facts that special phosphatases (such as the PR enzyme obtained from muscle and liver) have no inhibiting effect, and that the serine groups of the enzyme protein cannot be phosphorylated with phosphorylase *b* kinase in the presence of Mg^{2+} and ATP [67].

In potato phosphorylase, pyridoxal-5-phosphate has been identified by Lee [67] by the method of Petterson and Sober [82]. The perchloric acid extract of the enzyme undergoes the same spectral change when it is made alkaline (Fig. IV/3). Calculations on the basis of the extinction values gave a content of 1·9 to 2·1 moles of pyridoxal-5-phosphate per mole of enzyme. Pyridoxal-5-phosphate was also isolated by Lee from the perchloric acid

extract of the enzyme as a barium salt insoluble in ethanol, using the method of Baranowski *et al.* [6]; it was identified by various methods (electrophoresis, paper chromatography). In the case of potato phosphorylase, the separation of the apoenzyme from pyridoxal-5-phosphate was not unequivocal; it was also rather difficult since potato phosphorylase is extremely unstable at pH values below 5·5, and this pH level (24 dialysis in a 0·1 M citrate-cysteine buffer of pH 5·5) was unsatisfactory for the splitting of the bond between the apoenzyme and the prosthetic group. Splitting is incomplete at pH 4·5 though some recombination can be observed even at this pH level (24% of the activity being recovered). Recombination is not stimulated by 5′-AMP or cysteine. On carrying out the experiment at pH 4·0, the enzyme was very quickly inactivated (in 30 sec), and when the resolved enzyme was incubated with pyridoxal-5-phosphate, reactivation was only very slight (about 8%).

Pyridoxal-5-phosphate cannot be replaced by pyridoxine, pyridoxamine, pyridoxamine phosphate or pyridoxal in this process [67].

In rabbit muscle phosphorylase *a*, 8 g atoms of phosphorus were found per enzyme molecule. Four atoms of phosphorus are present in phosphoserine units and the other four atoms in pyridoxal-5-phosphate [6, 17, 57]. It is of interest that four combining sites for 5′-AMP exist in phosphorylase *a*, while in phosphorylase *b* the molecular weight of which is only half of that of phosphorylase *a*, two molecules of pyridoxal-5′-phosphate and two combining sites for 5′-AMP are present, phosphoserine groups being absent.

In the opinion of all the authors, pyridoxal-5-phosphate represents a prosthetic group quite indispensable for the catalytic activity.

According to the investigations of Cori [17], on treating muscle phosphorylase *a*, *b* and *b′* with ammonium sulphate (at a pH value between 3 and 4), it is possible to precipitate the apoenzyme, which can also be obtained in a crystalline state [48]. This apoenzyme is completely inactive but after incubation with pyridoxal-5-phosphate the catalytic activity of the enzyme is recovered. This recombination is a reaction of the second order [17].

The splitting of pyridoxal-5-phosphate takes place in two steps (cf. Fig. IV/6). In the first step, a Schiff base is formed which quickly rearranges to an aldimine structure. By removing pyridoxal-5-phosphate, the enzyme is inactivated and also undergoes some disaggregation. After incubation with pyridoxal-5-phosphate, reaggregation was detected by ultracentrifugal investigations. This proved that the combination of pyridoxal-5-phosphate to the enzyme protein is of fundamental importance for the development of the catalytically active, stable tetrameric form. The phenomenon of reaggre-

gation was observed also when pyridoxal or 5′-deoxypyridoxal was applied in high concentrations. However, the original enzyme activity was not recovered in either case.

This evidence points to the fact that the prosthetic role of pyridoxal-5-phosphate cannot be ascribed solely to its effect on the enzyme structure. Other groups of pyridoxal-5-phosphate may also participate in the catalytic step. This is pointed out by the fact that apophosphorylase *a* is also reactived by ω-methylpyridoxal-5′-phosphate (the 2-ethyl analogue of pyridoxal-5-phosphate), to at least 50%; indicating that it is possible to carry out slight modifications in the structure of the natural prosthetic group. Further, it can be presumed that for the recovery of phosphorylase activity, the 5-hydroxymethyl phosphate group of the pyridine ring is essential.

This was proved by Bresler and Firsov [13] who investigated the differential spectrum of muscle phosphorylase *b*. From the affinity values measured in the presence of glucose-1-phosphate, inorganic phosphate and AMP, they concluded that in the enzyme-substrate complex these substrates are combined to pyridoxal-5-phosphate which is present in its anionic form [13].

Pyridoxal-5-phosphate actually combines with phosphorylase *a* and phosphorylase *b* through its 4-formyl group as shown in Fig. IV/4 [26, 48, 57]. where N is the nitrogen atom of the protein, an ε-amino nitrogen of the lysyl residue of the peptide chain, X is of unknown structure, linked by a covalent bond to the 4-formyl C-atom.

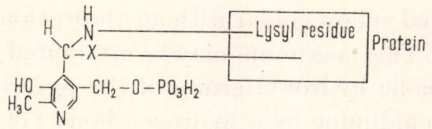


Fig. IV/4. Binding of pyridoxal-5-phosphate in the enzyme protein

The existence of the bond between the protein and the 4-formyl group of pyridoxal-5-phosphate has been confirmed experimentally. In the active enzyme this combination does not possess the character of a Schiff base as proved by the fact that the activity of phosphorylase *a* is not inhibited by high concentrations of isonicotinic hydrazide [48]. A similar conclusion has also been drawn by Kent [57] who investigated the spectral properties of phosphorylase *b* at various pH values (Fig. IV/5). Native phosphorylases *a* and *b* showed small absorption maxima at 330 mμ attributed to their

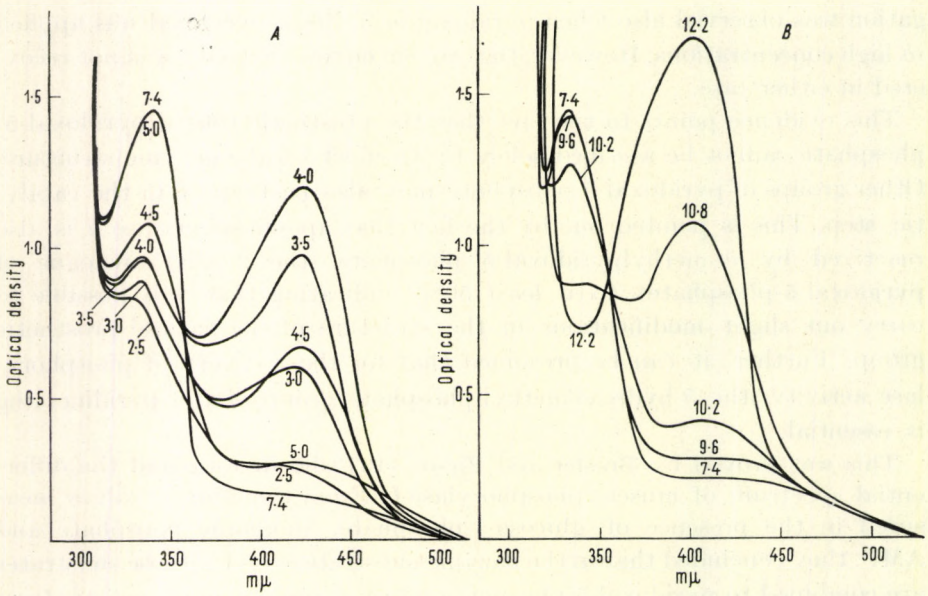


Fig. IV/5. Absorption spectrum of phosphorylase *b* at low (*A*) and at high (*B*) pH values [57]

pyridoxal-5-phosphate content. This maximum appears at a wavelength lower than that expected for the Schiff's base (due to its double bond in conjugation with the pyridine ring). On splitting the bond between pyridoxal-5-phosphate and protein by acid treatment (at pH 4·0–5·0) (Fig. IV/6), a transitional yellow colour with an absorption maximum at 415 mμ was observed [57]. This maximum may be attributed to the Schiff's base (II) in which the phenolic hydroxyl group of the pyridine ring is combined to the N atom of the aldimine by a hydrogen bond [74]. However, that structure is only transitional as it undergoes a reversible conversion into the stable pyridoxal-5′-phosphate (III) or into the active enzyme fraction, while in the native enzyme protein the aldimine bond is converted, by addition of XH, into a secondary amine structure (IV).

The discovery of Fischer *et al.* [26], revealing that on treating the Schiff's base (II) with sodium borohydride it can be quantitatively converted into a colourless pyridoxal derivative (IV) (cf. Fig. IV/6), represented an important advance in this field. Derivative IV proved to be resistant to treatment with acids or bases, and showed maximum absorption at 330 mμ. When the phosphorylase shows the spectral properties of form (I) it is not reduced by $NaBH_4$ in neutral solution. The reduced enzyme can be purified and crys-

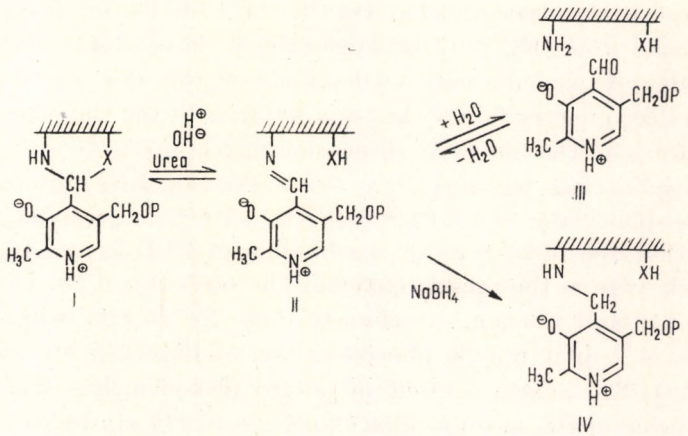


Fig. IV/6. $NaBH_4$ reduction of muscle phosphorylase

tallized, and retains nearly its full activity. Phosphorylase *b* can be converted into phosphorylase *a*, and conversely, in a quite normal way [26, 56]. In respect to AMP and glucose-1-phosphate, the native and the reduced enzyme showed the same optimum pH value and the same dissociation constants. This means that pyridoxal-5-phosphate, in contrast to all the known enzymes which contain pyridoxal-5-phosphate, participates in the activation of phosphorylase in a rather different mode, since a potential aldehyde group is indispensable in the enzymes.

The amino acid residue which links pyridoxal-5-phosphate to phosphorylase, has been determined by decomposing the reduced protein with chymotrypsin and proved to be ε-N-pyridoxyl-lysyl-phenylalanine [56].

According to quite recent data, the decomposition of the reduced phosphorylase by chymotrypsin yields not a dipeptide but a substituted tripeptide: ε-N-pyridoxyl-lys-lys-phenylalanine. The group denoted by X in Fig. IV/6 is thus far unknown; it may be a thiol, amino, imidazole or hydroxyl group. Urea and low concentrations of surface active agents promote the formation of the Schiff's base (II). This may mean that the group X in the peptide chain is not located in a vicinal position to the lysyl residue, and that in the natural structure (I) pyridoxal-5-phosphate which preserves the steric conformation of the protein [26] is of great importance.

Johnson and Graves [50] have investigated circular dichroism and optical rotatory dispersion of rabbit muscle phosphorylase to gain more information about PLP in this enzyme. Both phosphorylase *a* and *b* exhibit positive

Cotton effects in the absorption band of PLP [41]. Similar result was obtained by Torchinskii *et al.* [99, 100]. Kamogawa *et al.* have observed that potato phosphorylase displayed a positive maximum at the same wavelength [51]. The Kuhn dissymmetry factor $\Delta\varepsilon/\varepsilon$ was estimated from the observed circular dichroism and the observed absorption at 333 mμ to be $0{\cdot}89\times10^{-3}$ for potato phosphorylase, which is fairly close to the values for rabbit and lobster muscle phosphorylases, $1{\cdot}1\times10^{-3}$ and $0{\cdot}92\times10^{-3}$, respectively [50]. This indicates that the same type of the binding of PLP is present in potato phosphorylase as in the muscle enzyme. The positions of the two negative extremes, 219 and 209 mμ, are close to those for an alfa-helix, as well as in the case of rabbit muscle phosphorylase, as observed by Johnson and Graves [50]. The helical content of potato phosphorylase was estimated from the value of the mean residue ellipticity at 222 mμ to be about 29%. The value of reduced mean residue rotation at 231 mμ for the muscle enzyme [50] gave a helical content of about 19% for this enzyme protein. Although the two values of helical contents are based on different methods of determination, potato phosphorylase has probably more helical structure than the muscle enzyme [51].

(iii) Other Functional Groups

Holló *et al.* [44] established by kinetic investigations (described in detail in Section VI.3) the dependence of the apparent Michaelis constants on pH and temperature. From these data it is possible to determine the various functional groups. On the basis of the experimental data, imidazole and presumably the amino group, too, play a role in developing the potato phosphorylase substrate complex.

The presence of the imidazole and of the amino group established from the kinetic data was confirmed by the above-mentioned authors by means of photooxidation and acetylation [44]. The essential role of these groups in the enzyme reaction was proved by further experiments (photooxidation and acetylation will be described in detail in SectionVI.6 *(ii)*.

4. Detailed Structure of Phosphorylase

Until very recently, the investigation of enzyme structure was limited, in an over-simplified and schematic manner, solely to the active centre and to the substrate interaction. However, the active centre is only a very small

portion of the large molecule enzyme proteins. The steady increase of knowledge in this field, together with the development of technical ancillary instruments has made indispensable the stereochemical study of the correlations of the active site with the entire protein molecule as a necessary prerequisite of the full elucidation of enzyme-catalyzed reaction mechanisms.

For the time being, the study of the ternary and quaternary structure of enzyme proteins, particularly in the case of enzymes of high molecular weight, is limited mainly to examinations of the structure of protein solutions. This is quite remote from the actual knowledge of the build-up of enzyme proteins, but a number of remarkable results have been obtained in investigations into the correlation between the various structural properties and enzyme functions.

The number of papers referring to the detailed structure of phosphorylase are very small.

In a solution of muscle phosphorylase, the quaternary structure and the activating effect of glycogen substrate have been investigated [73, 106]. Wang *et al.* found that in the case of phosphorylase *a*, the activation

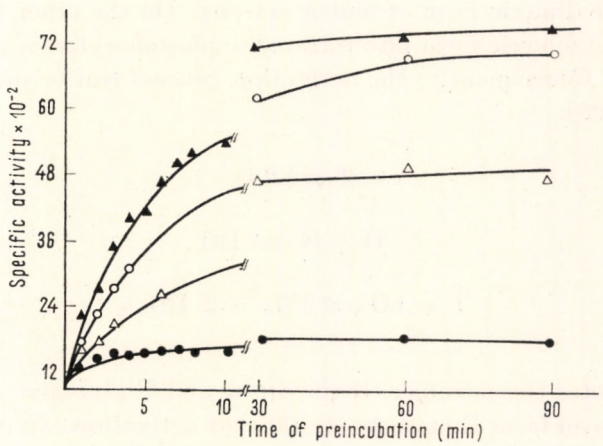


Fig. IV/7. Activation of phosphorylase *a* by glycogen [106]. Phosphorylase *a* (0·67 mg/ml) was preincubated in 0·03 M cysteine-0·04 N glycerophosphate, pH 6·8, at 20°C (●—●); in buffer with 0·1% glycogen (△—△); in buffer with 0·5% glycogen (○—○); and in buffer with 1% glycogen (▲—▲). At various intervals, 0·2 ml aliquots were removed and added to 0·2 ml of substrate, pH 6·8, at 20°C, containing 0·032 M glucose-1-phosphate and 2% glycogen. The inorganic phosphate released was measured after 30 sec of reaction.

depends on the length of the preincubation period and grows with increasing glycogen concentration (Fig. IV/7). It was proved in further experiments that no contamination by amylo-1,6-glucosidase, amylo-1,4-1,6-transglucosidase or α-amylase is responsible for the rise of activity. The activation effect of glycogen markedly increases with decrease of temperature; this is connected with the aggregation state of the enzyme. From this, it may be concluded that activation is possible only under conditions where the enzyme is present in a tetrameric form. In the case of phosphorylase *a* these conditions include a low ionic strength at 20°C, or a high ionic strength and 10^{-3} M AMP; in the case of phosphorylase *b*, low temperature, 10^{-3} M AMP and 10^{-2} M Mg^{2+} concentration [104, 106]. It has been proved experimentally that the preincubated dimer undergoes no activation by glycogen, and that the tetrameric form, when preincubated, approximately approaches the activity of the dimeric form. Hence, it appears highly probable that the activation process is correlated with the dissociation of the enzyme.

According to two earlier observations of Wang and Graves [105], the activity of phosphorylase *a* enzyme increases with the degree of dilution. This can only be explained by the dissociation of the less active tetrameric form into the dimeric form of higher activity. On the other hand, the conversion of the dimeric form into tetrameric phosphorylase *a* can be blocked by glycogen. Consequently, the activation process can be described as follows [103, 106]:

$$T \rightleftharpoons 2\,D \tag{1}$$

$$D + G \rightleftharpoons DG \tag{2}$$

$$T + nG \rightleftharpoons TG_n \rightarrow 2\ DG_{n/2} \tag{3}$$

where T denotes the tetramer, D the dimer and G glycogen.

It can be seen from Equations (1)–(3) that activation can only be carried out by shifting the tetramer/dimer equilibrium. If the activation process is in fact adequately described by Eqs (1) and (2), then the initial velocity of activation depends on the glycogen concentration, with respect to the velocity-limiting role of the complex glycogen-dimer. However, this appears to be improbable since the half activation period is of an order of magnitude of minutes while the conversion of the dimeric form into the tetrameric form can be blocked with glycogen instantaneously [105]. Dependence on the glycogen concentration may denote only the combi-

nation of the tetrameric form with glycogen, which process promotes enzyme dissociation Eq. (3). The preliminary kinetic data point to the fact that the degree of activation depends markedly on glycogen concentration, confirming that activation takes in fact place according to Eq. (3).

On examining the activating effect of various polysaccharides it was found [106] that these are competitive inhibitors of glycogen as a 'primer', or 'starter'. This indicates that primary combination and activation take place at two different sites of the enzyme (Table IV/5). Thus, activation is not simply the consequence of combination to the site where the primary is bound. Instead, a specific polysaccharide structure is required for the activation process.

Table IV/5

Activating Effect and Priming Capacity of Polysaccharides in the Case of Muscle Phosphorylase *a** [106]

Polysaccharides	Activation (%)	Priming capacity	Time for half-activation (min.)
Shellfish Glycogen	100**	100**	10
Potato amylopectin	94	39	2
Hydrolyzed amylose*** (DP_w 50)	93	17	0·5
β-Amylase limit dextrin (from amylopectin)	99	11	2

* The enzyme (0·5 mg/ml) was preincubated at 20°C with different polysaccharides (0·5%) in a 0·03 M cysteine – 0·04 M glycerophosphate buffer of pH 6·8.

In order to establish the activating effect, enzyme activity was measured at 20 °C in samples withdrawn from the reaction mixture at various intervals. The reaction mixture contained 0·016 M glucose-1-phosphate, 1% glycogen and 0·25% polysaccharide from the preincubated solution.

Priming capacity was examined at 20 °C, with a 0·5% polysaccharide and 0·016 M glucose-1-phosphate solution.

** Activation and priming capacity of these polysaccharides was referred to the 100% value of glycogen.

*** In order to promote dissolution, 2·5% dimethyl sulphoxide was employed. In the preincubation, the enzyme was not inhibited (or only to a minute extent) by the 2·5% dimethylsulphoxide additive.

In the case of potato phosphorylase, the structural changes of enzyme associates formed with various cyclo-oligosaccharides and with amylopectin have been investigated by Staerk and Schlenk [94]. In aqueous solution, cycloamyloses are capable of rearrangement to inclusion associates [91]. This is also proved by the fact that in this way their inhibitive power significantly decreases [92]. It was also observed that cyclo-amyloses of non-complex form exert a protecting effect in the proteolysis of the enzyme by trypsin. This can possibly be attributed to the fact that one molecule of cycloamylose is specifically associated with the active site of the enzyme.

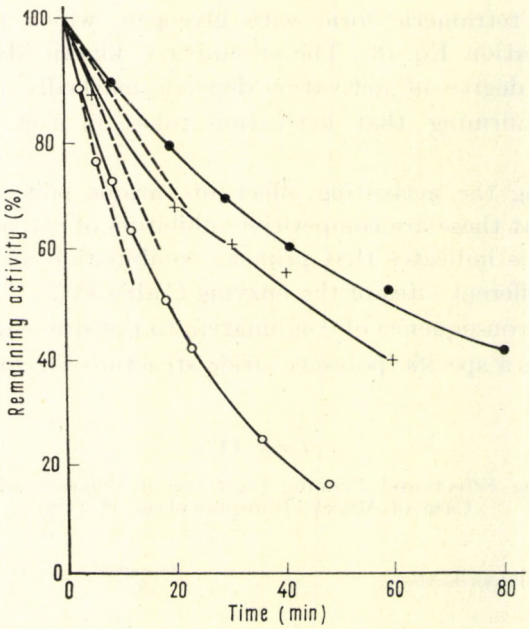


Fig. IV/8. Action of trypsin on potato phosphorylase protected by amylopectin and cyclohexaamylose [94]. 1·0 μM trypsin in citrate buffer (pH 6·9) with 20 μM potato phosphorylase. Initial velocity (v_0) was determined by the tangential method as indicated by the broken lines; ○ — ○: no complexing agent ($v_i = 8{\cdot}1 \times 10^{-8}$ M·min^{-1}); × — ×: 0·24 mM cyclohexaamylose ($v_i = 3{\cdot}2 \times 10^{-8}$ M·min^{-1}); ● — ●: 0·62 mM non-reducing end groups of amylopectin ($v_i = 2{\cdot}3 \times 10^{-8}$ M·min^{-1})

It can be seen in Fig. IV/8 that in the presence of amylopectin or of cyclohexaamylose, the decrease of the catalytic activity of the enzyme is slower than with the non-protected enzyme, though no full protection can be realized in practice. The authors mentioned applied various methods (such as electrophoresis, thin layer chromatography, finger print techniques, etc.) in order to distinguish the native and the protected enzyme products proteolyzed by trypsin. However, no differences between these products were observed [94].

The dehydrating effect of various solutions of high ionic strength is often employed for the study of changes in the fine structure of proteins. Structural changes can be followed indirectly by observing the degree of inactivation; the activation recovered on dialysis may serve as

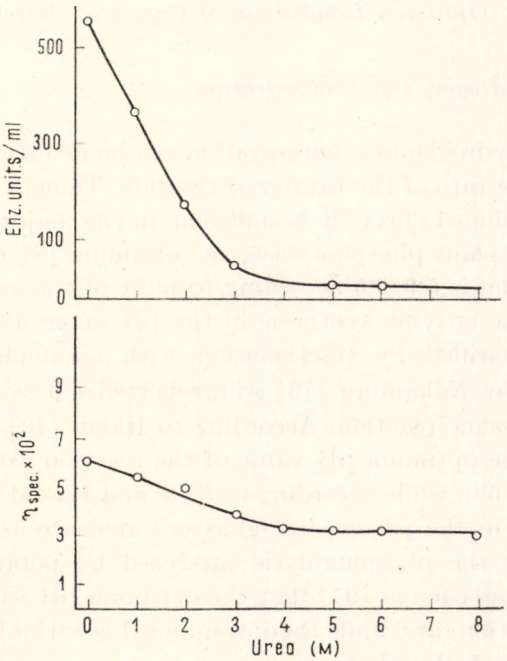


Fig. IV/9. Changes in enzyme activity and in values η_{spec} in the synthesis reaction catalyzed by potato phosphorylase, plotted against increasing urea concentrations [44]

a value pointing to the reformation of the original fine structure of the enzyme [1].

In general, urea is used in structural investigations as a dehydration agent.

Changes in the fine structure of enzyme protein with increasing urea concentration have been investigated by viscosimetric measurements by Holló *et al.* [44]. In these experiments, urea concentration was varied from 1 to 8 M at 15°C. Variations in viscosity values (measured with an Ostwald viscosimeter) and in enzyme activity are plotted against increasing urea concentration in Fig. IV/9.

In the urea concentration range 1–3 M, both the enzyme activity and viscosity appear to diminish, and the enzyme is practically inactivated at 3 M urea concentration; no changes in viscosity are perceivable on further raising the concentration of urea. When urea is removed by dialysis, enzyme activity is not recovered, indicating an irreversible structural change during the dehydration process.

5. Optimum Conditions of Catalytic Activity

(i) Effect of Hydrogen Ion Concentration

The effect of hydrogen ion concentration can be investigated most simply by measuring the rate of the catalyzed reaction. Though this method indicates only a combined effect, it is sufficient in the majority of cases.

In the case of plant phosphorylase, the optimum pH range of synthesis and decomposition is 5·9–6·5 according to most of the authors.

The rate of the enzyme synthesis in the pH range 5·5–6·3 with potato phosphorylase purified by fractionation with ammonium sulphate has been examined by Nakamura [76] who reported 5·9 as the optimum pH value for the enzyme reaction. According to Hanes [40], and Weibull and Tiselius [108], the optimum pH value of the reaction catalyzed by potato phosphorylase is 6·0, while according to Katz and Hassid [52] the optimum range is 6·2–6·3 in the presence of glucose-1-arsenate as donor substrate. On investigating the phosphorolysis catalyzed by potato phosphorylase, Suzuki and Hamada found [97] that the optimum pH value is 6·8 referred to the initial reaction rate, while the optimum pH is 8·0 for the maximum formation of glucose-1-phosphate.

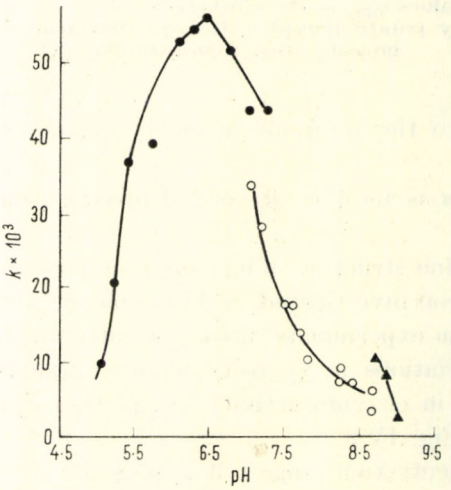


Fig. IV/10. Effect of pH on enzyme activity [66]. 1×10^{-2} M glucose-1-phosphate, 0·75% amylopectin and 54 units of enzyme were incubated in 0·1 M buffer at 30°C. *k:* Velocity constant. ●—●: citrate buffer; ○—○: tris buffer; ▲—▲: glycine buffer

The phosphorylase of Lee [66] in a 0·1 M citrate buffer showed a sharp optimum pH at 6·5 (Fig. IV/10).

Holló *et al.* investigated the optimum pH values of synthesis [43] and of phosphorolysis [47], in the pH range 5–8, using potato phosphorylase of high specific activity purified by DEAE-cellulose column chromatography (cf. Chapter V). They stated that the optimum pH region of enzyme catalysis is the same in both cases (pH 5·8–6·1), and is numerically in agreement with the majority of literature data published earlier.

According to Hamada [39], the optimum pH value of the synthesis catalyzed by rice phosphorylase is 6·2. In acidic solution, a marked decrease of enzyme activity is observed, but in alkaline solution the activity is reduced to a smaller extent.

An optimum pH level of 6·6–6·7 is given by Kurasawa *et al.* [62] for rice phosphorylase obtained from rice seeds in the milky ripening stage and fractionated with ammonium sulphate.

According to Datta [22], phosphorylase prepared from *Trapa bispinosa* has an optimum pH value of 6·2.

(ii) Effect of Temperature

Reaction rates usually increase with rise of temperature. This also holds for enzyme reactions between certain limits. However, on attaining a given temperature (optimum temperature), protein molecules begin to undergo deformation and later denaturation. Thus, the rate of the catalyzed reaction decreases, and the reaction completely stops at a given limiting temperature.

Literature data concerning optimum temperatures and Q_{10} values of enzymatic reactions catalyzed by plant phosphorylases are very scarce. According to Green and Stumpf [38], 61% of the original enzyme activity was lost in 3 minutes at 58°C, and 97% in 3 minutes at 68°C. At temperatures between 20 and 50°C, a value of 2·3 was given by Weibull and Tiselius [108] for Q_{10} for potato phosphorylase purified with ammonium sulphate. According to Hidy and Day [42], $Q_{10} = 2{\cdot}0$ between 10 and 20°C, and 1·3 between 40 and 50°C; while according to Nakamura [76], $Q_{10} = 2{\cdot}0$ in the temperature range 17–42°C.

On the basis of the data of Suzuki and Hamada [97], the optimum temperature range of phosphorolysis is 25–40°C, while an optimum range of 36–37°C has been given by Kurasawa *et al.* [62] for phosphorylase fractionated with ammonium sulphate from rice seeds in the milky stage of ripening.

From an investigation of the optimum temperature of synthesis at pH 6·3 with potato phosphorylase purified by zone electrophoresis, a value of 1·7 was obtained for Q_{10} in the temperature range 15–45°C by Lee [66].

Holló *et al.* investigated the optimum temperatures of enzymatic synthesis [43] and decomposition [47] and the Q_{10} values at pH 6·0 in the temperature range 15–60°C, employing potato phosphorylase purified by DEAE-cellulose column chromatography (data given in detail in Chapter V).

According to the above-mentioned authors, under the given conditions, the optimum temperatures of both the synthesis and the decomposition are 45–50°C. The denaturing effect of temperature appears only over 50 °C. In the range 30–45°C, the Q_{10} value is 1·4 for both types of reaction.

The high scatter of the Q_{10} values can be ascribed to differences in experimental conditions; reaction times, ionic strenghts and substrate concentrations were different in the determinations of the various data.

6. Inhibition

According to the up-to-date theory of inhibition, the following possibilities exist:

(a) all the substrate-binding sites in the active centre of the enzyme are occupied by the inhibitor;
(b) the site of the co-factor is occupied by the inhibitor;
(c) one of the substrate-binding sites is occupied by the inhibitor;
(d) instead of reacting with the substrate-binding groups, the inhibitor sterically impedes the bonding of substrates;
(e) the molecular structure of the protein is altered by the inhibitor;
(f) the inhibitor reacts with the substrate;
(g) the inhibitor reacts with the co-enzyme;
(h) the inhibitor reacts with the co-factor.

Within these types of inhibition, competitive, non-competetive and mixed inhibitions, or substrate, substrate-analogue, product and protein modifying types of inhibition may be distinguished. The majority of papers report on protein modifying inhibitions which may be carried out by physical methods (heat, irradiation, urea treatment, etc.), chemical methods (alkylation, acetylation, oxidation, iodination etc.) or enzymatically (by proteolytic enzymes).

(i) Inhibition by Physical Methods

The number of communications reporting inhibition of plant phosphorylase by physical methods is rather small.

Structural changes due to temperature rise and inactivation of enzymes as a result of such changes have already been discussed in Section IV. 1 *(ii)*.

Inhibition by irradiation has been examined mainly under in vivo conditions. Thus it is impossible to evaluate individual inhibitions.

The effect of γ-radiation on an aqueous solution of potato phosphorylase and on a lyophilized dry enzyme preparation has been investigated in vitro by Phillips and Griffits [85]. It was found that the mode of the enzyme reaction was not affected by γ-radiation; only the enzymatic activity was reduced. In the lyophilized dry samples, a slight protecting effect of the applied buffer was observed.

(ii) Inhibiton by Chemical Methods

Enzyme proteins are sensitive to chemical agents in general. However, only chemicals with a more or less specific effect are suitable for use, particularly those which react with the functional groups of the protein or which are capable of altering in a specific way the structure of the protein molecule.

Imidazole, amino and SH groups have been detected in the active centre of plant phosphorylase [44, 46].

The presence of imidazole as a functional group was confirmed by Holló *et al.* [44] by photo-oxidative inhibition carried out in the presence of methylene blue by the method of Weil *et al.* [109, 110]. To a solution of potato phosphorylase purified by DEAE-cellulose column chromatography (activity: 79·5 enz. units per ml; specific activity: 220 enz. units mg of protein) in 0·01 M citrate buffer of pH 7·0, methylene blue was added (conc. 0·1 mg/ml). The solution was thermostated at 20°C, and illuminated by a 300 watt incandescent lamp from a distance of 30 cm. In the samples withdrawn at given periods and in the control samples, the liberation of inorganic phosphate and the tyrosine and tryptophan contents were established (the latter two by the Folin–Ciocalteu method [28]). In order to exclude the interfering effect of methylene blue, it was removed from the samples by adsorption with cation exchanger Varion KS prior to the phosphate determinations.

The results of the photo-oxidative investigations are listed in Table IV/6.

Table IV/6

Changes in Enzyme Activity and Tyrosine and Tryptophan Content during Photo-oxidation [44]

Treatment of sample	Phosphorus extinction 650 mμ (1 cm cell)	Folin test extinction 750 mμ (1 cm cell)
Enzyme, without illumination or Methylene blue	0·270	0·21
Enzyme with Methylene blue, without illumination	0·260	0·22
Enzyme illuminated, without Methylene blue	0·205	—
Enyzme with Methylene blue, illuminated for		
5 minutes	0·205	—
10 minutes	0·205	—
30 minutes	0·140	0·21
60 minutes	0·105	—
100 minutes	0·050	0·24
130 minutes	0·035	—
210 minutes	0·025	0·22

It can be seen that as a result of photo-oxidation the imidazole group is quickly decomposed, and the catalytic activity of the enzyme shows a parallel decrease. According to the analytical data, the tryptophan and tyrosine contents did not alter during photo-oxidation. Consequently, it is highly probable that imidazole participates in the enzymatic reaction.

The functional character of the amino group has been determined by Holló *et al.* [44] with the use of the acetylation method described by Fraenkel-Conrat *et al.* [29]. Though the acetylation process is not unequivocally specific for the amino group (since the phenolic hydroxyl and free sulphhydryl groups may also react slightly), it is suitable for elucidating the role of the amino group when complemented by adequate control tests.

In the experiments of the above-mentioned authors, 5 mg of potato phosphorylase purified by DEAE-cellulose column chromatography (data in detail in the photo-oxidation test) was dissolved in 0·1 ml of 0·01 M citrate buffer of pH 7·0. The solution was cooled to 0°C and treated with 0·1 ml saturated sodium acetate solution. Within one hour, 5 μl of 0°C acetic anhydride was added and the solution diluted to 2 ml with 0·01 M citrate buffer of pH 7·0 and subjected to dialysis at 0°C against that buffer [44]. In the dialyzed sample, changes in enzymatic activity, protein content (Folin test) and amino group content (ninhydrin method) were followed (Table IV/7).

It appears from Table IV/7 that the enzyme activity decreased to about 30% of the initial level and the free amino groups contents to about 12% of

Table IV/7

Changes in Activity as a Result of the Acetylation of the Amino Group of the Enzyme [44]

Treatment of sample	Enzyme activity (Enz. unit/ml)	Folin test (750 mμ, 1 cm cell, Ext./ml)	Ninhydrin test (530 mμ, 1 cm cell, Ext./ml)
Prior to acetylation	347	13·4	38·5
After acetylation and dialysis	104	13·6	4·7

the initial value. The tyrosine-tryptophan content was unchanged. These data strongly support the presumption that the amino group participates in the enzyme reaction.

The presence of SH groups in potato phosphorylase and their participation in enzymatic synthesis has been reported by several authors [38, 46, 67].

According to the investigations of Lee [67], heavy metals (such as Ag^{+}, Cu^{2+}, Hg^{2+}) are strong inhibitors. On pre-incubating the enzyme with a glycerophosphate buffer of pH 6·5, a hundredfold excess of *p*-chloromercuribenzoate caused a 60% inhibition in one hour at 30°C, while a tenfold excess, brought about an almost complete inhibition in 5 hours. In contrast to muscle phosphorylase [71] this inhibition proved to be irreversible in that the activity was not recovered when the inhibited enzyme was treated with cysteine in excess. On repeating these experiments at 2°C, enzymatic activity was recovered only to a minute extent. The enzyme was not inhibited by 10^{-2} M iodoacetate [67].

According to Green and Stumpf [38], in the case of potato phosphorylase fractionated with ammonium sulphate, 80% inhibition was caused by $1 \times \times 10^{-4}$ M silver nitrate and 24% inhibition by $3{\cdot}7 \times 10^{-2}$ M sodium fluoride, while no inhibition was observed on applying zinc sulphate, mercuric chloride, lead acetate or iodoacetic acid.

Ram and Giri reported [89] that green grain phosphorylase can be completely inactivated with 10^{-3} M mercuric chloride and 10^{-2} M silver nitrate.

More detailed data of the role of the SH group are given by Holló *et al.* [46] who employed iodoacetamide (IAA), N-ethylmaleic imide (NEMI) and *p*-chloromercuribenzoate (*p*CMB) as inhibitors. Of these, *p*CMB proved to react with both free and latent SH groups, while NEMI and IAA were specific for free SH groups (though they did react to a small extent also with the latent SH groups). All the three inhibitors were applied in various concentrations in the enzyme reaction catalyzed by potato phosphorylase purified by DEAE-cellulose column chromatography (Table IV/8).

Table IV/8

Effect of Changes in the Concentration of Various Inhibitors on the Rate of Enzymatic Synthesis [46]

Type of inhibitor	Concentration of inhibitor (M)	Initial rate $v_0 \times 10^{-3}$ (M/min.)	Inhibition (%)
*p*CMB	$2{\cdot}0 \times 10^{-5}$	90	52·6
	$1{\cdot}5 \times 10^{-5}$	105	44·8
	$1{\cdot}0 \times 10^{-5}$	121	36·4
	$5{\cdot}0 \times 10^{-6}$	145	23·7
	$1{\cdot}0 \times 10^{-6}$	174	8·4
	$1{\cdot}0 \times 10^{-7}$	190	—
IAA	$2{\cdot}0 \times 10^{-2}$	128	32·7
	$1{\cdot}5 \times 10^{-2}$	139	27·1
	$1{\cdot}0 \times 10^{-2}$	132	20·0
	$5{\cdot}0 \times 10^{-3}$	166	8·8
	$1{\cdot}0 \times 10^{-3}$	174	8·4
	$1{\cdot}0 \times 10^{-4}$	190	—
NEMI	$2{\cdot}0 \times 10^{-2}$	162	14·8
	$1{\cdot}5 \times 10^{-2}$	167	12·0
	$1{\cdot}0 \times 10^{-2}$	170	10·5
	$5{\cdot}0 \times 10^{-3}$	172	9·1
	$1{\cdot}0 \times 10^{-3}$	174	8·4
	$1{\cdot}0 \times 10^{-4}$	190	—
Without inhibitors	—	190	—

It appears from Table IV/8 that *p*CMB which reacts with both types of SH groups, is the strongest inhibitor, while IAA and NEMI which react mainly with the free SH groups possess much weaker inhibiting effects (causing an inhibition identical to that of *p*CMB only in concentrations higher by about three orders of magnitude). Thus, the so-called latent SH groups presumably play a rather significant role in the development of the enzyme-substrate complexes. It may be possible that these groups stabilize the steric structure of the enzyme eventually by S–S (disulphide) bonds, and that the specific orientation is altered by the splitting of these bonds, resulting in an inhibition of enzymatic catalysis.

The time-dependence of the impeding effect of these inhibitors was also investigated by the authors (Fig. IV/11) because it may be assumed that the affinity of inhibitors for the SH groups of the enzyme is different, and that the degree of inhibition is a function of the duration of reaction.

It appears from Fig. IV/11 that on lengthening the inhibition period the degree of inhibition also increases, but *p*CMB remains the strongest inhibitor.

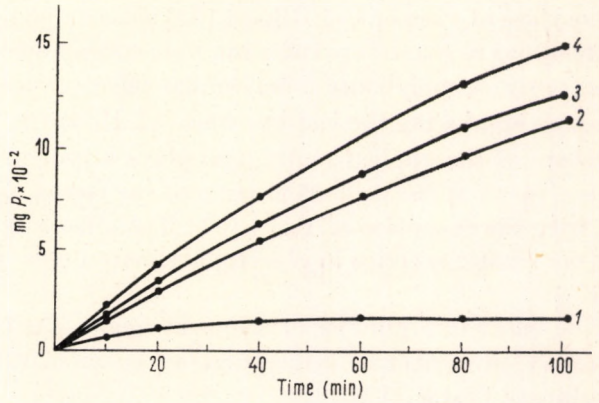


Fig. IV/11. Inhibition of the reaction catalyzed by potato phosphorylase, plotted against time with various SH inhibitors [46]. Concentrations of inhibitors: *1:* *p*-chloromercuribenzoate: 5×10^{-4} M; *2:* iodoacetamide: 1×10^{-1} M; *3:* N-ethylmaleinimide: 1×10^{-1} M; *4:* not inhibited

The experiments were carried out at 30°C at pH 6·0, enzyme concentration 17 Enz. units, donor substrate concentration 10 mM, acceptor substrate conc. 0·95 mM

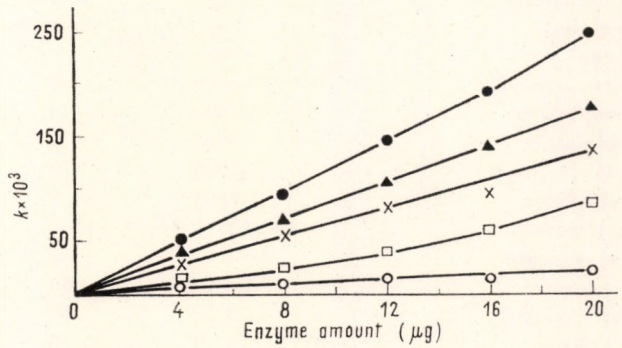


Fig. IV/12. Inhibiting action of heavy metals on the potato phosphorylase activity [66]. The reaction mixture contained 0·04 M glycerophosphate buffer pH 6·3, 1×10^{-2} M glucose-1-P and 0·75% amylopectin. ●—●: No inhibitor; ▲—▲: $AgNO_3$ (1×10^{-4} M); ×—×: $AgNO_3$ (1×10^{-3} M); □—□: $HgCl_2$ (1×10^{-4} M); ○—○: $CuSO_4$ (1×10^{-3} M)

The phosphorylase of Fischer and Hilpert [25] was completely inactivated when they attempted to inactivate the α-amylase contaminant with copper, silver, zinc, mercury or molybdate ions. Similar effects were also observed by Bailey *et al.* on employing the last two ions [5]. However, in the opinion of Lee, the nature of the applied buffer also plays a part in the inhibition process, and e.g. in 0·1 M citrate buffer, none of the tested ions are capable of inhibition, with the exception of Hg^{2+} [66]. The effects of various heavy metal ions on enzymatic activity in glycerophosphate buffer [66] are shown in Fig. IV/12.

The inhibiting effect of a number of chemical agents was investigated by Nakamura [75, 77] in syntheses carried out by enzyme fractionated with ammonium sulphate (Table IV/9).

Table IV/9

Effect of Various Inhibitors on the Enzymatic Synthesis Catalyzed by Potato Phosphorylase [75, 77]

Inhibitor type	Inhibitor concentration (M)	Inhibition (%)
Glucose	0·05	0
Phloridzin	$5{\cdot}8\times10^{-3}$	25
Sodium chloride	0·05	0
Sodium azide	0·02	0
Urethane	0·05	0
2,4-Dinitrophenol	1×10^{-3}	0
Sulphanylamide	0·05	0
Sulphathiazole	0·005	10
Penicillin	0·005	0
Chloromycetin	0·003	8
Silver nitrate	$1{\cdot}5–2{\cdot}0\times10^{-6}$	50
Copper sulphate	$3–8\times10^{-5}$	50
Zinc sulphate	7×10^{-5}	50
Mercuric chloride	$2{\cdot}3\times10^{-6}$	50
Lead acetate	5×10^{-4}	0
1-Naphthyl acetate	$1{\cdot}6–2{\cdot}4\times10^{-2}$	50
Adrenaline	$5–6\times10^{-4}$	50
Insulin	4 units/3·5 ml	10 activity
Tyrosine	0·004	0
p-Methylaminophenol sulphate	0·01	30
Pyrocatechol	0·01	11
Resorcinol	0·01	8
Hydroquinone	0·01	25
Pyrogallol	0·01	10
Phloroglucinol	0·01	9
Ephedrine	0·01	7

According to Rapp and Sliwinski [90], sodium fluorophosphate causes a competitive inhibition because, during the enzymatic reaction, monofluorophosphate competes with orthophosphate.

Potato phosphorylase fractionated with ammonium sulphate was inhibited by Leary and Clagett [64] with sodium 2,4-dichlorophenoxy acetate; it was found that the inhibition is non-competitive.

Similar effects were observed by Neely *et al.* [80] on subjecting phosphorylase obtained from the leaves and stem of red kidney beans to a treatment with 2,4-dichlorophenoxyacetic acid.

According to Ono [81], the synthesizing reaction of phosphorylase in green plant leaves is inhibited by calcium and potassium ions under in vivo conditions while no inhibition takes place under in vitro conditions. However, potassium cyanide causes inhibition in both cases.

Several authors have dealt with the inhibiting effect of phloridzin (2′-β-glucoside-phlorethine). According to Green and Stumpf [38], 18% inhibition is caused by phloridzin. Inhibition is similarly reported by Bailey and Whelan [4], and by Barker *et al.* [8], though in the experiments of the latter the synthesizing activity of potato phosphorylase was only slightly inhibited by phloridzin.

Fischer and Hilpert [25] are of the opinion that only the unpurified phosphorylase is inhibited by phloridzin, and that the enzyme preparations of earlier authors contained β-glucosidase as contaminant which converted phloridzin into phlorethin, a strong inhibitor. The enzyme preparation purified by Fischer and Hilpert could not be inhibited by phloridzin.

Inhibition experiments of substrate analogues are reported only in a few communications, though the elucidation of the functional groups of the substrates participating in the reaction is indispensable for the knowledge of the detailed mechanism of enzyme-catalyzed reactions. A number of authors have investigated the inhibition of glucose but the results are rather contradictory.

According to Green and Stumpf [38], glucose, maltose, sucrose and inulin have no inhibiting effects, while cyclohexa- and cycloheptaamyloses (Schardinger α-and β-dextrins) cause competitive inhibition in respect to primers.

Inhibition by cyclohexa-, cyclohepta- and cyclooctaamylose was also investigated by Staerk and Schlenk [94] in the synthesizing reaction using their purified enzyme (Fig. IV/13). It was found that the inhibition is competitive and that the affinity of the enzyme is altered in the following way on the basis of the constants K_i:

$$\text{cyclohexaamylose} > \text{cycloheptaamylose} > \text{cyclooctaamylose}.$$

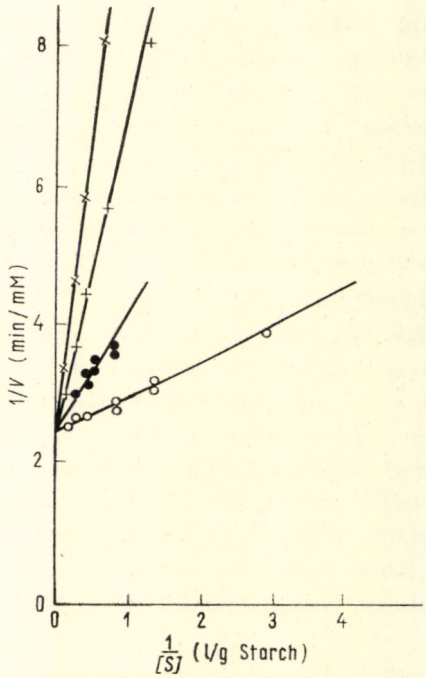


Fig. IV/13. Inhibiting action of various cycloamylose in the enzymatic synthesis catalyzed by potato phosphorylase [94]. Lineweaver–Burk diagram for different concentrations of starch. ○—○: No inhibitor; ×—×: 0·238 mM cyclohexaamylose; +—+: 0·238 mM cycloheptaamylose; ●—●: 0·238 mM cyclooctaamylose. Assays were carried out with 28·6 mM glucose-1-phosphate in 0·1 M citrate buffer (pH 6·3)

From an examination of the mechanism of inhibition, the hydroxy groups proved to be essential for the complex enzyme–inhibitor. This is confirmed by the fact that the enzymatic reaction is not inhibited by cycloheptaamylose-tetradecamethylether or by 2,3-dimethoxycycloheptaamylose.

Similarly to the decomposition by β-amylase, the competitive inhibition of the intrinsic members of the chain was observed by Pfannemüller [83] in the phosphorolytic degradation of amyloses of various degrees of polymerization.

According to Nakamura [77], 0·05 M glucose has no inhibiting effects. Similar results were obtained also by Sumner [95] and by Ram [89].

In contrast, it was found by Fischer and Hilpert [25], Arreguin-Lozano [3] and Porter [88] that glucose causes a competitive inhibition.

Holló *et al.* [45] investigated the inhibition of potato phosphorylase purified by DEAE-cellulose column chromatography, in the presence of glucose and various donor substrate analogues (such as 2-deoxy-D-glucose, 3-O-methyl-α-D-glucose and 6-O-methyl-glucose).

In their series of experiments the concentrations of glucose-1-phosphate (as donor substrate) amylopectin (as acceptor substrate) and the inhibitor were varied.

In this way, the initial reaction rate (v_0) in the inhibited and non-inhibited systems was established from the values of liberated inorganic phosphate.

It was found in these experiments that the phosphorolytic reaction of the enzyme is not inhibited by glucose and the above-mentioned glucose analogues [63]. The results were plotted by the double reciprocal graphical methods of Lineweaver and Burk [68] (Figs IV/14–IV/17).

It can be seen in Figs IV/14–IV/17 that competitive inhibition was caused by glucose and all the applied substrate analogues in respect to both the donor and the acceptor substrates. This is supported also by the identity of the values of V_{max} in all cases. The data K_i, K_m and V_{max} which can be established by the above method are listed in Table IV/10. The apparent Michaelis constants for both the donor and the acceptor substrates rise with increasing inhibitor concentration, indicating that in the presence of an

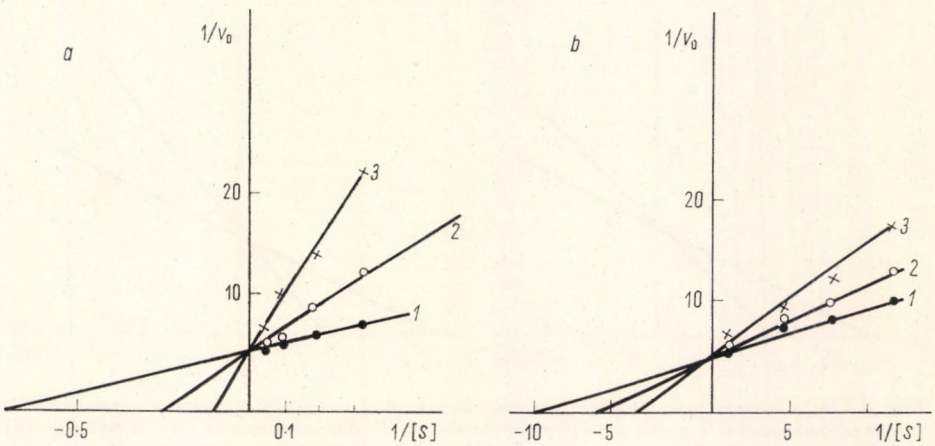


Fig. IV/14. Initial rate plotted against donor and acceptor substrate concentrations in the presence of D-glucose inhibitor. *1:* No inhibitor; *2:* 0·3 M D-glucose; *3:* 0·6 M D-glucose; *a:* D-glucopyranosyl phosphate concentration varying from 3 to 20 mM at a constant amylopectin concentration of 0·95 mM non-reducing end-group; *b:* amylopectin concentration varying from 0·095 to 0·95 mM non-reducing end-group at a constant D-glucopyranosyl phosphate concentration of 20 mM [45]

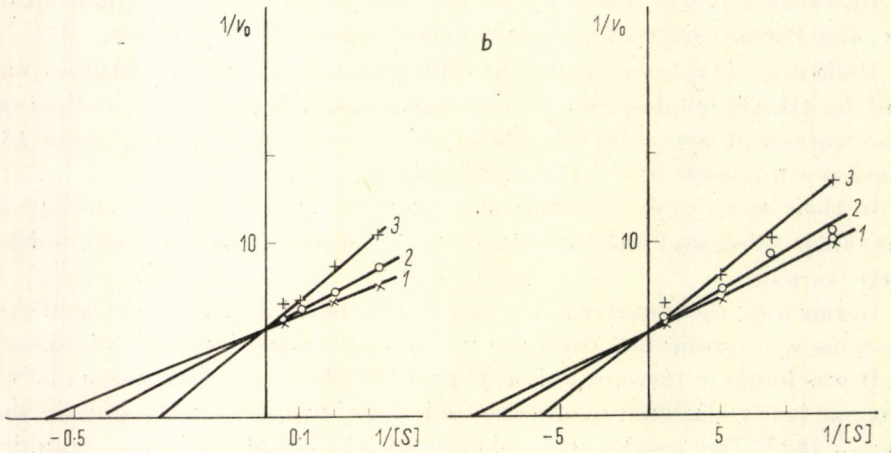


Fig. IV/15. Initial rate plotted against donor and acceptor substrate concentration in the presence of 2-deoxy-D-arabino-hexose inhibitor. *1:* No inhibitor; *2:* 0·3 M 2-deoxy-D-arabino-hexose; *3:* 0·6 M 2-deoxy-D-arabino-hexose; *a:* D-glucopyranosyl phosphate concentration varying from 3 to 20 mM at a constant amylopectin concentration of 0·95 mM non-reducing end group; *b:* amylopectin concentration varying from 0·095 to 0·95 mM of non-reducing end group at a constant D-glucopyranosyl phosphate concentration of 20 mM [45]

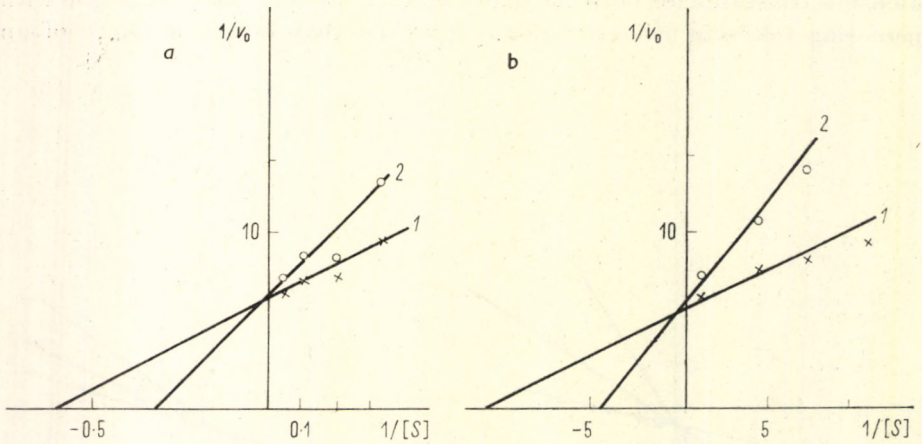


Fig. IV/16. Initial rate plotted against donor and acceptor substrate concentration in the presence of 3-O-methyl-D-glucose inhibitor. *1:* No inhibitor; *2:* 0·6 M 3-O-methyl-D-glucose; *a:* D-glucopyranosyl phosphate concentration varying from 3 to 20 mM at a constant amylopectin concentration of 0·95 mM non-reducing end group; *b:* amylopectin concentration varying from 0·095 to 0·95 mM non-reducing end group at a constant D-glucopyranosyl phosphate concentration of 20 mM [45]

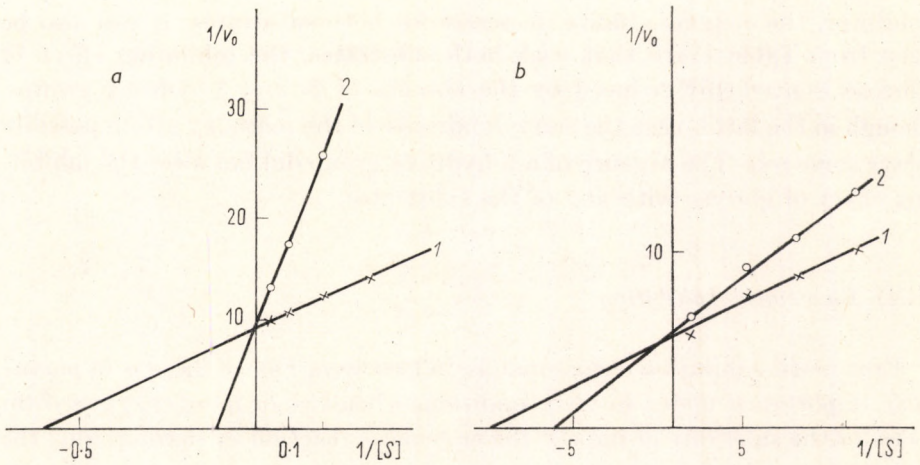


Fig. IV/17. Initial rate plotted against donor and acceptor substrate concentration in the presence of 6-O-methyl-D-glucose inhibitor. *1:* No inhibitor; *2:* 0·3 M 6-O-methyl-D-glucose; *a:* D-glucopyranosyl phosphate concentration varying from 3 to 20 mM at a constant amylopectin concentration of 0·95 mM non-reducing end group; *b:* amylopectin concentration varying from 0·095 to 0·95 mM non-reducing end group at a constant D-glucopyranosyl phosphate concentration of 20 mM [45]

Table IV/10

Values K_m, K_i and V_{max} Determined by the Graphical Method of Lineweaver and Burk, using Various Substrate Analogue Inhibitors [45]

Type of substrate analogue inhibitor	Inhibitor concentration (M)	Glucose-1-phosphate			Amylopectin		
		K_m	K_i	V_{max}	K_m	K_i	V_{max}
		(mM)		(μM/sec)	(mM)		(μM/sec)
D-glucose	0	1·64	—	0·204	0·220	—	0·204
	0·3	4·53	0·170	—	0·330	0·540	—
	0·6	10·00	0·115	—	0·500	0·405	—
2-Deoxy-D-arabino-D-hexose	0·0	1·60	—	0·200	0·097	—	0·202
	0·3	2·15	0·770	—	0·117	1·30	—
	0·6	3·33	0·520	—	0·168	0·79	—
3-O-Methyl-D-glucose	0·0	1·67	—	0·157	0·093	—	0·180
	0·6	3·20	0·650	—	0·213	1·57	—
6-O-Methyl-D-glucose	0·0	1·67	—	0·107	0·094	—	0·190
	0·3	9·10	0·130	—	0·145	0·52	—

inhibitor, the enzyme affinity decreases for both substrates. It can also be seen from Table IV/10 that with both substrates, the inhibiting effect of glucose is markedly reduced by the absence of 2- and 3-hydroxy groups, though in the latter case the steric hindrance of the methoxy group possibly plays some role. The absence of a 6-hydroxy group did not alter the inhibiting effect of glucose with any of the substrates.

(iii) Enzymatic Inhibition

Enzymatic inhibition occurs mainly in two ways. One of these is of particular importance under in vivo conditions when two or more enzymes compete for the substrate or impede the enzymatic reaction by decomposing the substrate.

In the case of unpurified plant phosphorylases the enzymatic reaction is inhibited mainly by α- and β-amylase, phosphatase, Q- and D-enzyme.

A case was reported by Porter [87] where the inhibition of the effect of potato phosphorylase was due to the enzyme contaminants α-amylase and phosphatase which reduced the rate of amylose synthesis. According to this author, on applying 0·005 M $HgCl_2$, the action of α-amylase is completely inhibited while 0·02 M NaF is sufficient to inhibit the phosphatase effect. Thus, by employing these agents, the inhibition by both enzymes can be compensated. This was proved by the fact that the enzymatic synthesis catalyzed by phosphorylase attained an equilibrium state of about 75%, and that neither glucose nor maltose were present in the reaction mixture [86].

Bénard *et al.* [11] has described the inhibiting effect of α-amylase (human saliva) during the enzymatic synthesis catalyzed by potato phosphorylase.

It was proved by Fujimura *et al.* [33] and Fuwa [34] in their research with maize phosphorylase that β-amylase is a strong inhibitor which completely suppresses the activity of phosphorylase. Similar results were observed by Bernstein [10], by Porter with the extracts of various cereals deprived of their germs [86] and with sweet potato [78, 93] as well. According to Porter [86], the effect of β-amylase can be effectively inhibited by heat treatment, while phenyl mercury acetate is suggested for the same purpose by Nakamura *et al.* [79].

It was reported by Whelan that the effect of phosphorylase is promoted by traces of α-amylase in that several primary chains are formed, while higher concentrations of α-amylase cause inhibition since the primary is decomposed [111].

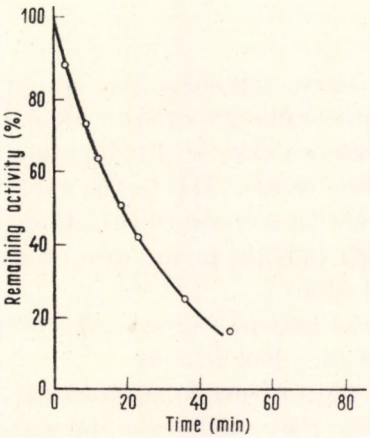


Fig. IV/18. Effect of trypsin on potato phosphorylase [94]. 1·0 μM trypsin in citrate buffer (pH 6·9) with 2·0 μM potato phosphorylase

The inhibiting action of Q-enzyme and of phosphatase was described by Bailey *et al.* [4]. These effects can be suspended by ammonium molybdate in the case of phosphatase and by mercuric chloride in that of Q-enzyme [4]. According to Barker *et al.* [7], the Q-enzyme is completely inactivated by a 30 minute heat treatment at 45–50°C, while Porter and Rees [88] observed that the chain length of synthesized amylose is diminished by the D-enzyme as well.

Another method of inhibition is a structural change effected by proteolytic enzymes. Investigations of this type have been conducted mainly with muscle phosphorylase.

The decomposition of muscle phosphorylase by trypsin leads to a phosphorylase *b'* which is active in the presence of AMP, and to partially decomposed peptides [23, 24]. However, this active fragment cannot be tetramerized to the original phosphorylase *a*. The complete enzyme is characterized by the presence of serylphosphate ester as well. It is of interest that the enzyme containing a phosphate ester is decomposed by trypsin, chymotrypsin, papain and bacterial proteases more quickly than phosphorylase *b* which does not carry such an ester group.

Proteolysis of potato phosphorylase by trypsin (Fig. IV/18) was described by Staerk and Schlenk [94] who observed that on the application of 1 μM of trypsin the catalytic activity decreases at a rate of $8{\cdot}1 \times 10^{8}$ M/min.

(iv) Activators

The data available concerning enzymatic activity are very scarce.

The activation of muscle phosphorylase with AMP is known from literature data. This role is known exactly. Binding takes place to the enzyme in the vicinity of the active centre [31]. In the case of plant phosphorylases, the effect of AMP has not been observed [67], though activation has already been noted by Fredrich [30–32] in the case of alga phosphorylase in the presence of AMP and Mn^{2+}.

According to the investigation of Green and Stumpf [38], potato phosphorylase is not activated by adenylic acid.

From a study of the effect of insulin, Nakamura [77] found that on raising the insulin concentration the activity also increases (10% activity increase was caused by 4 units/3·5 ml, and 20% activity increase by 20 units/3·5 ml).

Cornblath [18], and Cori and Illingworth [16] reported that adrenaline can be applied as an activator in the enzymatic synthesis catalyzed by animal phosphorylase. In marked contrast, according to Nakamura [77] adrenaline ($5–6 \times 10^{-4}$ M) showed 50% inhibition in the case of potato phosphorylase.

(v) Enzyme Stability, Storage

Investigations of enzyme stability were carried out first by Weibull and Tiselius [107]. Potato phosphorylase prepared by these authors was stored for 53 days at 4°C at various pH values, and activity values prior to and after storage were established. Percentages of residual activity after the experimental period are plotted against pH in Fig. IV/19. It appears from this Figure that the enzyme has the highest stability at pH 7·0 and becomes unstable at pH values below 6·5. On lyophilization, the enzyme becomes completely inactive.

According to Barker *et al.* [8], the use of a citrate buffer of pH 6–7, markedly reduces the degree of inactivation during the lyophilization process. Experimental data of Green and Stumpf [38], Weibull and Tiselius [108] and Hanes [40] show that on subjecting the enzyme to dialysis against distilled water, it is completely inactivated. This can be prevented, however, by applying potassium chloride [38, 40].

The stability of the enzyme under various experimental conditions has been investigated with potato phosphorylase prepared by Lee. The results are listed in Table IV/11 [66].

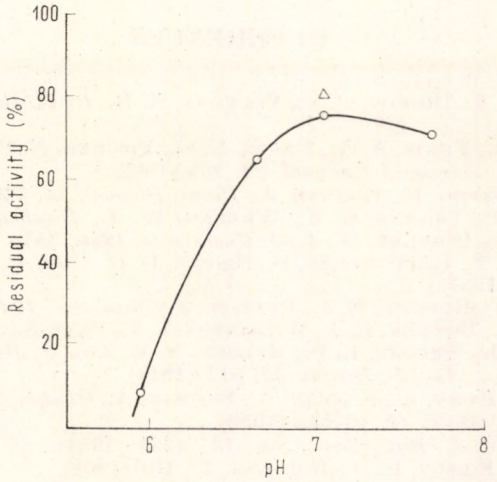


Fig. IV/19. Residual activition of potato phosphorylase (purified by ammonium-sulphate) after storage for 53 days at 4°C, at different pH values [107]

Table IV/11

Investigation of the Stability of Purified Potato Phosphorylase [66]

Incubation period	pH	Temperature (°C)	Decrease of enzyme activity (%)
5 min	6·3	50	10
5 min	6·3	55	30
5 min	6·3	60	90
5 min	6·3	65	100
17 h	5·9–7·7	30	0
17 h	7·7–8·7*	30	0
10 min	5·3	30	10
60 min	5·3	30	20
10 min	4·9	30	75
60 min	4·9	30	100
10 min	9·3**	30	20
60 min	9·3**	30	35

Incubations were carried out in 0·05 M citrate buffer, with the exception of the data denoted by * (0·05 M tris-phosphate buffer) and denoted by ** (0·05 M glycine buffer).

According to Kamogawa *et al.* [51], the crystalline potato phosphorylase, when suspended in a solution of ammonium sulphate (of pH 7·0) and kept at 5°C, is stable for several months. On freezing and thawing the enzyme, a marked activity decrease was experienced, and complete protein denaturation was caused by the low ionic strength.

REFERENCES

1. Anisimov, A. A., Boikov, P. Y., Volkova, A. B., *Prikl. Biokhim. i Mikrobiol. 1*, 206 (1965).
2. Appleman, M., Yunis, A. A., Krebs, E. G., Fischer, E. H., unpublished results referred to in: *Advanced Enzimol. 24*, 263 (1962).
3. Arreguin-Lozano, B., Bonner, J., *Plant. Physiol. 24*, 720 (1949).
4. Bailey, J. M., Thomas, G. J., Whelan, W. J., *Biochem. J. 49*, LVI (1951).
5. Bailey, J. M., Whelan, W. J., *J. Chem. Soc. 1950*, 3573.
6. Baranowski, T., Illingworth, B., Brown, D. H., Cori, C. F., *Biochim. Biophys. Acta 25*, 16 (1957).
7. Barker, S. A., Bourne, E. J., Peat, S., *J. Chem. Soc. 1949*, 1705.
8. Barker, S. A., Bourne, E. J., Wilkinson, I. A., Peat, S., *J. Chem. Soc. 1950*, 84.
9. Battell, M. L., Smillie, L. B., Madsen, N. B., *Can. J. Biochem. 46*, 609 (1968).
10. Bernstein, L., *Am. J. Botany 30*, 517 (1943).
11. Bénard, H., Horn, A., Kaswin, A., Seeman, A., *Compt. rend. soc. biol. 144*, 237 (1950); *Chem. Abstr. 44*, 10764a (1950).
12. Boyer, P. D., *J. Am. Chem. Soc. 76*, 4331 (1954).
13. Bresler, S., Firsov, L., *J. Mol. Biol. 35*, 131 (1968).
14. Chen, P. S., Toribara, T. Y., Jr., Warner, H., *Anal. Chem. 28*, 1757 (1956).
15. Cori, G. T., Cori, C. F., *J. Biol. Chem. 158*, 321 (1945).
16. Cori, G. T., Illingworth, B., *Biochim. Biophys. Acta 21*, 105 (1956).
17. Cori, C. F., Illingworth, B., *Proc. Natl. Acad. Sci. U. S. 43*, 547 (1957).
18. Cornblath, M., *Am. J. Physiol. 183*, 240 (1955).
19. Cowgill, R. W., *Abstr. IV. Intern. Congr. Biochem. 4* (1958).
20. Cowgill, R. W., Cori, C. F., *J. Biol. Chem. 261*, 133 (1955).
21. Damjanovich, S., Kleppe, K., *Biochim. Biophys. Res. Commun. 26*, 65 (1967).
22. Datta, J., *Ann. Biochem. Exptl. Med. 20*, 117 (1960); *Chem. Abstr. 61*, 12250f (1964).
23. Fischer, E. H., *J. Biol. Chem. 234*, 1698 (1959).
24. Fischer, E. H., Graves, D. J., Crittenden, E. S., Krebs, G., *J. Biol. Chem. 234*, 1698 (1959).
25. Fischer, E. H., Hilpert, H. M., *Experientia 9*, 176 (1953).
26. Fischer, E. H., Kent, A. B., Snyder, E. R., Krebs, E. G., *J. Am. Chem. Soc. 80*, 2906 (1958).
27. Fischer, E. H., Krebs, E. G., *J. Biol. Chem. 216*, 121 (1955).
28. Folin, O., Ciocalteu, V., *J. Biol. Chem. 73*, 627 (1927).
29. Fraenkel-Conrat, H., Bean, R. C., Lineweaver, H., *J. Biol. Chem. 177*, 385 (1949).
30. Fredrick, J. F., *Ann. N. Y. Acad. Sci. 88*, 385 (1960).
31. Fredrick, J. F., *Phytochemistry 2*, 413 (1963); *Chem. Abstr. 60*, 1984d (1964).
32. Fredrick, J. F., *Ann. N. Y. Acad. Sci. 121*, 634 (1964).
33. Fujimura, K., Kawabata, M., Ikeda, Sh., *Mem. Research Inst. Food Sci. Kyoto Univ.* No. *15*, 23 (1958); *Chem. Abstr. 53*, 506b (1959).
34. Fuwa, H., *Arch. Biochem. Biophys. 70*, 157 (1957).
35. Gold, A. M., *Biochemistry 7*, 2106 (1968).
36. Green, A. A., *J. Biol. Chem. 158*, 315 (1945).
37. Green, A. A., Cori, G. T., *J. Biol. Chem. 151*, 21 (1943).
38. Green, D. E., Stumpf, P. K., *J. Biol. Chem. 142*, 355 (1942).
39. Hamada, J., *Nippon Kogaku Zasshi 80*, 182 (1959); *Chem. Abstr. 59*, 14241h (1963).
40. Hanes, C. S., *Proc. Roy. Soc. London B 129*, 174 (1940).
41. Hedrick, J. L., *Arch. Biochem. Biophys. 114*, 216 (1966).
42. Hidy, P. H., Day, H. G., *J. Biol. Chem. 160*, 273 (1945).
43. Holló, J., László, E., Hoschke, Á., *Die Stärke 17*, 377 (1965).
44. Holló, J., László, E., Hoschke, Á., *Die Stärke 18*, 337 (1966).
45. Holló, J., László, E., Hoschke, Á., *Carbohydr. Res. 10*, 49 (1969)
46. Holló, J., László, E., Hoschke, Á., Varga, P., *Carbohydr. Res. 8*, 416 (1968).
47. Holló, J., László, E., Juhász, J., *Die Stärke 19*, 246 (1967).

48. ILLINGWORTH, B., JANSZ, H. S., BROWN, D. H., CORI, C. F., *Proc. Natl. Acad. Sci. U. S. 44*, 1180 (1958).
49. JANSZ, H. S., unpublished results.
50. JOHNSON, G. F., GRAVES, D. J., *Biochem. J. 5*, 2906 (1966).
51. KAMOGAWA, A., FUKUI, T., NIKUNI, Z., *J. Biochem. 63*, 361 (1968).
52. KATZ, J., HASSID, W. Z., *Arch. Biochem. 30*, 272 (1951).
53. KELLER, P. J., *Thesis*, Washington Univ. 1953.
54. KELLER, P. J., *J. Biol. Chem. 214*, 135 (1955).
55. KELLER, P. J., CORI, G. T., *Biochim. Biophys. Acta 12*, 235 (1953).
56. KENT, A. B., *Thesis*, Washington Univ. 1959.
57. KENT, A. B., KREBS, E. G., FISCHER, E. H., *J. Biol. Chem. 232*, 549 (1958).
58. KREBS, E. G., FISCHER, E. H., *Biochim. Biophys. Acta 20*, 150 (1956).
59. KREBS, E. G., FISCHER, E. H. in: *Adv. in Enzymology* (NORD, F. F.) Vol. 24, p. 267, J. Wiley, New York, 1962.
60. KREBS, E. G., KENT, A. B., FISCHER, E. H., *J. Biol. Chem. 231*, 73 (1958).
61. KUDO, A., SHUKUYA, R., *J. Biochem. (Tokyo) 55*, 254 (1964); *61*, 912e (1964).
62. KURASAWA, H., IGAUE, J., HAYAKAWA, T., OGAMI, H., *Nippon Nogei Kagaku Kaishi 33*, 393 (1959); *Chem. Abstr. 54*, 9005d (1960).
63. LÁSZLÓ, E., *Thesis* for the title of Cand. Chem., Budapest, 1968 (in Hungarian).
64. LEARY, J. B., CLAGETT, C. O., *Proc. N. Dakota Acad. Sci. 5*, 47 (1951); *Chem. Abstr. 47*, 1626 (1953).
65. LEE, Y. P., *Fred. Proc. 18*, 271 (1959).
66. LEE, Y. P., *Biochim. Biophys. Acta 43*, 18 (1960).
67. LEE, Y. P., *Biochim. Biophys. Acta 43*, 25 (1960).
68. LINEWEAVER, H., BURK, D., *J. Am. Chem. Soc. 56*, 658 (1934).
69. MADSEN, N. B., *J. Biol. Chem. 223*, 1067 (1956).
70. MADSEN, N. B., CORI, C. F., *Biochim. Biophys. Acta 15*, 516 (1954).
71. MADSEN, N. B., CORI, C. F., *J. Biol. Chem. 223*, 1055 (1956).
72. MADSEN, N. B., GURD, F. R. N., *J. Biol. Chem. 223*, 1075 (1956).
73. METZGER, B., HELMREICH, E., GLASER, L., *Proc. Natl. Acad. Sci. U. S. 57*, 994 (1967).
74. METZLER, D. E., IKAWA, M., SNELL, E. E., *J. Am. Chem. Soc. 76*, 648 (1954).
75. NAKAMURA, M., *J. Agr. Chem. Soc. Japan 24*, 5 (1950); *Chem. Abstr. 47*, 1858c (1953).
76. NAKAMURA, M., *J. Agr. Chem. Soc. Japan 26*, 260 (1952); *Chem. Abstr. 48*, 757e (1954).
77. NAKAMURA, M., *J. Agr. Chem. Soc. Japan 26*, 267 (1952); *Chem. Abstr. 48*, 757g (1954).
78. NAKAMURA, M., *J. Biochem. (Tokyo) 46*, 799 (1959); *Chem. Abstr. 54*, *54*, 1641h (1960).
79. NAKAMURA, M., YAMAZAKI, K., MARUO, B., *J. Agr. Chem. Soc. Japan 24*, 299, 302 (1951); *Chem. Abstr. 45*, 6669e (1951).
80. NEELY, W. B., BALL, C. D., HAMNER, C. L., SELL, H. M., *Plant Physiol. 25*, 525 (1950).
81. ONO, R., *Botan. Mag. (Tokyo) 66*, 182 (1953); *Chem. Abstr. 48*, 8883i (1954).
82. PETERSON, E., SOBER, H. A., *J. Am. Chem. Soc. 76*, 169 (1954).
83. PFANEMÜLLER, B., *Die Stärke 20*, 351 (1968).
84. PHILLIP, G., GRAVES, D. J., *Biochemistry 7*, 2093 (1968).
85. PHILLIPS, G. O., GRIFFITS, W., *Radiation Res. 26*, 363 (1965).
86. PORTER, H. K., *Biochem. J. 47*, 476 (1950).
87. PORTER, H. K., *J. Exper. Bot. 4*, 44 (1953).
88. PORTER, H. K., REES, W. R., *Plant Physiol. 29*, 514 (1954).
89. RAM, J. S., GIRI, K. V., *Arch. Biochem. Biophys. 38*, 231 (1952).
90. RAPP, G. W., SLIWINSKI, R. A., *Arch. Biochem. Biophys. 60*, 379 (1956).
91. SCHLENK, H., SAND, D. M., *J. Am. Chem. Soc. 83*, 2312 (1961).
92. SCHLENK, H., SAND, D. M., *190th Meeting Am. Chem. Soc. Chicago 1961*, Abstract 18C No. 46
93. SHEN KUNG-MOU, SHEN YUEN-KANG, *Sheng Li Hsüe Pao, 21*, 185 (1957); *Chem. Abstr. 53*, 13220i (1959).
94. STAERK, J., SCHLENK, H., *Biochim. Biophys. Acta 146*, 120 (1967).

95. SUMNER, J. B., CHOU, T. C., BEVER, A. T., *Arch. Biochem. 26*, 1 (1950).
96. SUTHERLAND, E. W., WOSILAIT, W. D., *J. Biol. Chem. 218*, 459 (1956).
97. SUZUKI, T., HAMADA, J., *Nippon Kagaku Zasshi 77*, 1681 (1956); *Chem. Abstr. 53*, 3321d (1959).
98. TISELIUS, A., PEDERSEN, K. O., SVEDSBERG, T., *Nature 140*, 848 (1937).
99. TORCHINSKII, Y. M., LIVANOVA, N. B., PIKHELGAS, V. Y., *Biochim. Biophys. Acta 110*, 619 (1965).
100. TORCHINSKII, Y. M., LIVANOVA, N. B., PIKHELGAS, V. Y., *Mol. Biol. 1*, 28 (1967).
101. VELICK, S. F., WICKS, L. F., *J. Biol. Chem. 190*, 741 (1951).
102. VULFSON, P. L., SKOLISHEVA, L. K., *Biokhimiya 32*, 582 (1967).
103. WANG, J. H. C., BLACK, W. J., *J. Biol. Chem. 243*, 4641 (1968).
104. WANG, J. H., GRAVES, D. J., *J. Biol. Chem. 238*, 2386 (1963).
105. WANG, J. H., GRAVES, D. J., *Biochemistry 3*, 1437 (1964).
106. WANG, J. H., SHONKA, M. L., GRAVES, D. J., *Biochemistry 4*, 2296 (1965).
107. WEIBULL, C., TISELIUS, A., *Arkiv Kemi, Mineral. Geol. 19 A*, 1 (1945).
108. WEIBULL, C., TISELIUS, A., *Arkiv Kemi, Mineral. Geol. A 19*, 25 (1945).
109. WEIL, L., BUCHERT, A. R., MAHER, J., *Arch. Biochem. Biophys. 40*, 241 (1952).
110 WEIL, L., GORDON, W. G., BUCHERT, A. R., *Arch. Biochem. Biophys. 33*, 90 (1951).
111. WHELAN, W. J., in *Method of Enzymology* (COLOWICK, S. P. and KAPLAN, N. O.) Vol. 1, p. 193. Academic Press, New York, 1955.
112. WOSILAIT, W. D., *J. Biol. Chem. 233*, 597 (1958).
113. WOSILAIT, W. D., SUTHERLAND, E. W., *J. Biol. Chem. 218*, 469 (1956).
114. YUNIS, A. A., FISCHER, E. H., KREBS, E. G., *J. Biol. Chem. 235*, 3163 (1960).

V. MECHANISM OF ENZYME ACTION

Research carried out in recent years has proved that the mechanism of the action of enzymes responsible for the synthesis of the (1,4)-α-D-glucosidic bonds (phosphorylase, disproportionating enzyme, starch synthetase, amylomaltase, amylosaccharase etc.) can be expressed by a general equation as follows [43]:

$$G - OX + HOR \rightleftharpoons G - OR + HOX$$

where G — OX denotes the glucose donor, α-D-glucopyranose-1-phosphate in the case of plant phosphorylase.

The acceptor substrate HOR is a 'primary' chain consisting of at least three D-glucose units linked by α-1,4 bonds [3, 17, 57, 59]. In the case of potato phosphorylase, the above equation can be written in the form:

$$G - 1 - P + G - (G)_n - G \rightleftharpoons G - G - (G)_n - G + P.$$

Enzymatic action consists, in essence, in a transfer, by the enzyme, of the D-glucosyl radical of the donor to the non-reducing end of the acceptor substrate [5, 7, 9, 21] followed by the linkage of further glucosyl radicals to the acceptor by bonds of similar type. In the course of this reaction, phosphate ions (OX) are liberated. The new bonds formed are all α-1,4--glucosidic bonds. Thus, it is possible to attain the degree of polymerization of native amylose. On denoting the 'primary' by $(C_6H_{10}O_5)_n$, the reaction catalyzed by potato phosphorylase becomes:

$$1.\ (C_6H_{10}O_5)_n + \text{G-1-P} \rightarrow (C_6H_{10}O_5)_{n+1} + \text{inorganic P}$$

$$2.\ (C_6H_{10}O_5)_{n+1} + \text{G-1-P} \rightarrow (C_6H_{10}O_5)_{n+2} + \text{inorganic P}$$

and so on [12].

This reaction leads to an equilibrium. It can be seen from the previous equation that the equilibrium value depends solely on the ratio of inorganic phosphate to ester phosphate, being quite independent of the concentration

of polysaccharide, because the number of reducing glucose end groups does not alter in the course of the reaction.

$$K = \frac{[(C_6H_{10}O_5)_{n+1}] \cdot [\text{inorganic phosphate}]}{[(C_6H_{10}O_5)_n] \cdot [\text{G-1-P}]}.$$

From the equilibrium constant K, the ratio of inorganic phosphate to ester phosphate can be calculated at a given pH. In the case of potato phosphorylase, this ratio was investigated by Hanes [21] in a series of experiments at 25°C (Fig. V/1).

In the case of muscle phosphorylase between DP 3 and 150, the ratio of inorganic phosphate to ester phosphate was 6·14 at pH 6 and 3·55 at pH 6·8, according to Cori *et al.* [9].

The equilibrium ratio of inorganic phosphate to ester phosphate is markedly affected by the hydrogen ion concentration [10, 20, 22]. This is quite reasonable since phosphoric acid is a weaker acid than glucose-1-phosphoric acid. The dissociation constants measured for phosphoric acid are

$$K_1 = 1{\cdot}07 \times 10^{-2} \quad \text{and} \quad K_2 = 1{\cdot}57 \times 10^{-7}$$

and for glucose-1-phosphoric acid ($ROPO_3H_2$)

$$K_1 = 7{\cdot}8 \times 10^{-2} \quad \text{and} \quad K_2 = 7{\cdot}4 \times 10^{-7}.$$

According to Trevelyan, this latter K_2 value equals $3{\cdot}09 \times 10^{-7}$[55].

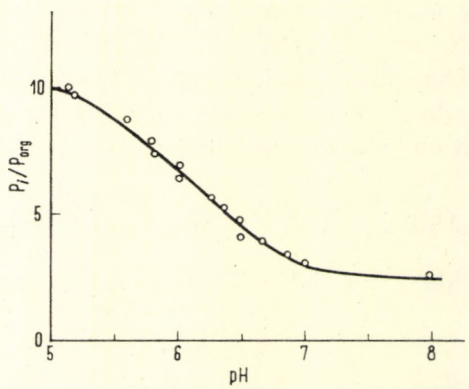


Fig. V/1. Equilibrium constant of the effect of potato phosphorylase according to Hanes [21]

The calculated ratio of the two acids for either monovalent or bivalent ions in the pH range 5–7 in the equilibrium state, proves to be approximately constant [9, 21, 22]. At an ionic strength extrapolated to 0, Trevelyan [55] gives $K = 11{\cdot}35 - \pm 0{\cdot}07$.

However, the above data require correction because the phosphorylase preparations used in the measurements were contaminated with amylase. Thus, the stability of the mole concentration of the 'primary' could not be maintained and possible changes could not be taken into account by the various authors.

Data published recently, based on the use of enzymes free of amylase, can be considered as realistic equilibrium constants. Since the equilibrium constants also depend on experimental conditions, the technique of the determination will be precisely described here [25]. Holló *et al.* employed five different temperatures (15, 20, 25, 30 and 35°C) and five different pH values (5·0, 5·5, 6·0, 7·0 and 8·0). In the synthesis experiments, the composition of the reaction mixture was as follows: 5 ml of 0·2 M citrate buffer, 1 ml of glucose-1-phosphate solution (0·05 mmole/ml), 2 ml amylopectin solution (10 mg/ml) and 2 ml of enzyme solution (210 enz.units). The various reaction mixtures were kept in a thermostat to an accuracy of ±0·1°C. Samples were withdrawn at certain intervals and the reaction was followed by measuring the inorganic phosphate content. When equilibrium was attained, the exact concentration of inorganic phosphate was established by five parallel measurements (Table V/1).

Table V/1

Dependence of Inorganic Phosphate Content on pH and Temperature at Equilibrium of the Synthesis Reaction [25]

Temperature (°C)	Equilibrium amount of inorganic phosphate mg in 0·5 ml of reaction mixture				
	at pH 5·0	5·5	6·0	7·0	8·0
15	0·0590	0·0580	0·0555	0·0515	0·0505
20	0·0645	0·0639	0·0610	0·0565	0·0540
25	0·0665	0·0655	0·0625	0·0575	0·0530
30	0·0575	0·0560	0·0525	0·0500	—
35	0·0635	0·0625	0·0580	0·0550	0·0530

In the phosphorolysis experiments, the reaction mixtures contained 2 ml of 0·2 M phosphate buffer, 5 ml of amylopectin solution (16·2 mg/ml) and 3 ml of enzyme solution (315 enz.units). The pH values and temperatures were the same as employed above, and the equilibrium concentrations of ester phosphate were established in a similar way (Table V/2).

Table V/2

Dependence of Equilibrium Concentration of Ester Phosphate on pH and Temperature, in Phosphorolysis [25]

Temperature (°C)	Equilibrium amount of ester phosphate mg in 1 ml of reaction mixture				
	at pH 5·0	5·5	6·0	7·0	8·0
15	0·081	0·087	0·126	0·139	0·233
20	0·085	0·096	0·114	0·164	0·176
25	0·094	0·119	0·142	0·185	0·193
30	0·102	0·114	0·133	0·171	0·198
35	0·101	0·113	0·134	0·160	0·199

Prior to calculating the equilibrium constants, the following considerations appear to be necessary: the equilibrium constants can be calculated from the values of the thermodynamic activity of the reaction components, e.g. the equilibrium constant of the synthesis is

$$K = \frac{a_{H_2PO_4^{(-)}} \cdot a_{G_{n+1}}}{a_{C_6H_{11}O_5OPO_3H^{(-)}} \cdot a_{G_n}}$$

where G_n denotes the initial 'primary' molecule.

In the above equation, the quotient $a_{G_{n+1}}/a_{G_n}$ is unity, since on employing pure enzyme preparations, no alterations take place in the number of non-reducing glucose terminal units, i.e. the activity of the C_4-hydroxy group of the terminal units remains unchanged.

Under the applied measurement conditions, the activity coefficients of inorganic phosphate and of glucose-1-phosphate are nearly identical [55]. Thus, at a given constant ionic strength, it is possible to replace thermodynamic activities by the concentration values:

$$K = \frac{[\text{inorganic phosphate}]}{[\text{glucose-1-phosphate}]}.$$

The equilibrium constants of the synthesis, calculated from the measured data are listed in Table V/3.

On comparing the values of Tables V/3 and V/4 it can be seen that, within experimental error, the equilibrium constants of the synthesis and the reciprocal values of the equilibrium constants of phosphorolysis are identical. From this it follows that the dependence on temperature and pH of the equilibrium amounts of inorganic phosphate and ester phosphate are the same for both reactions.

Table V/3

Dependence of the Equilibrium Constants of the Synthesis on pH Values and Temperature [25]

Temperature (°C)	Equilibrium constants				
	at pH 5·0	5·5	6·0	7·0	8·0
15	13·40	11·40	9·20	5·60	4·95
20	12·40	10·65	8·90	5·35	4·70
25	11·70	10·15	8·35	4·80	4·40
30	11·00	9·50	7·90	4·60	—
35	10·55	9·15	7·45	4·35	4·00

In phosphorolysis, in turn, the equilibrium constants presented in Table V/4 can be calculated.

Table V/4

Dependence of Equilibrium Constants of Phosphorolysis on pH and Temperature [25]

Temperature (°C)	Equilibrium constants				
	at pH 5·0	5·5	6·0	7·0	8·0
15	0·075	0·0875	0·111	0·159	0·202
20	0·081	0·094	0·118	0·172	0·208
25	0·086	0·105	0·123	0·188	0·217
30	0·094	0·106	0·127	0·173	0·232
35	0·094	0·108	0·130	0·189	0·244

1. Synthesis of α-1,4-bonds by Phosphorylase

It has already been mentioned in discussing the equilibrium data (cf. preceding Section) that on employing an enzyme preparation free of amylase, no changes occur in the molar concentration of the acceptor during the synthesis of the α-1,4-bonds. Accordingly, the reaction catalyzed by phosphorylase can be more easily surveyed and followed mathematically. In the present section the reaction order of the synthesis of α-1,4-bonds, the factors affecting the reaction rates, the mechanism of the synthesis, the specificity of phosphorylase, the formation and decomposition of the enzyme-substrate complex, and the thermodynamic data will be discussed.

(*i*) *Order of the Reaction*

The reaction rate of the synthesis of amylose catalyzed by potato phosphorylase is determined most often from the amount of inorganic phosphate liberated in unit time from glucose-1-phosphate. In the case of muscle phosphorylase, it was found by Cori *et al.* [9] that the synthesis follows first order kinetics. For first order reactions, the reaction rate is expressed by the equation:

$$v = \frac{1}{t} \cdot \ln \frac{A}{A - x}$$

where A is the equilibrium amount of inorganic phosphate at a given pH, x the amount of inorganic phosphate liberated during the reaction and t the reaction period in minutes.

Results similar to those of Cori have been obtained by Hidy and Day[23], and by Weibull and Tiselius [56], for potato phosphorylase enzyme of low specific activity, fractionated with ammonium sulphate, at low conversion percentages and in two reaction periods.

According to Nakamura [46], for an enzyme prepared in a way similar to that used in the previous experiments, the reaction rate was of zero order in the initial part of the synthesis, until the ratio of inorganic phosphate to organic phosphate plus ester phosphate attained the value of 0·2.

The reaction order in the synthesis and decomposition was investigated by Arreguin *et al.* [2] for an enzyme purified by lead acetate. The enzymatic reaction was described by the equation

$$\underset{\text{Glucose-1-phosphate}}{A} \rightleftharpoons \underset{\text{Amylose}}{B} + \underset{\text{Inorganic phosphate}}{C}$$

i.e. the phosphorolysis can be described by a first order equation and the synthesis by a second order equation. The reaction rate of the synthesis could be expressed by the equation

$$\frac{dx}{dt} = k(a - x) - k_1 \cdot x \cdot \frac{x}{n}$$

where a is the initial concentration of glucose-1-phosphate, x the concentration of glucose-1-phosphate reacted up to time t, $\frac{x}{n}$ the concentration of amylose formed during time t, n the number of glucose units in the amylose formed and k and k_1 the first and second order rate constants in the two reaction directions.

At equilibrium (when $dx/dt = 0$), the following equation holds:

$$k(a - x_e) = k_1 \cdot x_e \cdot \frac{x_e}{n}$$

where x_e is the equilibrium concentration of glucose-1-phosphate.

By extracting k from both equations and conducting some simple mathematical operations, a 'first-second', order rate constant can be obtained by which, according to the authors mentioned, the synthesis process can be characterized.

The 'first–second' order rate constant is given by

$$k = \frac{x_e}{t(2a - x_e)} \ln \frac{ax_0 + x(a - x_e)}{a(x_e - x)}$$

According to Holló *et al.* [24], the synthesis of α-1,4-bonds, up to a 90% conversion of glucose-1-phosphate, can be described by a reaction equation of the first order (Table V/5).

Table V/5

Conversion of Glucose-1-phosphate as a Function of Time
(pH 6·0, 30°C, 22·5 Enz. units/ml, 0·01 M glucose-1-phosphate) [24]

Duration of reaction (min)	Conversion of glucose-1-phosphate (%)	Rate constant $k_1(\text{min}^{-1})$
2·5	17·5	$7{\cdot}6 \times 10^{-2}$
5·0	32·0	$7{\cdot}3 \times 10^{-2}$
10·0	52·1	$7{\cdot}5 \times 10^{-2}$
20·0	77·4	$7{\cdot}4 \times 10^{-2}$
30·0	89·8	$7{\cdot}6 \times 10^{-2}$
40·0	91·8	$7{\cdot}0 \times 10^{-2}$
50·0	92·3	$5{\cdot}0 \times 10^{-2}$
60·0	92·6	$4{\cdot}2 \times 10^{-2}$
100·0	93·8	$2{\cdot}5 \times 10^{-2}$

(ii) Effect of Hydrogen Ion Concentration on Reaction Rates

In general, enzymes are active only in certain defined pH ranges, and in the majority of cases a definite optimum pH can be observed as the combined result of several factors. Changes in pH influence the ionization state of the various ionizable groups in the enzyme protein. Catalytic activity

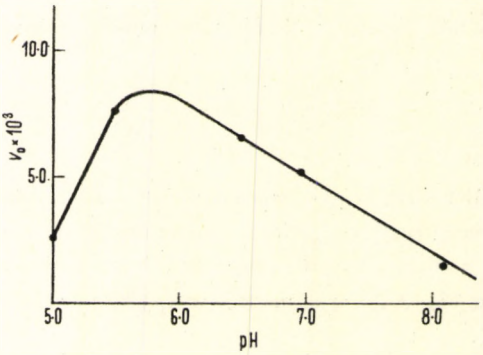


Fig. V/2. Dependence of the initial rate of the synthesis reaction on the pH [24]

is affected in general by a relatively narrow pH range possibly determined by the ionic form of the active centre [15].

The investigation of the dependence of pH can even be utilized for the determination of these ionizing groups which will be discussed later. As regards the effect of pH, different data were given for enzymes of various degrees of purity (cf. Chapter IV).

Literature data, with the exception of the most recent reports, do not refer to the dependence of well definable initial rates on the pH. According to Holló *et al.* [24], the dependence of the synthesis on hydrogen ion concentration is characterized by the curve shown in Fig. V/2. This Figure clearly indicates that the optimum range is pH 5·8–6·1. The pH values between which the reaction rate is at least half the optimum value (pH_{50}) are 5·2 and 7·5, i.e. an interval of 2·3 pH units. For muscle phosphorylase, the optimum pH value was 6·8, while pH_{50} covered 2·7 units [8]. Among the enzymes which decompose and synthesize starch potato phosphorylase showed the minimum pH_{50} value [41]. For comparison it may be stated that with wheat β-amylase, reaction rates corresponding to 50% of the optimum value can be observed over 6·2 pH units [45]. Consequently, potato phosphorylase is extremely sensitive to changes in hydrogen ion concentration.

(iii) Effect of Temperature on Reaction Rates

As discussed in Section IV.5 *(ii)*, at a certain temperature proteins begin to be denatured. As a consequence, the rate of catalyzed reactions diminishes, and at a certain temperature the reactions completely stop.

Several data are known of the effect of temperature in the case of potato phosphorylase (cf. Chapter IV).

Differences in the data of various authors are due on the one hand to differences of purity of the enzyme preparations, and on the other hand to the appreciable dependence of the effect of temperature on the experimental conditions. The ionization state of the various functional groups and, to an even greater extent, the alterations in the enzyme structure caused by denaturation by heat, are strongly affected by the applied experimental conditions. Thus, on carrying out kinetic investigations, utmost care must be taken as to the identity of experimental conditions, and the details, these experimental conditions must always be given.

In a study of the dependence on temperature in the synthesis of α-1,4--bonds, the following technique was applied by Holló *et al.* [24]. Reaction mixtures containing 0·1 M glucose-1-phosphate, 1·5% amylopectin, 7·5 enzyme units per ml and a citrate buffer of pH 6 were incubated for 5 minutes at temperatures from 15 to 60°C. Parallel to this run, another series without glucose-1-phosphate was kept for 5 minutes at the same temperatures (in order to observe the denaturing effect of temperature), cooled, adequate amounts of glucose-1-phosphate added, and the mixture incubated again for 5 minutes.

The measured data are shown in Fig. V/3.

It can be seen that the optimum temperature range is 45–50°C and that the denaturing effect of temperature appears only at temperatures over 50°C. In the temperature range 30–45°C, Q_{10} has a value of 1·40.

The investigation of the effect of temperature can be utilized for the elucidation of the energetic conditions of the various partial processes of enzymatic catalysis. This will be discussed later (cf. Chapter VI).

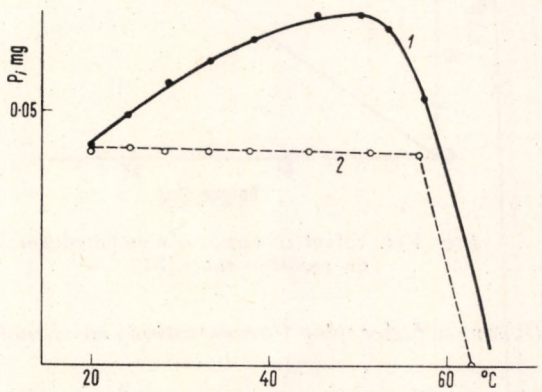


Fig. V/3. Dependence of the initial rate of the synthesis reaction on temperature [24]. *1:* Without incubation; *2:* with incubation

(iv) Effect of Enzyme Concentration on Reaction Rates

For the major part of enzymatic reactions, the correlation

$$v = k[E]$$

holds, i.e. with increasing enzyme concentration, the rate of the catalyzed reaction increases in a linear manner. This is an important relation since conclusions as to the presence of inhibitor or activator contaminants in the enzyme preparations can be drawn from a relatively simple series of experiments.

In the case of potato phosphorylase, the effect of enzyme concentration on reaction rates at two or three different enzyme concentrations was examined by Weibull and Tiselius [56], and by Hidy and Day [23]. According to both communications, reaction rates increase linearly with enzyme concentration. Hoschke [31] has published more detailed data on the effect of enzyme concentration (Fig. V/4). It can be seen from the values measured at 30°C and pH 6 at various enzyme concentrations that on raising the enzyme concentration the initial rates show a linear increase. This indicates that the enzyme preparation used did not contain any inhibitor or activator contaminant.

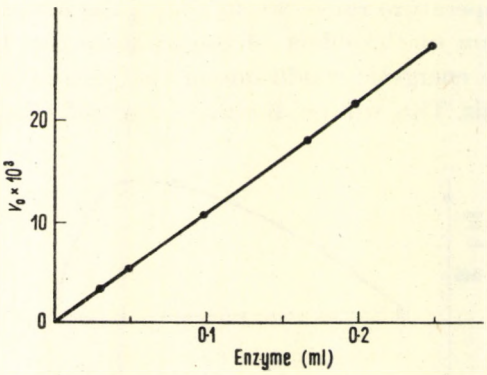


Fig. V/4. Effect of enzyme concentration on reaction rates [31]

(v) Effect of Donor and Acceptor Concentrations on Reaction Rates

In chemical reactions, reaction rates generally increase with increasing component concentrations. In reactions catalyzed by enzymes however the increase of reaction rates is limited by the formation, conversion or de-

composition of the enzyme-substrate complex. Reactions of this latter type can be described by the Michaelis–Menten theory.

Changes in reaction rates at 38°C and pH 6·2 were measured by Hidy and Day [23] at various concentrations of glucose-1-phosphate and soluble starch. According to Weibull and Tiselius [56], in the case of potato phosphorylase prepared by fractionation with ammonium sulphate, the Michaelis constant for glucose-1-phosphate is $2{\cdot}6 \times 10^{-3}$ M and for inorganic phosphate $6{\cdot}2 \times 10^{-3}$ M. The K_m values (Table V/6) were also determined

Table V/6

K_m-values Obtained with Various Polysaccharides [56]

Concentration of glucose-1-phosphate (mM/ml)	Type of polysaccharide	K_m (mg/ml)
0·0285	Zullkowski starch	0·99
0·0285	Hanes starch	0·98
0·00285	Zullkowski starch	0·93
0·00143	Maltohexaose	0·65

by these authors at various concentrations of polysaccharide and glucose-1-phosphate. The experiments were conducted at 38°C and at pH 6·0.

From the experimental data the authors concluded that the K_m values referring to glucose-1-phosphate and polysaccharide are independent of their relative concentration, and that these substrates are not linked to the same sites in the enzyme molecule.

It is impossible to evaluate the results of these experiments in an unequivocal way, with the exception of maltohexaose, because the non-reducing terminal groups in soluble starch are only poorly defined, and because the employed enzyme preparation was impure.

Detailed and well-defined experimental results were reported by Holló *et al.* [24], on the basis of a series of experiments carried out at 30°C, pH 6·0, four different concentrations of glucose-1-phosphate (16·5,8·27, 4·14 and 1·65 mM and four different concentrations of amylopectin (0·95, 0·23, 0·095 and 0·07 mM non-reducing terminal groups). The final volumes were 4 ml, with a citrate concentration of 0·1 M and with a content of 30 enzyme units. The experimental results were plotted according to Lineweaver and Burk. The effect of the concentration of glucose-1-phosphate on reaction rates can be studied in Fig. V/5, and that of the 'primer' in Fig. V/6. It appears from these Figures that in the synthesis of the α-1,4-bonds

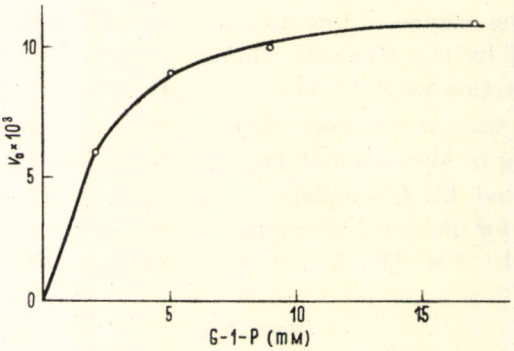


Fig. V/5. Effect of the concentration of glucose-1-phosphate on reaction rates [24]

both substrates (glucose-1-phosphate and amylopectin as 'primer') behave as normal substrates in that they are combined to the enzyme molecule. Under the applied experimental conditions, the apparent Michaelis constants were for glucose-1-phosphate: $4{\cdot}6 \times 10^{-3}$ M and for the 'primer': $6{\cdot}6 \times 10^{-5}$ M.

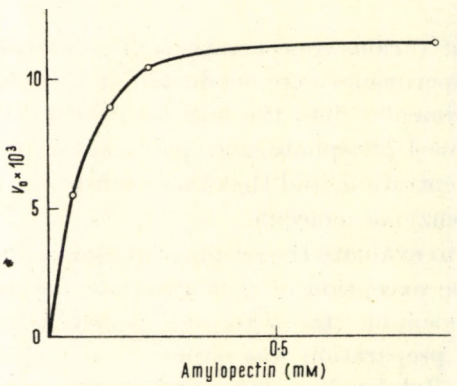


Fig. V/6. Effect of amylopectin concentration on reaction rates [24]

(vi) Effect of Substrate Structure on the Formation of α-1,4-bonds

As shown earlier, α-1,4-bonds are synthesized from glucose-1-phosphate. However, for this synthesis the presence of an initial substance, a 'primer' or 'starter' is indispensable. The smallest maltooligosaccharide possessing a 'starter' power of this type contains three units of D-glucose bound by α-1,4-

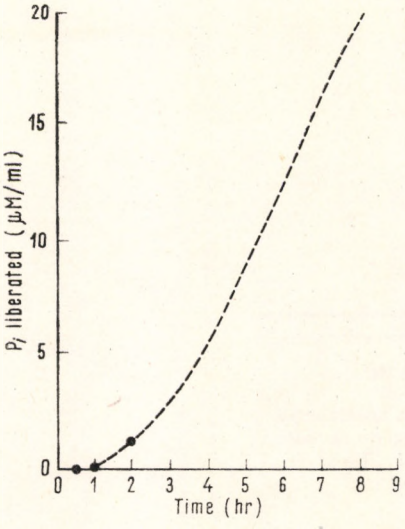


Fig. V/7. Effect of potato phosphorylase on non-purified glucose-1-phosphate [38]

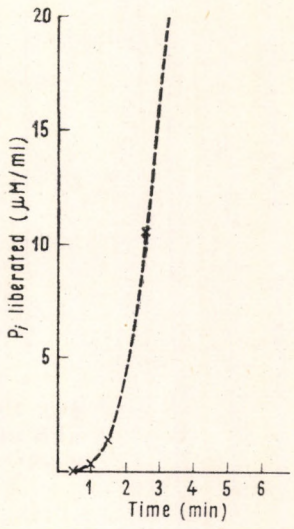


Fig. V/8. Effect of muscle phosphorylase on non-purified glucose-1-phosphate [38]

-bonds [3, 17, 57, 59]. In contrast, Nakamura [47] reported that no 'starter' is required for the activity of lima beans phosphorylase and batata phosphorylase, and Kurasawa *et al.* [40] gave the same report on the activity of rice phosphorylase. These erroneous observations are due to carbohydrate contaminants in the enzyme preparations [18, 33, 38] and glucose-1-phosphate [1, 38] used. The glucose-1-phosphate was purified by various methods (adsorption, ion exchange, enzymatic treatment) [38]. Synthesis with potato phosphorylase (Fig. V/7) or with muscle phosphorylase (Fig. V/8) was investigated in the presence of glucose-1-phosphate purified in the mentioned way, without employing any 'starter' substance. It can be seen that with potato phosphorylase, reaction rates referred to the control series were not decreased by any of the applied treatments while in the case of muscle phosphorylase, though the lag period became considerably longer, the enzymatic reaction could not be completely stopped. The treatment of potato phosphorylase with amyloglucosidase resulted in a complete liberation from the 'starter' (Fig. V/9), proving that there is no 'de novo' amylose from synthesis pure glucose-1-phosphate [38].

Whereas maltotriose is a weak starter, maltotetraose, -pentaose, -hexaose, amylose and amylopectin show starter effects of increasing strength [59]. The linear amylodextrins produce their maximum 'starter' effect at $DP = 20$ [5].

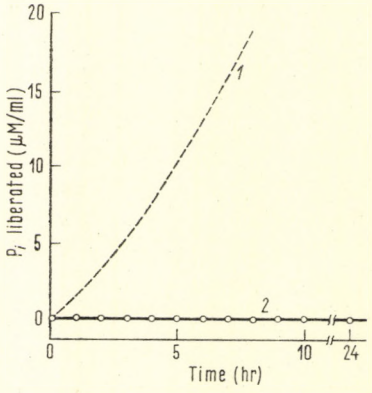


Fig. V/9. Effect of a treatment with amyloglucosidase on potato phosphorylase [38]. *1:* Treated; *2:* untreated

Amylopectin has stronger effects as a 'starter' than amylose, due to its higher ratio of non-reducing terminal groups [5, 53]. The effect of various 'starter' activators in the synthesis of amylose with potato phosphorylase (Table V/7) has been investigated by Nakamura [46].

For a complete 'starter' activity, the acceptor molecule must contain at least three such glucose units linked by α-1,4-bonds, with a non-substituted C_4 atom in the non-reducing glucose terminal unit [53]. The β-limit dextrin

Table V/7

Effect of Various Carbohydrate Starters on Amylose Synthesis (Referred to Effect of Soluble Potato Starch Starters = 100%) [46]

Starter	% of activation
Soluble potato starch	100
Potato starch	65
Potato amylose	45
Potato amylopectin	100
Rice starch	65
Rice amylose treated with isoamylase	80
Glycogen	30
Dextrin	85
Dextran	0
Dextran hydrolyzed by acid	0
Dextran decomposed by β-amylase	0
Maltose	0

of amylopectin which carries on its external chain for only 2–3 D-glucose units did not show any acceptor substrate properties [53].

On substituting a phosphate group onto the C_6 atom of the non-terminal glucose unit of maltotetraose, Posternak found [51] that the product possessed only an extremely weak 'starter' power in respect to that of unsubstituted maltotetraose. However, no further decrease in activity was experienced when the reducing terminal group of phosphotetraose was oxidized by hypoiodite. Similar results were obtained on substituting maltohexaose. According to French [17], the phloridzin glycosides of maltodextrin may also act as 'starters'.

All the authors agree that it is impossible to replace glucose-1-phosphate as donor substrate by other sugar phosphates such as β-D-glucose-1-phosphate, α-L-glucose-1-phosphate, D-glucose-6-phosphate, D-fructose-1-phosphate, D-fructose-1,6-diphosphate, maltose-1-phosphate and D-xylose-1--phosphate [11, 44, 52, 58, 61], or by glycuronic-1-phosphate [4], fructose-6-phosphate and ribose-5-phosphate [38].

Despite the great number of above-listed data, no uniform picture exists regarding the available substrates. In modern enzyme research it is a fundamental prerequisite to define exactly the structural and other characteristics of substrates which enable them to participate in the given reaction. Data for this purpose are furnished by Holló *et al.* who have attempted to establish the required construction of substrates and the functional groups of substrates [29]; they have prepared special glucose-1-phosphate derivatives [41] and investigated their role, and have examined the inhibiting effect of special glucose derivatives.

Prior to investigating the role of substituted derivatives of glucose-1--phosphate, the problem must be solved as to what extent the stability of the phosphate-ester bond is altered by substitution. Therefore, the rate

Table V/8

Rate Constants of the Hydrolysis of Glucose-1-phosphate Derivatives in 0·25 N HCl (min^{-1})

Temperature (°C)	Glucose-1-phosphate	6-Deoxy-glucose--1-phosphate	3-O-Methyl-glucose--1-phosphate
40	$4{\cdot}07\times10^{-3}$	$1{\cdot}49\times10^{-2}$	—
45	$7{\cdot}67\times10^{-3}$	$4{\cdot}26\times10^{-2}$	$6{\cdot}90\times10^{-3}$
50	$1{\cdot}46\times10^{-2}$	$5{\cdot}06\times10^{-2}$	$1{\cdot}12\times10^{-2}$
55	$2{\cdot}72\times10^{-2}$	$8{\cdot}28\times10^{-2}$	$2{\cdot}53\times10^{-2}$
60	$4{\cdot}15\times10^{-2}$	$1{\cdot}95\times10^{-1}$	$4{\cdot}25\times10^{-2}$

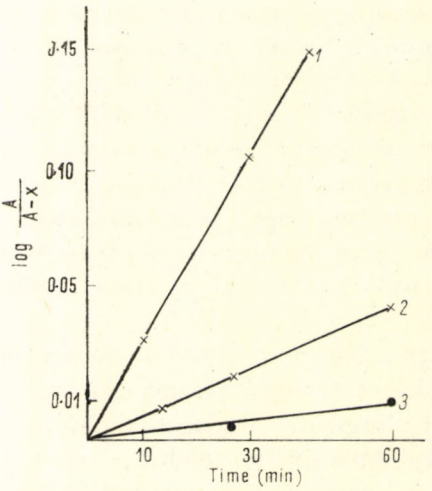


Fig. V/10. Enzyme reaction of glucose-1-phosphate derivatives [29]. *1:* Glucose-1-phosphate; *2:* 6-deoxy--glucose-1-phosphate; *3:* 3-O-methyl-glucose-1-phosphate

constants of the hydrolysis of glucose-1-phosphate, 3-O-methyl-glucose-1--phosphate and 6-deoxy-glucose-1-phosphate were compared in 0·25 N hydrochloric acid at various temperatures (the hydrolysis takes place by a first order process.) The measured data are listed in Table V/8 [30]. It can be seen that the rates of hydrolysis for glucose-1-phosphate and for 3-O-methyl-glucose-1-phosphate are nearly the same while the 6-deoxy derivative undergoes hydrolysis 3–4 times more quickly.

The calculated activation energies of the hydrolysis for the three mentioned compounds are:

Glucose-1-phosphate	24 600 cal/mole
6-deoxy-glucose-1-phosphate	25 500 cal/mole
3-O-methyl-glucose-1-phosphate	25 600 cal/mole

i.e. they are practically identical.

Enzymatic Reactions of Glucose-1-phosphate Derivatives. Samples of the three compounds (glucose-1-phosphate, 6-deoxy-glucose-1-phosphate and 3-O-methyl-glucose-1-phosphate) were compared under the conditions of activity measurements (cf. p. 157). Figure V/10 was plotted from the measured inorganic phosphate, and hence the following rate constants were calculated [29]:

Glucose-1-phosphate	$5{\cdot}50 \times 10^{-3}$ min^{-1}
6-deoxy-glucose-1-phosphate	$1{\cdot}49 \times 10^{-3}$ min^{-1}
3-O-methyl-glucose-1-phosphate	$5{\cdot}36 \times 10^{-4}$ min^{-1}

It appears from these values that the absence of the 6-hydroxy group causes a 3·7-fold decrease and the blocking of the 3-hydroxy group a 10-fold rate decrease.

It follows from the discussions in Section IV. 6 *(ii)* that quite contradictory data have been published on the inhibiting effect of glucose. The inhibiting effect of glucose was unequivocally proved by Holló *et al.* [28] though the inhibition constants obtained were markedly above the usual values. The inhibiting effect is unequivocal since the rate of the enzymatic reaction is not affected by similar concentrations of fructose, mannose and maltose. Figures V/11 and V/12 indicate that the inhibition is also competitive with respect to the donor and acceptor substrates.

Similarly, competitive inhibition was also observed in the cases when one of the hydroxy groups of glucose was absent or was substituted. The Lineweaver–Burk diagrams obtained for 2-deoxy-glucose are shown in Figs V/13 and V/14, those for 3-O-methyl-glucose in Figs V/15 and V/16 and those referring to 6-O-methyl-glucose in Figs V/17 and V/18.

The kinetic constants of the inhibition experiment carried out with glucose analogues are summarized in Table V/9 [28].

The competitive nature of the inhibition effects has been proved already by the data given in Table IV/10 (p. 71). The values in Table V/9 indicate,

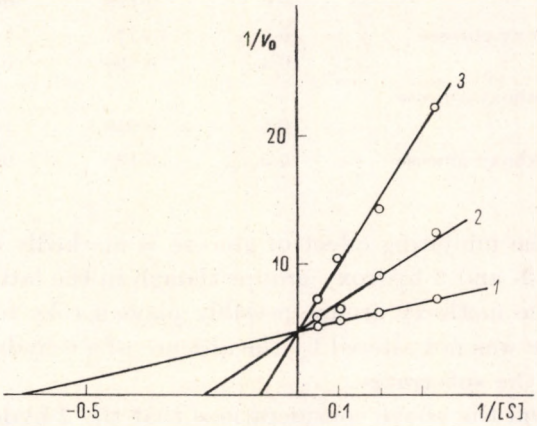


Fig. V/11. Inhibitor effect of glucose plotted against donor concentrations [28]. *1:* In absence of inhibitor; *2:* 0·3 M glucose concentration; *3:* 0·6 M glucose concentration

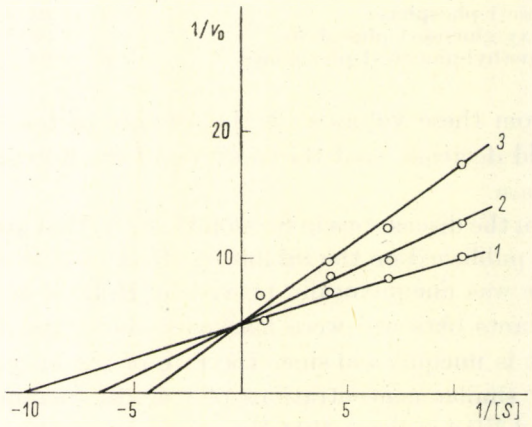


Fig. V/12. Inhibitor effect of glucose plotted against acceptor concentrations [28]. *1:* In absence of inhibitor; *2:* 0·3 M glucose concentration *3:* 0·6 M glucose concentration

Table V/9

K_i-values Obtained by Graphical Method with Various Substrate Analogues [28]

Inhibitor	Inhibitor concentration (M)	K_i (for Glucose-1-phosphate)	K_i (for amylopectin)
Glucose	0·3	0·170	0·540
	0·6	0·115	0·405
2-Deoxy-glucose	0·3	0·77	1·30
	0·6	0·520	0·79
3-Methoxy-glucose	0·3	—	—
	0·6	0·650	1·57
6-Methoxy-glucose	0·3	0·130	0·52

in turn, that the inhibiting effect of glucose is markedly decreased by the absence of the 2- and 3-hydroxy groups though in the latter case the steric hindrance of the methoxy groups possibly plays a role, too. The inhibiting effect of glucose was not altered by the absence of a 6-hydroxy group in the case of any of the substrates.

It follows from the above considerations that the 2-hydroxy group plays an essential role in the enzymatic reaction in the case of both substrates studied, while the role of the 3- and 6-hydroxy groups cannot be unequivocally proved by the literature data reported so far.

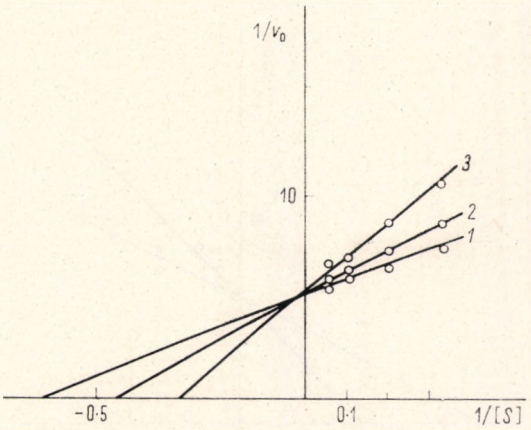


Fig. V/13. Inhibitor effect of 2-deoxy-glucose plotted against glucose-1-phosphate concentrations [28]. *1:* In absence of inhibitor; *2:* 0·3 M 2-deoxy-glucose (2-deoxy-D-arabino-hexose); *3:* 0·6 M 2-deoxy-glucose

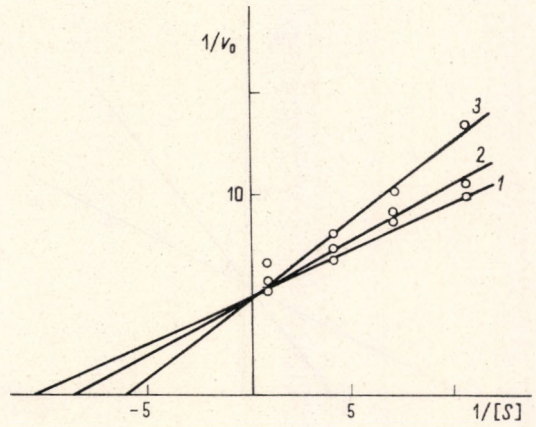


Fig. V/14. Inhibitor effect of 2-deoxy-glucose plotted against amylopectin concentrations [28]. *1:* In absence of inhibitor; *2:* 0·3 M 2-deoxy-glucose (2-deoxy-D-arabino-hexose); *3:* 0·6 M 2-deoxy-glucose

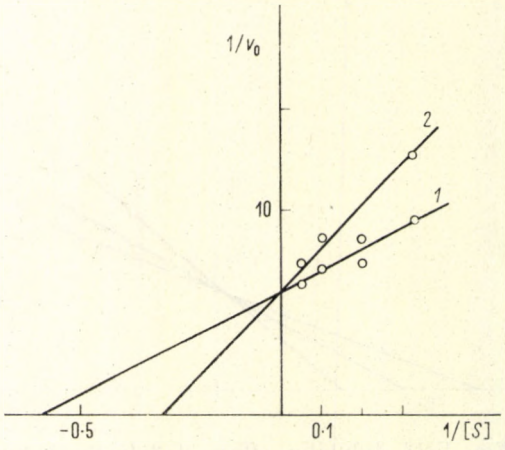


Fig. V/15. Inhibitor effect of 3-O-methyl-D--glucose plotted against glucose-1-phosphate concentrations. [28]. *1:* In absence of inhibitor; *2:* with 0·6 M 3-O-methyl-D-glucose

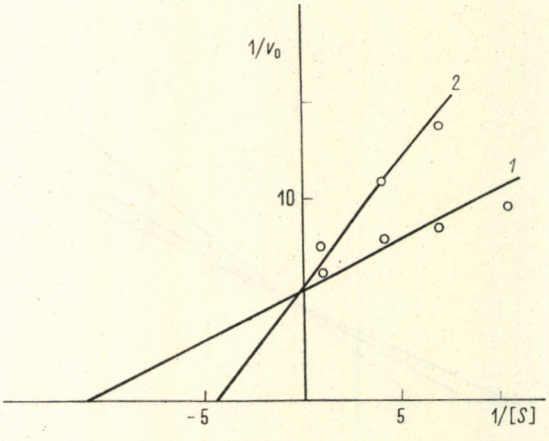


Fig. V/16. Inhibitor effect of 3-O-methyl-D-glucose plotted against amylopectin concentrations [28]. *1:* In absence of inhibitor; *2:* with 0·6 M 3-O--methyl-D-glucose

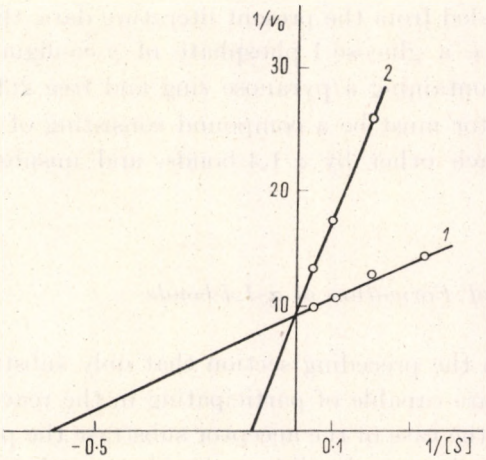


Fig. V/17. Inhibitor effect of 6-O-methyl-D-glucose plotted against glucose-1-phosphate concentrations [28]. *1:* In absence of inhibitor; *2:* with 0·3 M 6-O-methyl-D-glucose

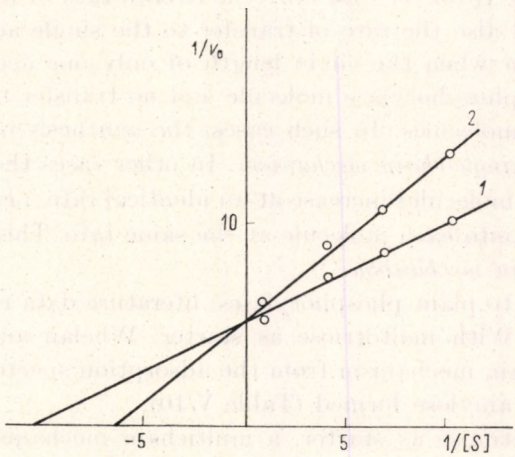


Fig. V/18. Inhibitor effect of 6-O-methyl-D-glucose plotted against amylopectin concentrations [28]. *1:* In absence of inhibitor; *2:* with 0·3 M 6-O-methyl-D-glucose

It can be concluded from the present literature data that the donor substrate can only be a glucose-1-phosphate of β-configuration, of dextrorotatory nature, containing a pyranose ring and free 2(3)- and 6-hydroxy groups. The acceptor must be a compound consisting of at least 3 glucose units linked to each other by α-1,4-bonds, and unsubstituted on its 2-hydroxy group.

(vii) Mechanism of Formation of α-1,4-bonds

It was shown in the preceding section that only substrates of a strictly defined structure are capable of participating in the reaction catalyzed by phosphorylase. In the case of the acceptor substrate the prerequisite is that this substrate must be a maltooligosaccharide constructed by α-1,4-bonds from at least 3 glucose units or an oligo- or polysaccharide which carries the 3 glucose units at the end of its non-reducing chain.

It was also proved that with acceptors of different structure and molecular weight, the introduction of new glucose units into the chain takes place at various rates. However, not only the overall rate of insertion of glucose units varies but also the rate of transfer to the single acceptor molecules. Cases may occur when the chain length of only one acceptor molecule is increased by a phosphorylase molecule and no transfer takes place on the other acceptor molecules. In such cases, the synthesis reaction occurs according to the *single-chain mechanism*. In other cases the chain lengths of all the acceptor molecules increase at an identical rate, i.e. the glucose units are transferred onto each molecule at the same rate. This latter is denoted as the *multichain mechanism*.

With respect to plant phosphorylases, literature data refer to both types of mechanism. With maltotriose as starter, Whelan and Bailey [58] deduced single-chain mechanism from the adsorption spectrum of the iodine complex of the amylose formed (Table V/10).

With maltotetraose as starter, a multichain mechanism was unambiguously observed by the same authors. Three experimental data serve as evidence [58].

(a) The synthesis reaction is bimolecular with respect to the enzyme and to maltotetraose, as proved by the fact that a linear correlation exists between the reciprocal value of the reaction rate and the square of the dilution rate of the enzyme and of maltotetraose (Fig. V/19).

(b) During synthesis, the maximum adsorption of the iodine-complex of the amylose formed increases to a great extent in the course of the reaction

Table V/10

Changes in the Maximum Adsorption of the Iodine-complex on Employing Maltotriose as Starter

Chain length	λ_{max} ($m\mu$)
8·3	620
12	620
19	620
25	—
97	635
195	640

(Fig. V/20), i.e. the chain length of the product shows a monotonous increase.

(c) Similar conclusions can be drawn by investigating the spectra of identical amounts of amyloses obtained by applying various amounts of 'starters' (Fig. V/21). At a high 'starter' concentration a lower mean chain length is attained; this is reflected in the shift of the adsorption spectra to shorter wavelengths. The shape of the curves indicates a homogeneous distribution of the molecular weight of the amylose formed pointing to a multichain mechanism.

An extremely homogeneous product distribution was also observed by Husemann *et al.* [36] in the case of amyloses synthesized with phosphorylase. Homogeneity was determined by fractionation methods and by the quotient of the weight and the number average molecular

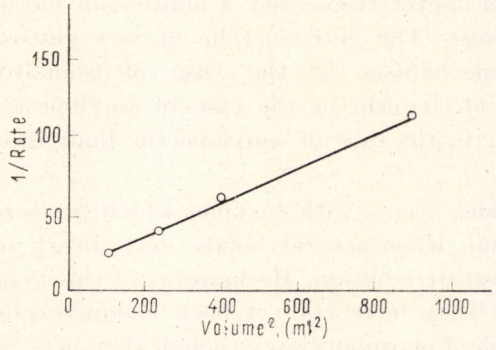


Fig. V/19. Reaction rates in presence of maltotetraose [58]

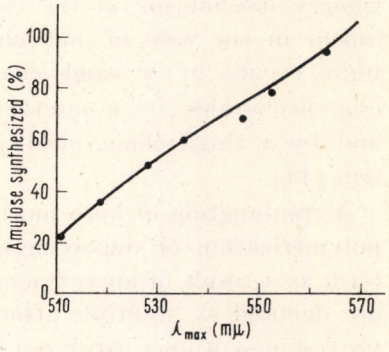


Fig. V/20. Changes in the adsorption of the amylose–iodine complex during synthesis [58]

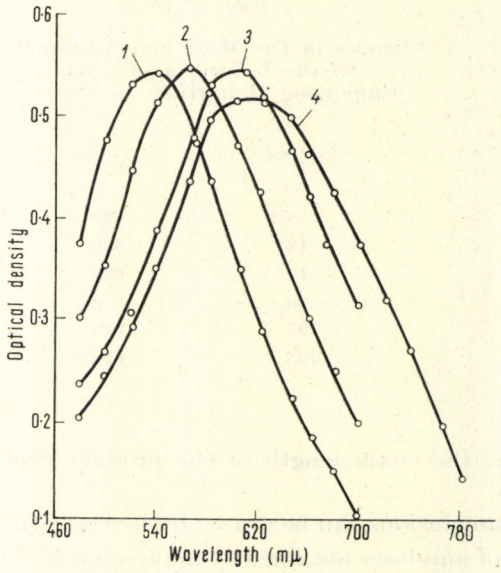


Fig. V/21. Adsorption curves of the amylose-iodine complex in syntheses carried out with various 'primers' [58]. *1:* 2 mg 'primer'; *2:* 1 mg 'primer'; *3:* 0·5 mg 'primer'; *4:* 0·2 mg 'primer'

weights. This value may be 1·05, indicating an extraordinary homogeneous nature.

On the basis of the measurement of the iodine spectrum, De Souza and Cardini drew the conclusion that the chain lenghtening takes place by a single-chain mechanism in the case of maltotriose, a transitionary mechanism in the case of maltotetraose and a multichain mechanism in the case of maltoheptaose. The introduction of new glucose units occurs by a single-chain mechanism in the case of isomaltooligosaccharides, by a multichain mechanism in the case of amylopectin and by a single-chain mechanism in the case of amylopectin limit dextrin [14].

A combination of both mechanisms occurs with enzymes which catalyze polymerization or depolymerization, when several bonds may break or form as a result of an enzyme-substrate collision. Mechanisms of this type are denoted as 'multiple attacks'. They have not yet been unequivocally proved in syntheses catalyzed by plant phosphorylases, though they may be inferred from the experimental results of De Souza and Cardini [14]. Different overall depolymerization reaction rates can be observed on applying

identical molar concentrations of acceptor structures (amylose molecules containing exclusively α-1,4-bonds). A recent communication by Pfannemüller [50] reports alterations in the various kinetic constants over quite a wide range of molecular weights. As appears from Table V/11, in the low

Table V/11

Synthesis of Amylose by Means of Amylose- and Maltodextrin Starters of Low Molecular Weight [50]

Molecular weight (DP_n)	$V'_{max} \times 10^{-2}$ (M/min)	$K'_m \times 10^{-5}$ (M)
4	8·10	3·49
6	9·22	3·24
8	8·82	3·55
25·5	6·24	1·25
43·5	7·89	2·61
97·0	6·12	1·81
139·0	6·66	1·88
260·0	5·40	1·80
357·0	3·84	1·07
480·0	3·63	1·09
970·0	3·33	1·00

molecular weight range, the maximum rates and apparent Michaelis constants depend to only a small extent on the chain length of amylose. However, investigations at higher molecular weight show a monotonous decrease in the values of the constants, with the exception of a few data. In the opinion of the author, the inactive binding between the internal glucose units of amylose molecules and enzyme molecules is responsible for this phenomenon which will be discussed in detail in connection with phosphorolysis (cf. p. 112).

It is of interest that the maximum rates and apparent Michaelis constants depend not only on the molecular weight but also on the concentration of the 'starter'. This effect is particularly noticeable in the case of high molecular weights [50].

2. *Phosphorolytic Splitting of α-1,4-bonds*

Phosphorolytic splitting catalyzed by α-1,4-glucan-phosphorylase is very widespread in living organisms as an extremely important phase of the metabolism of starch and glycogen. From this it follows that the investigation of the effect of various reaction conditions and of the mechanism of splitting by phosphorolysis is of great importance from both theoretical and practical aspects.

(i) Order of Reactions in the Decomposition by Phosphorolysis

Without any experimental data, Arreguin *et al.* claim that phosphorolytic decompositions are first order reactions [2]. Experimental evidence [26], however, points to the conclusion that it is impossible to describe the entire decomposition process on the basis of a common order of reaction (Table V/12). The phenomenon can presumably be attributed to product inhibition,

Table V/12

Degrees of Conversion and Rate Constants in Phosphorolysis [26]

Degrees of conversion (%)	$k \times 10^{-2}$ (min^{-1})
4·3	8·35
15·3	8·39
20·8	8·29
33·6	8·23
50·5	8·15
62·0	6·31

in that the rate of decomposition is decreased by the glucose-1-phosphate formed [37].

(ii) Effect of Hydrogen Ion Concentration on the Rate of Phosphorolysis

It has been shown earlier that the dissociation state of the functional groups of the enzyme, the structure of the enzyme molecule and the degree of dissociation of the ionic substrates are affected by the hydrogen ion concentration. This is also the case with phosphorolysis, and even the bind-

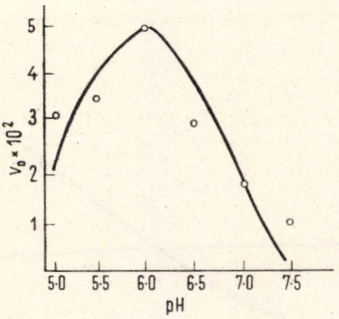


Fig. V/22. Effect of pH on the initial rates of phosphorolysis [26]

ing of ionic groups may occur since dibasic glucose-1-phosphate is formed from the tribasic phosphoric acid.

Consequently, the investigation of the dependence on hydrogen ion concentration is of primary importance. Values measured at 35°C are shown in Fig. V/22 [26], indicating that the optimum pH range is 5·8–6·2, with pH_{50} 5·1–6·8, an interval of 1·7 pH units, even smaller than the data obtained in synthesis.

(iii) Effect of Temperature on the Rate of Phosphorolysis

The effect of temperature has already been discussed in connection with synthesis where it was emphasized that the measured values hold only under the actual conditions of measurement. The effect of temperature was investigated by Holló *et al.* under the following experimental conditions: 0·2 ml of 0·4 M phosphate buffer of pH 6·5, 0·5 ml of amylopectin solution of a concentration of 4 μmole/ml, and 30 units of enzyme. The reaction mixtures were incubated for 30 minutes in a temperature range of 15 to 60°C, then the enzyme activity was stopped by adding 0·4 ml 6 N sulphuric acid, and the phosphorus content of the glucose-1-phosphate formed was measured [26].

Parallel to the above series of experiments, reaction mixtures containing no phosphate buffer were pre-incubated for 30 minutes at the given temperatures. After the reaction periods, the reaction mixtures were quickly cooled, the prescribed amounts of phosphate buffer added, and the mixtures again preincubated for 30 minutes at 35°C. Enzyme activity was stopped similarly with 6 N sulphuric acid, and the contents of ester-phosphorus established [26].

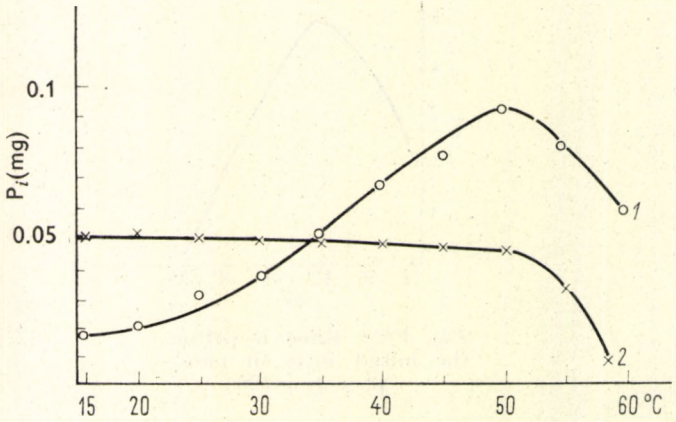


Fig. V/23. Effect of temperature on the initial rates of phosphorolysis [26]. *1:* Without incubation; *2:* with incubation

The data shown in Fig. V/23 indicate that under the given conditions, the optimum temperature of the reaction is about 50°C. Above 50°C, the reaction rate decreases, and the enzyme protein begins to deteriorate. This is disclosed by the rapid decrease of the curve plotted with the pre-incubated enzyme samples. In the temperature range 30–45°C, the value of Q_{10} equals 1·46.

(iv) Effect of Substrate Concentration on the Reaction Rate

According to the Nakamura theory (to be discussed in Chapter VI.5) phosphorolysis takes place by a molecular mechanism resembling that of amylolysis. However, in the case of phosphorolysis, the hydroxy group is

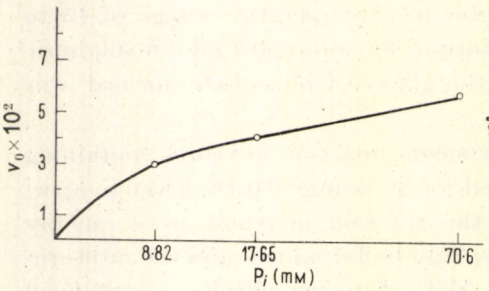


Fig. V/24. Effect of the concentration of inorganic phosphate on the initial rates of phosphorolysis [26]

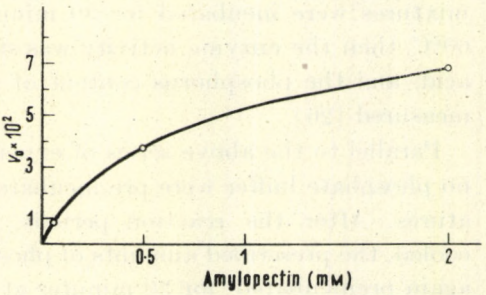


Fig. V/25 Effect of amylopectin concentration on the initial rates of phosphorolysis [26]

replaced by a monovalent phosphate ion which is linked to the activated substrate molecule in a non-enzymatic process. Thus, the rate-increasing effect of the phosphate cannot be described by the Michaelis–Menten theory.

The data measured by Holló *et al.* [26] and presented in Figs V/24 and V/25 indicate that in both cases Michaelis–Menten kinetics are valid, and thus that point of the Nakamura hypothesis cannot be considered as proved.

(v) Effect of Substrate Structure on Phosphorolysis

It has been shown earlier that the phosphate group can be replaced by arsenate which is of a similar structure. However, a marked rate decrease takes place [39, 45]. The possibility of decomposition by phosphorolysis was investigated in the case of native and synthetic amylose, amylopectin and glycogen.

When natural amylose is decomposed by phosphorylase, the conversion into glucose-1-phosphate is about 70% in the presence of inorganic phosphate [49]. Similarly to β-amylolysis, the enzymatic attack of a molecule begins on the non-reducing terminal group (single-chain mechanism), and the enzymatic reaction is stopped by the anomalous bonds present in the substrate [16].

Amylose synthesized with phosphorylase [48] can be completely decomposed with potato phosphorylase and β-amylase. According to Gilbert [18], amylose prepared under anaerobic conditions and treated with oxygen shows an extremely low phosphorolytic limit.

The external chains of amylopectin are relatively easily decomposed by phosphorylase with the formation of about 30–60% D-glucose-1-phosphate; the phosphorylase-limit dextrin carries after the branchings still 4 or 5 units of D-glucose [42].

Glycogen is hardly attacked by plant phosphorylase. This is a marked difference between plant and animal phosphorylases.

The sulphuric acid esters of starch (degree of substitution = 1) and the carboxymethyl starch (degree of substitution = 0·9) cannot be decomposed by phosphorylase [33]. Methyl amylose of a degree of substitution = 0·2 is decomposed at a reduced rate. When the substrate is substituted by a polymer of different steric structure (e.g. by mannane), the product is fully resistant to the enzyme, while the alteration of the shape of the molecule (in the case of expanded or contracted amylose) does not affect the rate of enzymatic decomposition [32]. The 6-hydroxy group plays a significant role

in the enzymatic reaction since in the presence of inorganic phosphate 6-deoxy-amylose cannot be decomposed to the corresponding glucose-1-phosphate [60].

(vi) Mechanism of the Phosphorolysis of α-1,4-bonds

Like in the case of synthesis various data have been reported on the single-chain and multichain mechanisms describing the depolymerization rate of the different molecules.

According to earlier literature data [34], the mechanism of decomposition of synthetic amylose differs from that of natural amylose as proved by the investigation of the degree of polymerization of the residual amylose prepared during the decomposition procedure.

The course of decomposition of synthetic amylose of a mean polymerization degree of 3000 by weight can be examined by means of Table V/13, the data of which point to a monotonous decrease of molecular weight during the decomposition process.

Table V/13

Decomposition of Synthetic Amylose by Potato Phosphorylase at 37°C in a 0·8% Solution [34]

Sample number	Decomposition (%)	Polymerization degree by weight	
		measured[1]	calculated[2]
I	0	3000	—
II	24·5	2500	2260
III	41·0	1900	1770
IV	67·5	1300	975

[1] By viscosity measurement of the tricarbanilate derivative [6].
[2] Calculated from the degree of decomposition on presuming a multichain mechanism.

In the course of the phosphorolytic decomposition of natural amylose (amylose extracted at 65–68°C from potato starch according to Meyer), the degree of polymerization is altered as shown by Table V/14. It can be seen that no changes occur in the polymerization degree up to a decomposition of 75%. Consequently, the phosphorolysis of natural amylose takes place according to a single-chain mechanism, while that of synthetic amylose follows the multichain mechanism (Fig. V/26).

It has been proved by later investigations that this difference is only an apparent one. Husemann *et al.* [35] confirmed that the two types of amy-

Table V/14

Decomposition of Native Amylose by Potato Phosphorylase at 37°C in a 0·8% Solution, at pH 6·6 [34]

Sample number	Decomposition (%)	Polymerization degree by weight	
		measured[1]	calculated[2]
I	0	2150	—
II	32·5	2050	1450
III	55·0	2220	970
IV	74·5	2080	550

[1] By viscosity measurement of the tricarbanilate derivative [6].
[2] Calculated from the degree of decomposition presuming a multichain mechanism.

lose differ from each other, not in structural or conformation properties, but rather in their association and solubility properties. Marked differences exist in the heterodispersity and distribution of molecular weight of the two types. The tricarbanilates of the two amylose types were fractionated by these authors from dimethyl formamide with methanol containing calcium chloride [13]; the amounts of the various fractions were determined by gravimetry while the molecular weights were established by viscosimetry in dioxan.

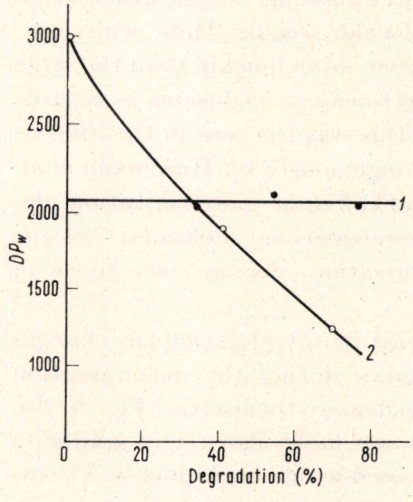


Fig. V/26. Changes in the degree of polymerization in the phosphorolysis of natural (*1*) and synthetic amylose (*2*) [34]

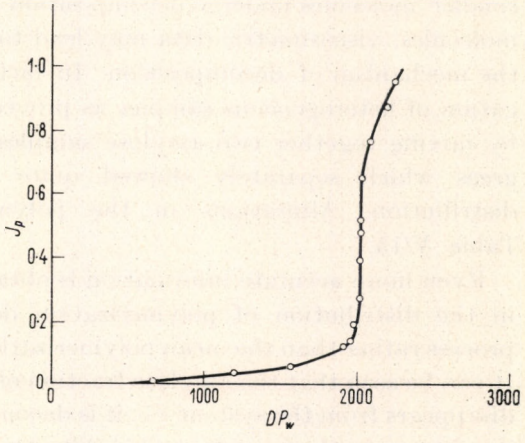


Fig. V/27. Integral molecular weight distribution of synthetic amylose [35]

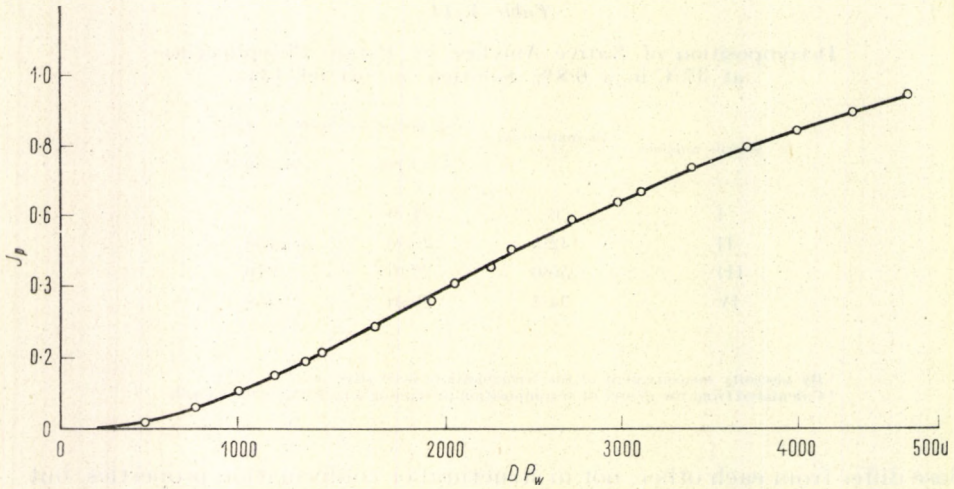


Fig. V/28. Integral molecular weight distribution of natural amylose [35]

The distribution curve of the integral polymerization degree of a synthetic amylose sample is presented in Fig. V/27 and that of a natural amylose sample in Fig. V/28, indicating that synthetic amylose consists of extremely homogeneous molecules quite in contrast to the extreme heterogeneity of natural amylose [35].

The viscosity of a polymer of heterogeneous molecular weight distribution is determined by the fractions of high molecular weight. Thus, when the smaller molecules undergo decomposition much more quickly than the large molecules, viscosimetric data may lead to erroneous conclusions as regards the mechanism of decomposition. In fact, this was the case in the investigation of heterogeneous samples as proved ingeniously by Husemann *et al.* by mixing together two amylose samples of different polymerization degrees which separately showed quite homogeneous molecular weight distribution. Alterations in the polymerization degree are given in Table V/15.

Even more accurate information is obtained by investigating the changes in the distribution of polymerization degrees during the decomposition process rather than the mean polymerization degrees themselves (Fig. V/29). It can be seen that the amylose fraction of lower molecular weight gradually disappears from the system, i.e. it is decomposed much more quickly. Viscosity, however, shows only a slow alteration, and thus an apparent single-chain mechanism is experienced. With homogeneous fractions, the phosphoroltyic decomposition of amylose takes place monotonously, always

Table V/15

Decomposition of the Mixture of Amyloses (Polymerization Degrees 2060 and 3700, in a 0·65% Solution) by Potato Phosphorylase [21]

Sample number	Decomposition (%)	Polymerization degree by weight	
		measured[1]	calculated[2]
I	—	2870	—
II	32·0	2470	1950
III	51·0	2170	1400
IV	77·5	2170	650

[1] By viscosity measurement of the tricarbanilate derivative [6].
[2] Calculated from the degree of decomposition presuming a multichain mechanism.

according to the multichain mechanism. The possibility of a 'multiple attack' is not excluded by the experimental data, though no direct evidence is to be found in the literature in this respect.

Interesting conclusions can be drawn from the dependence of overall reaction rates on the degrees of polymerization and on concentration. It was found that amyloses of different polymerization degree but identical molar concentration decompose at different rates [50]. The initial rates characterizing the decomposition of amyloses of polymerization degrees 970,

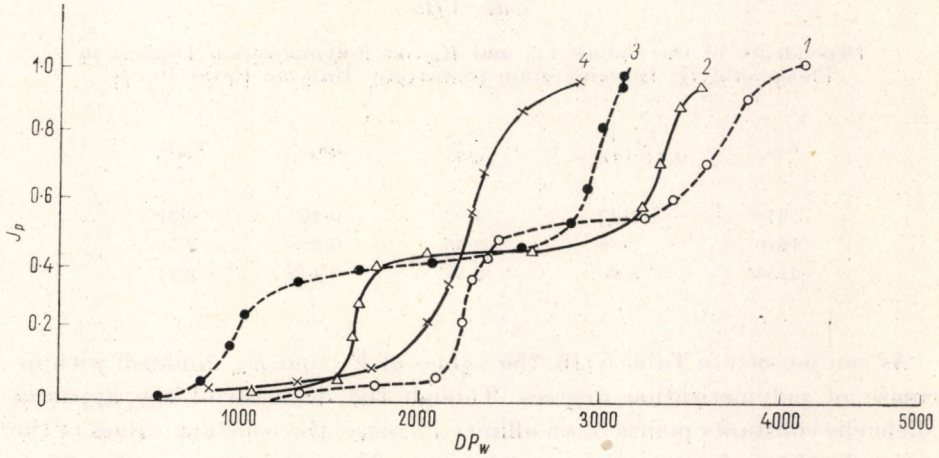


Fig. V/29. Changes in the molecular weight distribution of a mixture of synthetic amyloses (DP_w 2060 and 3700) during phosphorolysis [35].
1: Without degradation; *2:* at 32% degradation; *3:* at 51% degradation; *4:* at 77·5% degradation

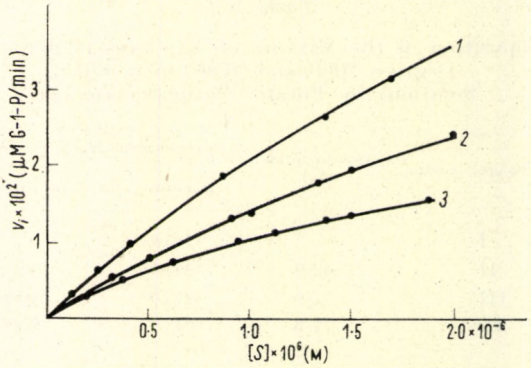


Fig. V/30. Phosphorolysis of synthetic amylose. *1:* DP_n 970; *2:* DP_n 1610; *3:* DP_n 2155 at 25°C and pH 7·0 [50]

1610 and 2155 are presented in Fig. V/30. It can be seen that, similarly to decompositions by β-amylase [54], at an identical molar concentration of terminal groups, the phosphorolytic decomposition is the faster, the shorter the chain length. Related to this, the constants corresponding to the Michaelis kinetics also change. The apparent Michaelis constants and maximum rates obtained by the Lineweaver–Burk graph are listed in Table V/16.

Table V/16

Dependence of the Values V'_m and K'_m on Polymerization Degrees in Phosphorolytic Decomposition (Substrate: Enzyme Ratio 10 : 1)

DP_n	$V'_m \times 10^2$ (μM G-1-P/min)	K'_m (μM)	$1/K'_m$	V'_m/K'_m
970	11·35	4·92	0·203	2·31
1610	7·20	3·35	0·298	2·51
2155	5·26	2·35	0·426	2·24

As can be seen in Table V/16, the values of V'_m and K'_m diminish with increase of polymerization degrees. Though the decrease of the apparent Michaelis constants points to an affinity increase, the constant values of the ratios V'_m/K'_m indicate rather an inhibition. This inhibition is competitive (though this cannot be read directly from the corresponding Figures, since in that case, inhibitor concentration and terminal group concentration can not be separated from each other), and is due to the formation of an inac-

tive enzyme-substrate complex, an inactive bond of the internal glucose units of the polymer chain.

In the decomposition by β-amylase, according to Thoma [54], inactive binding increases with the f(P)-number of the chain members. The values of V'_m and K'_m show a similar change:

$$V'_m = \frac{V_m}{1 + \mathrm{f(P)} \cdot \dfrac{K_m}{K_i}} \tag{1}$$

$$K'_m = \frac{K_m}{1 + \mathrm{f(P)} \cdot \dfrac{K_m}{K_i}} \cdot \tag{2}$$

After rearrangement, (2) becomes

$$\frac{1}{K'_m} = \frac{1}{K_m} + \frac{1}{K_i}\mathrm{f(P)} \,. \tag{3}$$

When the values of $1/K'_m$ are plotted against the polymerization degrees according to Eq.(3), a straight line is obtained (Fig. V/31), with a slope of $1/K_i$ denoting the binding affinity per glucose unit. According to Pfannemüller [50], this value is $5{\cdot}4 \times 10^{-3}$ M in the case of potato phosphorylase, in fair agreement with the value $2{\cdot}35 \times 10^{-3}$ established by Thoma in the case of β-amylase [54].

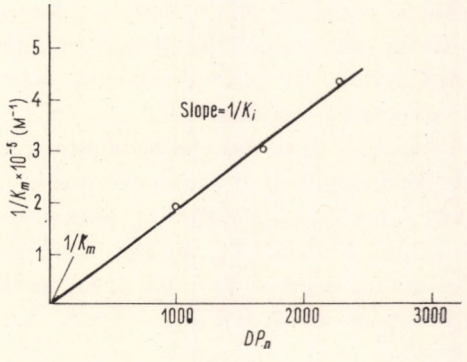


Fig. V/31. Dependence of the apparent Michaelis constant on the degrees of polymerization [50]

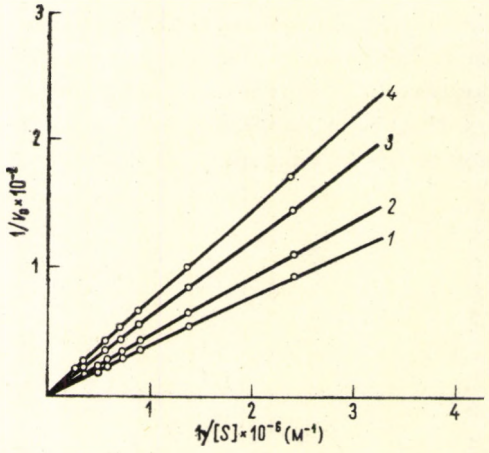


Fig. V/32. Phosphorolysis of amylose at various concentrations of α-cyclodextrin [50]. *1:* In absence of cyclodextrin; *2:* with $0{\cdot}3\times10^{-4}$ M cyclodextrin; *3:* with $0{\cdot}6\times10^{-4}$ M cyclodextrin; *4:* with $1{\cdot}0\times10^{-4}$ M cyclodextrin

According to Eq. (3), the intercept in Fig. V/31 indicates the K_m value of the shortest maltodextrin which is still decomposable by phosphorylase (about 6×10^{-5} M). For the sake of comparison it may be mentioned that in the case of phosphorolytic decomposition, with amylopectin as substrate, Holló *et al.* [27] obtained a value corresponding to $0{\cdot}55\times10^{-3}$ M non-reducing terminal units; in the case of synthesis, with maltohexaose as starter, this value was $0{\cdot}65\times10^{-3}$ [56].

In our opinion, Eq. (3) and the values calculted from it hold only in the case when the Michaelis constant (K_m) does not depend on the degree of polymerization. However, this cannot be proved directly since it is impossible to make any distinctions by kinetic methods between the effects due to inhibition or to structural reasons.

The inhibiting effect of the internal chain members was proved by Pfannemüller [50] by the investigation of the decomposition, in the presence of cyclodextrins, of two amyloses of different polymerization degrees. The dependence of the values V'_m and K'_m on the chain length in the case of amyloses of polymerization degrees 970 and 2155, in the absence of cyclodextrin and in the presence of 0·3, 0·6 and $1{\cdot}0\times10^{-4}$ M α-cyclodextrin, are presented in Fig. V/32.

Figure V/32 shows the Lineweaver–Burk diagram of a competitive inhibition of typical shape. The value V'_{max} is independent of the inhibitor

concentration. It can be calculated from the data that in the phosphorylase, 1·03 molecule of cyclodextrin is bound per active centre. Similarly to β--amylase, potato phosphorylase can also be competetively inhibited by cyclodextrin, differing thus from animal phosphorylases where no inhibition is observable at all. In Table V/17 the kinetic constants obtained at two different polymerization degrees and in different inhibitor concentrations are summarized.

Table V/17

Values of V'_m and K'_m Plotted against Concentration of Cyclodextrin in the Phosphorolytic Decomposition of Amyloses of DP_n 970 and 2155 [50]

Concentration of α-cyclodextrin (M)	Amylose DP_n 970		Amylose DP_n 2155		
	$V'_m \times 10^2$ (μM glucose-1-phosphate/min)	$K'_m \times 10^6$ (M)	$V'_m \times 10^2$ (μM glucose-1-phosphate/min)	$K_m \times 10^6$ (M)	$\frac{K'_m 970}{K'_m 2155}$
—	10·0	3·42	6·9	2·76	1·24
$0{\cdot}3 \times 10^{-4}$	10·0	4·65	6·9	3·85	1·21
$0{\cdot}6 \times 10^{-4}$	10·0	6·30	6·9	5·49	1·15
$1{\cdot}0 \times 10^{-4}$	10·0	8·0	6·9	6·77	1·18

The effect of competitive inhibition is clearly demonstrated by the data in Table V/17. The value of K'_m rises with increasing concentrations of cyclodextrin while it diminishes with the increase of the substrate chain length at an identical inhibitor concentration.

The investigation of phosphorolytic decomposition [50] carried out in the presence of glucose, maltose, maltotriose and maltotetraose confirmed the competitive inhibitor effect of the internal chain members. These oligomers do not undergo spontaneous decomposition. Consequently, a binding affinity of phosphorylase may be deduced from their inhibitor properties.

These oligomers, with the exception of glucose which discloses slight inhibitor effects, show a typical competitive inhibition in the Lineweaver–Burk diagram (just as in Fig. V/32). The K'_m values obtained with and without the inhibitor, and the calculated K_i values referring to the various maltooligosaccharides are presented in Table V/18. For the sake of comparison, the mean inhibition per glucose residue is expressed as the quotient K_i/glucose.

It appears from Table V/18 that the K_i/glucose inhibitor concentration is of the same order of magnitude in the case of β-cyclodextrin and maltotetraose. In contrast, a K_i/glucose value of $3{\cdot}0 \times 10^{-3}$ M was reported by

Table V/18

Inhibitor Constants of α-cyclodextrin and of some Maltooligomers in the Phosphorolytic Decomposition of Amyloses DP_n 970 and 2155 [50]

Inhibitor	Inhibitor concentration (M)	K_i (M)		K_i/glucose (M) DP_n 970
		DP_n 970	DP_n 2155	
a-cyclodextrin	$0{\cdot}6\times10^{-4}$	$0{\cdot}71\times10^{-4}$	$0{\cdot}61\times10^{-4}$	
	$1{\cdot}0\times10^{-4}$	$0{\cdot}75\times10^{-4}$	$0{\cdot}69\times10^{-4}$	$4{\cdot}38\times10^{-4}$
	mean:	$0{\cdot}73\times10^{-4}$	$0{\cdot}65\times10^{-4}$	
Maltotetraose	$0{\cdot}5\times10^{-4}$	$1{\cdot}5\times10^{-4}$	$1{\cdot}41\times10^{-4}$	
	$1{\cdot}0\times10^{-4}$	$1{\cdot}59\times10^{-4}$	$1{\cdot}47\times10^{-4}$	$6{\cdot}18\times10^{-4}$
	mean	$1{\cdot}55\times10^{-4}$	$1{\cdot}44\times10^{-4}$	
Maltotriose	$0{\cdot}5\times10^{-2}$	$0{\cdot}81\times10^{-2}$		
	$1{\cdot}0\times10^{-2}$	$0{\cdot}77\times10^{-2}$		$2{\cdot}37\times10^{-2}$
	mean	$0{\cdot}79\times10^{-2}$		
Maltose	$0{\cdot}25\times10^{-2}$	$0{\cdot}36\times10^{-2}$		
	$0{\cdot}5\times10^{-2}$	$0{\cdot}34\times10^{-2}$		$0{\cdot}7\times10^{-2}$
	mean	$0{\cdot}35\times10^{-2}$		
Glucose	$2{\cdot}0\times10^{-2}$	$8{\cdot}52\times10^{-2}$		$8{\cdot}52\times10^{-2}$

Thoma and Koshland for α-cyclodextrin in the decomposition by β-amylase. The ten-times lower affinity can be ascribed to the fact that in these experiments the substrate was starch in which the number of active terminal groups is significantly higher, with respect to the inhibitor, than in the case of high molecular substrates. This means that in the presence of amylose of high molecular weight, the inhibitor effect becomes more pronounced.

The binding affinity of phosphorylase to maltotriose is lower by about 200 than that to maltotetraose, quite in accordance with the weaker starter-activity of maltotriose [59].

Though maltose can not serve as a substrate of phosphorylase, it may cause a competitive inhibition. The deficient starter affinity of maltose is based on the fact that only some of its enzyme-binding groups are active or that the coordination of glucose-1-phosphate to the binding sites does not take place. Glucose exerts only a weak inhibition which is however non-competitive with respect to the terminal group of the acceptor, and is bound by a quite different mechanism.

The investigation of the dependence of reaction rates on substrate concentration led to rather interesting results. In the case of enzyme reactions, in general, the dependence of substrate concentration is utilized for the

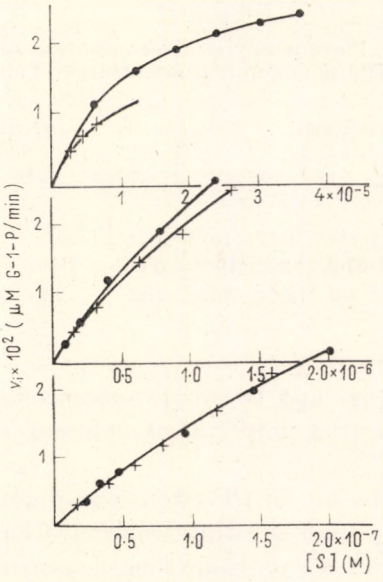


Fig. V/33. Determination of the Michaelis constant in various ranges of substrate concentration [50]. ●—●: DP_n 1020; ×—×: DP_n 2500

determination of the Michaelis constant and of the maximum rates. However, in the case of substrates of long chain molecular weight, due to their complicated structural properties, this correlation can be applied only with caution. As can be seen from Fig. V/33, the changes of reaction rate were established [50] in three different concentration ranges (10^{-5}, 10^{-6} and 10^{-7} M) of two amyloses of different polymerization degrees (1020 and 2500).

In the case of these two amyloses, the differences in reaction rates are the smaller the lower the substrate concentration in the investigated solution. The V'_m and K'_m values corresponding to the various concentration effects, established from the adequate Lineweaver–Burk diagram, are given in Table V/19.

The data in Table V/19 indicate extremely great differences in the mole ratios of the enzyme and the substrate in all the three measurement ranges. For the sake of simplicity, it was presumed that the protein consists exclusively of phosphorylase of a molecular weight of 200 000. Since the enzyme is not crystalline, a high enzyme molarity was obtained. In order to take into account the different phosphorylase concentrations in the

Table V/19

Dependence of the Phosphorolytic Decomposition of Amyloses DP_n 1020 and 2500 at Various Concentration Values [50]

Average substrate conc. (M)		DP_n 1020			DP_n 2500			$\frac{K'_m\ 1020}{K'_m\ 2500}$
		$V'_m \times 10^2$	$V'_{m,\,rel} \times 10^2$	K'_m(M)	$V'_m \times 10^2$	$V'_{m,\,rel} \times 10^2$	K'_m(M)	
10^{-5}	650 : 1	3·08	102·7	$1{\cdot}12 \times 10^{-5}$	1·78	59·5	$0{\cdot}64 \times 10^{-5}$	1·74
10^{-6}	10 : 1	7·70	27·2	$2{\cdot}2 \times 10^{-6}$	5·35	18·9	$1{\cdot}68 \times 10^{-6}$	1·31
10^{-7}	0·25 : 1	5·08	3·5	$2{\cdot}56 \times 10^{-7}$	4·65	3·2	$2{\cdot}20 \times 10^{-7}$	1·16

three series of experiments and to obtain commensurable values of V'_m, the data must be converted into values referred to identical enzyme activity ($V'_{m,\,rel}$).

In Table V/19, the ratios K'_m for the two types of amylose are listed. This ratio decreases markedly from substrate concentrations 10^{-5} M to 10^{-7} M, proving the dependence of K'_m on chain length, because with the decrease of substrate concentration, the inactive binding of the enzyme is gradually more and more suppressed. This can be understood if it is assumed that the affinity of the enzyme for the end of chain is higher than that for the intrinsic units of anhydroglucose. If only few amylose molecules are present (10^{-7} M) in comparison to phosphorylase, the binding of enzyme is preferred by the terminal groups; with increasing excess of substrate inactive binding plays a role to an increasing extent.

It appears from the table as well that the values of V'_m and K'_m for both types of amylose are markedly lower with the decrease of substrate concentrations (from 10^{-5} to 10^{-7} M). This is even more conspicuous in Fig. V/34 where the values $1/K'_m$ obtained by means of $1/V'_m$, and the values K'_m obtained by means of V'_m, are plotted in a doubly logarithmical scale against the reciprocal values of substrate concentration.

In Figure V/34, the shape of the curve for $1/V'_m$ and $1/K'_m$ shows a strong concentration dependence at low and medium substrate concentrations; at higher concentrations the curve has a turning-point. In contrast, the ratio V'_m/K'_m is throughout independent of substrate concentration, and is given by a straight line with a slight tendency to decrease (Fig. V/34). The dependence of the kinetic data on concentration shows a striking similarity to competitive inhibition. In solutions of higher concentration, owing to the formation of a gradually stronger coil, relatively fewer glucose residues of the high molecular amylose molecule remain available for the inactive

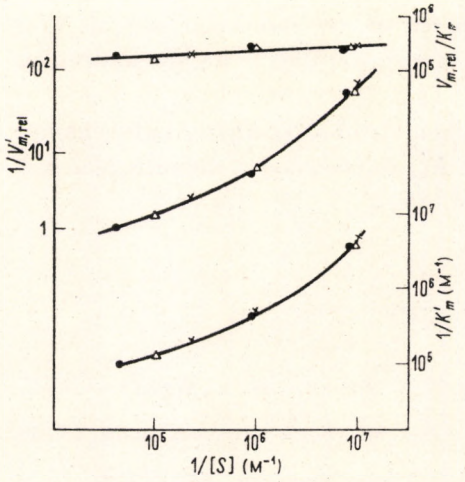


Fig. V/34. Dependence of kinetic constants on amylose concentration [50]. ●—●: DP_n 1020; ×—×: DP_n 1680; △—△: DP_n 2500

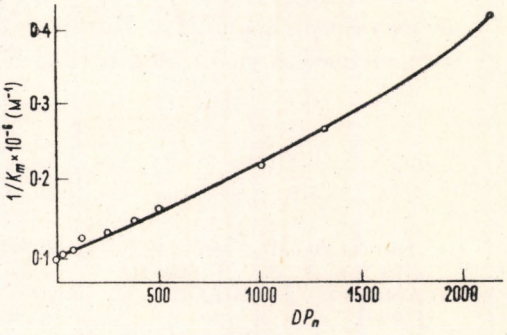


Fig. V/35. Dependence of Michaelis constants on degrees of polymerization in the range of low molecular weights [50]

Table V/20

Kinetic Data for the Phosphorolytic Decomposition of Low Molecular Weight Amyloses [50]

DP_n	$V'_m \times 10^3$ (μM G-1-P min^{-1})	$K'_m \times 10^6$ (M)	$1/K'_m \times 10^{-6}$ (M^{-1})	$V'_m/K'_m \times 10^{-4}$
6	4·26	9·65	0·103	0·441
25·5	9·09	18·45	0·054	0·443
43·5	6·37	8·4	0·119	0·760
97·0	5·88	8·2	0·122	0·717
139·0	3·85	7·0	0·143	0·550
260·0	3·7	7·24	0·138	0·510
357·0	3·55	6·64	0·151	0·530
480·0	3·92	6·06	0·165	0·647
970·0	2·65	5·06	0·197	0·504
1610·0	1·66	3·77	0·265	0·440
2155·0	1·16	2·25	0·445	0·516

enzyme binding than in a more diluted solution under good solvation conditions.

If the observed effects are correlated with the high molecular character of the products (effect of molecular shape and coil-like state on the inactive binding of the enzyme or the small number of terminal groups against the inactive binding sites), then the dependence of the values of V'_m and K'_m on

the concentration must gradually disappear at low molecular weight. The dependence of kinetic data on the polymerization degree at low molecular weight is shown in Table V/20.

In the range DP_n 25–48, it is rather difficult to observe a systematic change of the values V'_m and K'_m. However, the K'_m values show a definite decrease with increasing chain length (Fig. V/35) [50].

REFERENCES

1. Abdullah, M., Fischer, E. H., Quershi, M. Y., Slessor, K. N., Whelan, W. J *Biochem. J. 97*, 9P (1965).
2. Arreguin, B., Miranda, S., Cetina, R., *Bol. inst. quim univ. nal auton. 15*, 48 (1963).
3. Bailey, J. M., Whelan, W. J., Peat, S., *J. Chem. Soc. 1950*, 3692 (1956).
4. Barker, S. A., Bourne, E. J., *Quart. Rev. Chem. Soc. London 7*, 56 (1953); *Chem. Abstr. 48*, 3426e (1954).
5. Bourne, E. J., Sitch, D. A., Peat, S., *J. Chem. Soc. 1949*, 1548 (1949).
6. Burchard, W., Husemann, E., *Makromolekulare Chem. 44/46*, 358 (1961).
7. Chou, M. J., *J. Biol. Chem. 180*, 771 (1949).
8. Cori, G. T., Colowick, S. P., Cori, F. C., *J. Biol. Chem. 127*, 771 (1939).
9. Cori, C. F., Cori, G. T., Green, A. A., *J. Biol. Chem. 151*, 39 (1943).
10. Cori, G. T., Cori, C. F., Schmidt, G., *J. Biol. Chem. 129*, 629 (1939).
11. Cori, C. F., Schmidt, G., Cori, G. T., *Science 89*, 464 (1939).
12. Cori, G. T., Swanson, N. A., Cori, C. F., *Fed. Proc. 4*, 234 (1954).
13. Cumberbirch, R. J. E., Harland, W. G., *J. Textile Inst. 49*, 679 (1958).
14. De Souza, B. C., Cardini, C. E., *An. Asoc. Quim. Argent. 55*, 271 (1967).
15. Dixon, M., Webb, E. C., *Enzymes*, Longmans Co. Ltd. London (1958).
16. French, D., Knapp, D. W., Pazur, J. H., *J. Am. Chem. Soc. 72*, 1866 (1950).
17. French, D., Wild, G. M., *J. Am. Chem. Soc. 75*, 4990 (1953).
18. Gilbert, G. A., *Die Stärke 5*, 95 (1958).
19. Gilbert, G. A., *Nature, 207*, 857 (1965).
20. Hanes, C. S., *Nature, 145*, 348 (1940).
21. Hanes, C. S., *Proc. Roy. Soc. London B 129*, 174 (1940).
22. Hanes, C. S., Maskell, E. J., *Biochem. J. 36*, 76 (1942).
23. Hidy, P. H., Day, H. G., *J. Biol. Chem. 160*, 273 (1945).
24. Holló, J., László, E., Hoschke, Á., *Die Stärke 17*, 377 (1965).
25. Holló, J., László, E., Hoschke, Á., *Acta Chim. Hung. 50*, 351 (1966).
26. Holló, J., László, E., Juhász, J., *Die Stärke 19*, 246 (1967).
27. Holló, J., László, E., Juhász, J., *Die Stärke 19*, 285 (1967).
28. Holló, J., László, E., Hoschke, Á., *Carbohydr. Res. 10*, 49 (1969).
29. Holló, J., László, E., Hoschke, Á., Turán, A., *Die Stärke*, in the press.
30. Holló, J., László, E., Turán, A., *Acta Chim. Hung.* in the press.
31. Hoschke, Á., *Dissertation*, Budapest, 1965.
32. Husemann, E., Fritz, B., Lippert, R., Pfannemüller, B., *Die Makromol. Chem. 26*, 199 (1958).
33. Husemann, E., Lindemann, E., *Die Stärke 6*, 141 (1954).
34. Husemann, E., Pfannemüller, B., *Die Makromol. Chem. 49*, 214, (1961).
35. Husemann, E., Pfannemüller, B., *Die Makromol. Chem. 69*, 74, (1963).
36. Husemann, E., Pfannemüller, B., Burchard, W., *Die Makromol. Chem. 59*, 1 (1963).
37. Husemann, E., Pfannemüller, B., *Die Stärke, 20*, 1 (1968).
38. Kamogawa, A., Fukui, T., Nikuni, Z., *Biochemistry 63*, 361 (1968).
39. Katz, J., Hassid, W. Z., *Arch. Biochem. 30*, 272 (1951).

40. KURASAWA, H., IGAUE, I., HAYAKAWA, T., OGAMI, H., *Nippon Nogei Kagaku Kaishi 33*, 393—8 (1959) *Chem. Abstr. 54*, 9005d.
41. LÁSZLÓ, E., Dissertation, Budapest, 1967.
42. LIDDLE, A. M., MANNERS, D. J., WRIGHT, A., *Biochem. J. 80*, 304 (1961).
43. MANNERS, D. J., *Adv. Carbohydrate Chem. 17*, 371 (1962).
44. MEAGHER, W. R., HASSID, W. Z., *J. Am. Chem. Soc. 68*, 2135 (1946).
45. MEYER, K. H., SPAHR, P. F., FISCHER, E. H., Helv. Chim. Acta *36*, 1924 (1963).
46. NAKAMURA, M., *J. Agr. Chem. Soc. Japan 26*, 260 (1952). *Chem. Abstr. 48*, 757e (1954).
47. NAKAMURA, M., *Nature*, 171, 795 (1953).
48. PEAT, S., PIRT, S. J., WHELAN, W. J., J. Chem. Soc. *705*, 714 (1952).
49. PEAT, S., THOMAS, G. J., WHELAN, W. J., J. Chem. Soc. *1952*, 722 (1952).
50. PFANNEMÜLLER, B., *Die Stärke, 20*, 351 (1968).
51. POSTERNAK, T., *J. Biol. Chem. 188*, 317 (1951).
52. POTTER, A. L., SOWDEN, J. C., HASSID, W. Z., DOUDOROFF, M., *J. Am. Chem. Soc. 70*, 1751 (1948).
53. SWANSON, N. A., CORI, C. F., *J. Biol. Chem. 172*, 815 (1948).
54. THOMA, J. A., *J. Mol. Biol. 2*, 169 (1960).
55. TREVELYAN, W. E., MANN, P. F. E., HARRISON, J. S., Arch. Biochem. Biophys. *39*, 419 (1952).
56. WEIBULL, G., TISELIUS, A., *Arkiv. Kemi. Mineral. Geol. A19*, 1 (1945).
57. WEIBULL, C., TISELIUS, A., *Arkiv. Kemi. Mineral. Geol. A19*, 25 (1945).
58. WHELAN, W. J., *Methods in Enzymol. 1*, 192 (1955).
59. WHELAN, W. J., BAILEY, J. M., *Biochem. J. 58*, 560 (1954).
60. WHISTLER, R. L., PASCHALL, E. F., *Starch, Chemistry and Technology;* Vol. 2. p. 331. Academic Press, New York 1967.
61. WOLFROM, M. L., SCHMIDT, C. F., BROWN, A. E., *J. Am. Chem. Soc. 65*, 255 (1943.)

VI. MOLECULAR MECHANISM OF THE ACTION OF PHOSPHORYLASE

The knowledge of the molecular mechanism of enzyme reactions is the most important problem in present enzyme research. These investigations concern the problems of the formation of enzyme-substrate complexes; of their activation; of the elucidation of the chemical nature of the catalyzed reaction; of the determination of the functional groups participating in the binding of substrates, and in the catalytic step; of establishing the optimum structure of enzymes required for the catalysis; etc.

1. Binding of Substrates to Phosphorylase

The affinity of enzymes for various substrates is different. Affinity is actually expressed by the reciprocal of the dissociation constant of the

Table VI/1

Apparent Michaelis Constants and Affinities

Substrate	K_m (mM)	Affinity (M^{-1})	Conditions		Ref.
			pH	°C	
Glucose-1-phosphate	2·6	380	6·0	38	[24]
	1·5	670	—	—	[19]
	3·5	290	6·3	30	[16]
	2·7	370	6·0	35	[14]
Inorganic phosphate	6·2	160	6·0	38	[24]
	7·5	130	6·3	30	[16]
	18·2	55	6·0	35	[14]
Primers					
Maltohexaose	0·66	1500	6·0	38	[24]
Amylopectin[1]	0·19	5300	6·0	30	[14]
In phosphorolysis					
Amylose (*DP* 1020)[2]	0·011	9000	7·0	25	—
Amylopectin[1]	0·55	1800	6·0	30	[14]

[1] Concentration referred to non-reducing terminal groups.
[2] In the case of amylose, the value of K_m is dependent on the degree of polymerization and on the concentration (cf. Section V.2.(vi.).

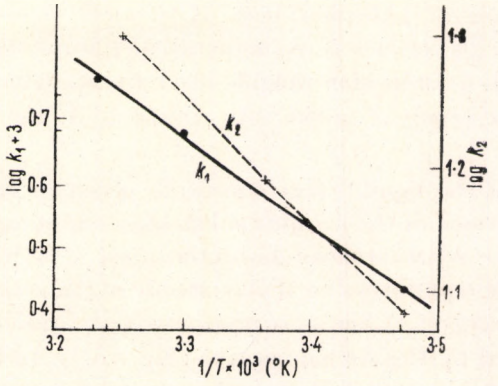


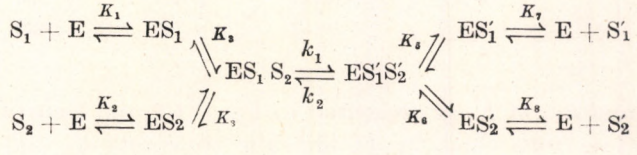
Fig. VI/1. Arrhenius plot of synthesis and decomposition catalyzed by potato phosphorylase [14]. k_1 and k_2 are the rate constants of phosphorolysis and synthesis, respectively

enzyme-substrate complex or, when this constant is not known, of the apparent Michaelis constant. The Michaelis constants available in the literature are given in Table VI/1, together with the affinity data calculated by us (this Table contains only the literature data which could be expressed in molar concentrations).

It appears from these data that this enzyme shows the greatest affinity for amylopectin, particularly under the conditions of the synthesis reaction, and a minimum affinity for inorganic phosphate.

Thus, the problem emerges obviously as to whether the binding of the various substrates is affected by the single substrates if such great differences in affinities exist.

For the determination of the 'relative sites of binding' of the various substrates, the Florini–Westling method [7] was employed. With this method, it can be decided whether the various substrates are all bound to the active centre of the enzyme at the same site or whether their binding is affected by each other. For a reversible reaction with two substrates, the following scheme can be presented:



where, in the case of phosphorylase, E denotes the enzyme molecule, S_1 is amylopectin, glycogen etc., S_2 is inorganic phosphate, S_1' amylopectin, glycogen etc. with a molecular weight lower by one glucose unit etc. and S_2' is glucose-1-phosphate. k is the rate constant, K the equilibrium constant.

By determining the equilibrium constants given in the above scheme, the binding conditions of the various substrates can be established:

The equilibrium constants can be determined if a 'rapid equilibrium' exists in the single equilibria and if the 'steady state' is excluded. The equilibrium interconversion of the two products is the rate-determining step. This can be proved by the Arrhenius plot (Fig. VI/1). In the case of potato phosphorylase a linear correlation exists in the examined range both for the synthesis and for the decomposition. Thus, the above scheme in fact holds.

With regard to the conditions of binding of the single substrates, interesting conclusions can be drawn from the separately determined equilibrium constants. In the case of potato phosphorylase, these determinations were carried out by Holló *et al.* [15] at 35°C at pH 6·0.

The results of these measurements are listed in Table VI/2.

Table VI/2

Effect of the Concentration of Amylopectin (Ap) and of Inorganic Phosphate (P_i) on v_0, $1/v_0$ and $[E]$ v_0 at pH 6·0 and 35°C [15]

P_i concentration (mM)	Ap-concentration (mM)	$v_0 \times 10^{-3}$ (μM/min)	$1/v_0$ (min/M)	$[E]v_0$ ([E] min/μM)
18·7	0·118	3·38	308	2500
	0·237	5·15	194	1650
	0·53	10·75	93	792
	1·18	13·65	72	623
50	0·118	3·97	252	2140
	0·237	6·30	159	1350
	0·53	12·75	78	667
	1·18	17·40	57	487
75	0·118	5·67	176	1500
	0·237	9·55	105	891
	0·53	15·90	63	534
	1·18	20·15	50	420

The determination of K_1 is presented in Fig. VI/2 and that of K_2 in Fig. VI/3. Figure VI/4, from which the equilibrium constants K_3 and K_4 can be

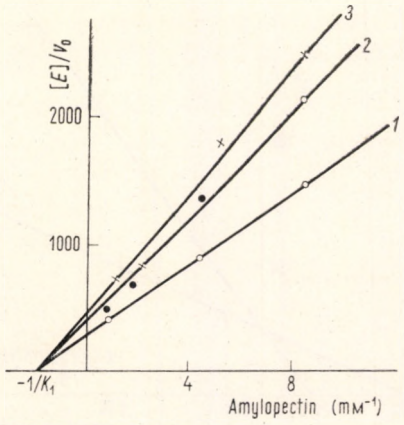


Fig. VI/2. Determination of equilibrium constant K_1 [15]. *1:* 1·18 mM amylopectin; *2:* 0·53 mM amylopectin; *3:* 0·237 mM amylopectin

determined, has been plotted from the values $1/V_{max}$ read from Figs VI/2 and VI/3.

For these equilibrium constants, the following values were obtained:

$K_1 = 0{\cdot}55$ mM; $K_4 = 0{\cdot}54$ mM; $K_2 = 18{\cdot}2$ mM; $K_3 = 18{\cdot}8$ mM.

Further equilibrium constants can be determined by investigating the synthesis reaction. Obviously, only values determined under identical conditions (35°C, pH 6·0) are suitable for comparison [15].

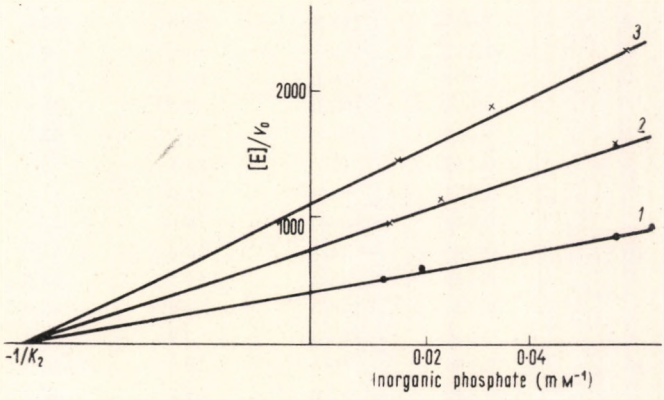


Fig. VI/3. Determination of equilibrium constant K_2 [15]. *1:* 1·18 mM amylopectin; *2:* 0·53 mM amylopectin; *3:* 0·237 mM amylopectin

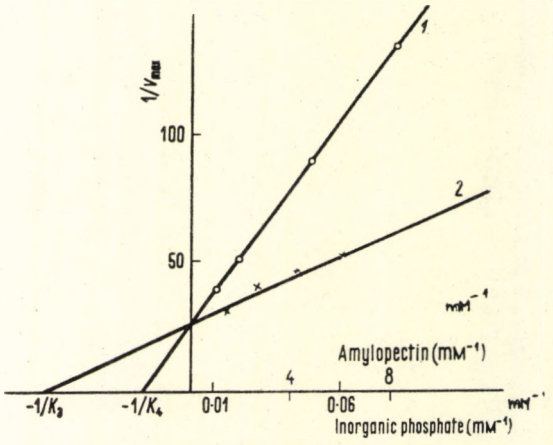


Fig. VI/4. Determination of equilibrium constants K_3 and K_4 [15]. *1:* Maximal velocity (V_{max}) for P_{inorg}; *2:* for amylopectin

The initial rates calculated from the quatities of inorganic phosphate formed are shown in Table VI/3.

Table VI/3

Effect of the Concentration of Glucose-1-phosphate (G-1-P) and of Amyolpectin (Ap) on v_0, $1/v_0$ and $[E]$ v_0 at pH 6·0 and 35°C [15]

G-1-P concentration (mM)	Ap-concentration (mM)	$v_0 \times 10^{-3}$ (μM/min)	$1/v_0$ (min/μM)	$[E]v_0$ ($[E]$ min/μM)
3	0·095	43·8	22·8	84·5
	0·142	62·3	16·0	59·2
	0·237	70·0	14·3	52·9
	0·950	108·0	9·35	34·9
5	0·095	54·5	18·3	67·7
	0·142	70·8	14·1	52·7
	0·237	88·0	11·3	41·8
	0·950	158·0	6·0	23·2
10	0·095	64·8	15·4	57·0
	0·142	85·2	11·7	43·3
	0·237	106·0	9·45	35·0
	0·950	183·0	5·45	20·4
20	0·096	80·5	12·4	45·8
	0·142	87·0	11·5	42·5
	0·237	116·5	8·6	31·8
	0·950	189·0	5·3	19·6

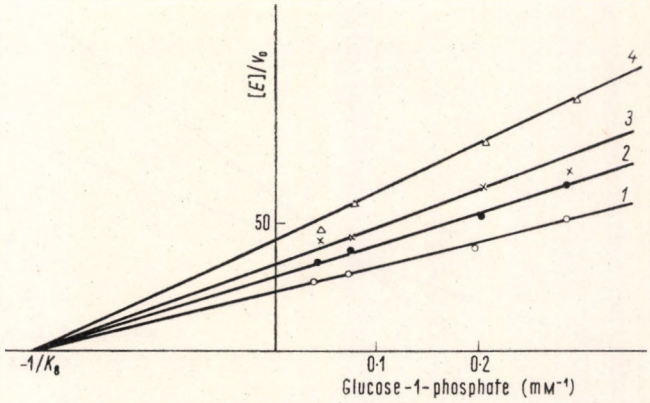


Fig. VI/5. Determination of equilibrium constant K_7 [15]. *1:* 20 mM glucose-1-phosphate; *2:* 10 mM glucose-1-phosphate; *3:* 5 mM glucose-1-phosphate; *4:* 3 mM glucose-1-phosphate

Values of K_7 were determined on the basis of Fig. VI/5, and values of K_8 from Fig. VI/6.

Figure VI/7 for the determination of the constants K_5 and K_6 has been plotted from the values of $1/V_{max}$ read from Figs VI/5 and VI/6.

The following values were obtained for the equilibrium constants: $K_7 =$ $= 0{\cdot}185$ mM; $K_6 = 0{\cdot}195$ mM; $K_8 = 2{\cdot}75$ mM; $K_5 = 2{\cdot}90$ mM.

By comparing these eight equilibrium constants with each other (for the time being we do not know whether they are in fact the dissociation

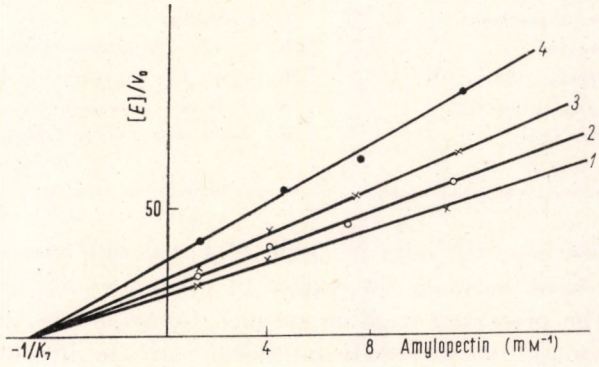


Fig. VI/6. Determination of equilibrium constant K_8 [15]. *1:* 0·95 mM amylopectin; *2:* 0·237 mM amylopectin; *3:* 0·142 mM amylopectin; *4:* 0·095 mM amylopectin

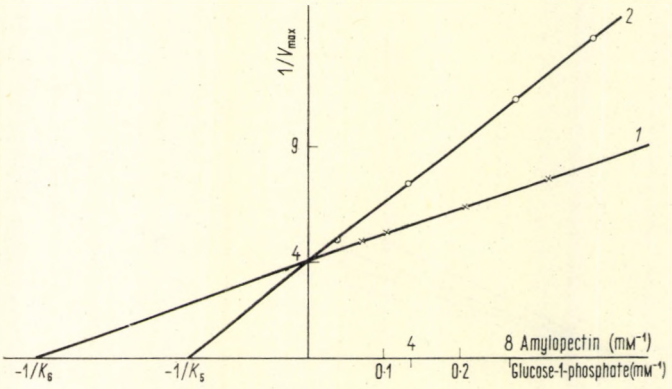


Fig. VI/7. Determination of equilibrium constants K_5 and K_6 [15]. *1:* Maximal velocity (V_{max}) for; glucose-1-phosphate; *2:* for amylopectin

constants of the enzyme-substrate complexes), it appears that the equilibrium constants for related reactions are approximately identical. Consequently, the substrates are bound to the enzyme molecule at different sites and do not interfere with the binding of each other in a competitive or steric way.

The eight equilibrium constants have also been determined for liver phosphorylase [18]. The literature data are summarized in Table VI/4.

Table VI/4

Equilibrium Constants in the Case of Liver Phosphorylase [18]

In the case of decomposition:	In synthesis:
K_1 = 13 mM glycogen[1]	K_5 = 0·28 mM glucose-1-phosphate
K_2 = 11 mM inorganic phosphate	K_6 = 2·9 mM glycogen[1]
K_3 = 0·82 mM inorg. phosphate	K_7 = 14 mM glycogen[1]
K_4 = 0·93 mM glycogen[1]	K_8 = 1·3 mM glucose-1-phosphate

[1] Glycogen concentration is expressed as the molar equivalent of its glucose residues.

It can be seen from the data in Table VI/4 that differences in the order of magnitude exist between the values of related equilibrium constants according to the presented reaction scheme. Consequently, in the case of liver phosphorylase, one substrate interferes with the binding of another substrate. A similar phenomenon was also observed in the case of muscle phosphorylase [17], i.e. another significant difference was found between phosphorylases of plant and animal origin.

2. Formation and Decomposition of Enzymes–Substrate Complexes

In the case of phosphorylase, the kinetic constants obtained by the Dalziel method [5] have been used to study the individual reaction steps of the enzymatic process [15].

On the basis of the given reaction scheme, the following rate equations can be written:

for the decomposition

$$\frac{[E]}{v_0} = \frac{1}{k_1} + \frac{K_4}{k_1[S_1]} + \frac{K_3}{k_1[S_2]} + \frac{K_3 \cdot K_4}{k_1[S_1]\,[S_2]}$$

and for the synthesis

$$\frac{[E]}{v_0} = \frac{1}{k_2} + \frac{K_6}{k_2[S_1']} + \frac{K_5}{k_2[S_2']} + \frac{K_5 \cdot K_6}{k_2[S_1']\,[S_2']}$$

On employing the Dalziel reductions, the above equations can be converted into the following forms:

for the decomposition

$$\frac{[E]}{v_0} = \Phi_0 + \frac{\Phi_1}{[S_1]} + \frac{\Phi_2}{[S_2]} + \frac{\Phi_{12}}{[S_1]\,[S_2]}$$

for the synthesis

$$\frac{[E]}{v_0} = \Phi_0' + \frac{\Phi_1'}{[S_1']} + \frac{\Phi_2'}{[S_2']} + \frac{\Phi_{12}'}{[S_1']\,[S_2']}$$

On plotting $[E]/v_0$ against $1/[S_2]$ at constant S_1 according to the above equation, a straight line is obtained of slope $\Phi_2 + \Phi_{12}/[S]$ and intercept $\Phi_0 + \Phi_1/[S_1]$. By plotting the x-axis intercepts against $1/[S_1]$, all the four coefficients ($\Phi_0, \Phi_1, \Phi_2, \Phi_{12}$) can be determined from the intercepts and slopes of the lines. When the same plots are made for the other substrate the values of K_8 are obtained, and as a control the coefficients can again be determined from the intercepts and the slopes.

The data of the experiment with potato phosphorylase described in Section VI.1 were used for the determination of the various constants. The slopes and intercepts of the straight lines shown in Figs VI/2, VI/3 and VI/5, VI/6 were established (Table VI/5).

Table VI/5

Values of the Intercepts and Slopes of the Plots in Figs VI/2, VI/3, VI/5 and VI/6 [15]

No. of Fig.		$[E]/v_0$ ([E] min/μM)	$1/v_0$ (min/μM)	Slope ([E] min/ml)
Fig. VI/2	*1*	270	31·80	154
	2	350	41·40	206
	3	450	53·00	241
Fig. VI/3	*1*	325	38·30	6 570
	2	440	51·80	8 470
	3	750	88·20	16 800
Fig. VI/5	*1*	17	4·60	2·90
	2	19	5·30	3·60
	3	23	6·21	4·52
	4	29	7·84	5·27
Fig. VI/6	1	17	4·60	48
	2	28	7·56	72
	3	34	9·20	90.20
	4	45	12·10	118·50

The values of this table are illustrated in Figs VI/8–VI/11 [15].

The Dalziel constants read from Figs VI/8–VI/11, and the equilibrium and rate constants calculated from them are summarized in Tables VI/6 and VI/7. For the sake of comparison, the values of the corresponding constants obtained in the case of liver phosphorylase are also given [18].

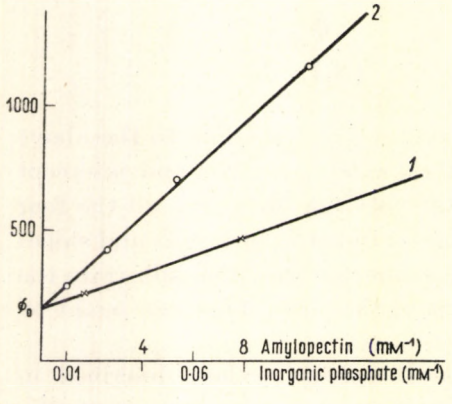


Fig. VI/8. Plot of axis intersections in phosphorolysis [15]. *1:* Inorganic phosphate; *2:* amylopectin

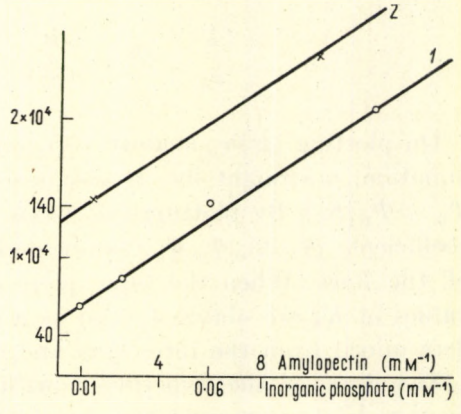


Fig. VI/9. Plot of slope in phosphorolysis [15]. *1:* Inorganic phosphate; *2:* amylopectin

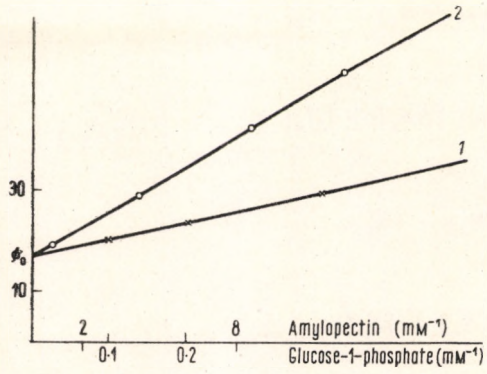


Fig. VI/10. Plot of axis intersections in synthesis [15]. *1:* Glucose-1-phosphate; *2:* amylopectin

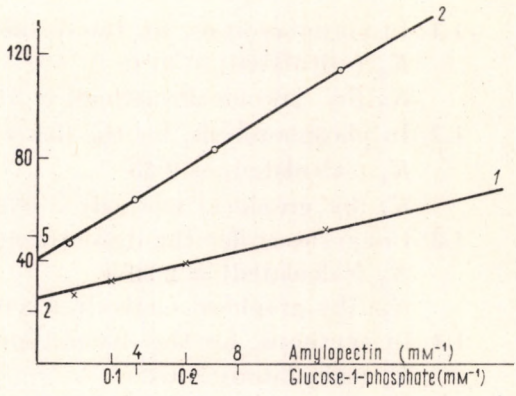


Fig. VI/11. Plot of slope in synthesis [15]. *1:* Glucose-1-phosphate; *2:* amylopectin

Table VI/6

Dalziel Constants of Potato and Liver Phosphorylase

Constant	Potato phosphorylase [15]	Liver phosphorylase [18]
Φ_0	220 [*E*] min/μM	0·0725 mg protein·min/μM
Φ_1	118 [*E*] min/ml	67 min·mg
Φ_2	4600 [*E*] min/ml	60 min·mg
Φ_{12}	2050 [*E*] min μM/ml^2	0·8 M·min·mg
Φ'_0	14·5 [*E*] min/μM	0·027 mg protein·min/μM
Φ'_1	2·9 [*E*] min/ml	80 min·mg
Φ'_2	43·0 [*E*] min/ml	7.5 min·mg
Φ'_{12}	7·2 [*E*] min·μM/ml^2	0·11 M·min·mg

It appears from the data of these tables that the given equation is suitable for the description of the enzyme reaction. Accordingly, in both reactions catalyzed by phosphorylase, four enzyme substrate dimers and two ternary complexes participate, and the equilibrium interconversion of the two ternary complexes is the rate determining step.

All these characteristics correspond to a reaction scheme of type Dalziel I. Further evidence pointing to a reaction mechanism of type I is as follows.

1. The dissociation constants of the ternary complex can be calculated from the Dalziel constants, and can also be determined by the graphical method of Lineweaver and Burk. If these values obtained by different methods are identical, the correctness of reaction type I is proved. In the case of potato phosphorylase, the following values were obtained [14]:

1.1. In phosphorolysis, for the dissociation $[ES_1] + [S_2]$
K_3 (calculated) = 21·0
K_3 (by graphical method) = 21·3
1.2. In phosphorolysis, for the dissociation $[ES_2] + [S_1]$
K_4 (calculated) = 0·55
K_4 (by graphical method) = 0·527
1.3 In synthesis, for the dissociation $[ES_1'] + [S_2']$
K_5 (calculated) = 2·96
K_5 (by graphical method) = 3·03
1.4. In synthesis, for the dissociation $[ES_2'] + [S_1']$
K_6 (calculated) = 0·20
K_6 (by graphical method) = 0·19
2. Correlations between the Dalziel constants:

If the value calculated from the relationship $\Phi_0 = \frac{\Phi_1 \cdot \Phi_2}{\Phi_{12}}$ is identical with the value of Φ_0 obtained graphically, the correctness of reaction type I is proved:

Φ_0(calculated) = 278
Φ_0(graphically) = 220

and, similarly,

Φ_0'(calculated) = 17·4
Φ_0'(graphically) = 14·5.

Table VI/7

Kinetic Constants of Potato and Liver Phosphorylase

Constant	Potato phosphorylase [15]	Liver phosphorylase [18]
K_1	0·55 mM	13 mM
K_2	18.20 mM	11 mM
K_3	21·00 mM	0·82 mM
K_4	0·55 mM	0·93 mM
K_5	2·90 mM	0·28 mM
K_6	0·20 mM	2·9 mM
K_7	0·19 mM	14·0 mM
K_8	2·75 mM	1·3 mM
k_1	$4{\cdot}50 \times 10^{-3}$ μM/min	13·8 μ M/min/mg
k_2	$6{\cdot}90 \times 10^{-2}$ μM/min	37·2 μM/min/mg

Further relationships can be established between the various dissociation constants. These can be used to distinguish between alternatives within the reaction type I.

In the case of decomposition $K_1K_3 = K_2K_4$ ($11{\cdot}7 \sim 10$)
In the case of synthesis $K_5K_7 = K_6K_8$ ($0{\cdot}535 \sim 0{\cdot}55$).

These data are characteristic of the reaction types Ia and Ib. A decision as which of these is actually present can be made by employing the relationships:

in the case of decomposition	$K_1 = K_4$ ($0{\cdot}55 = 0{\cdot}55$)
	$K_2 = K_3$ ($18{\cdot}2 \sim 21{\cdot}3$)
in the case of synthesis	$K_6 = K_7$ ($0{\cdot}20 \sim 0{\cdot}185$)
	$K_5 = K_8$ ($2{\cdot}9 \sim 2{\cdot}75$).

On the basis of the relationships discussed above, it is certain that mechanism Ib is characteristic for the description of the synthesis and decomposition catalyzed by phosphorylase.

Besides the properties established for reaction mechanism of type I, this type is characterized also by the following features:

1. The order of binding of the various substrates is completely irrelevant from the aspect of the formation of the two ternary complexes.

2. The same affinity can be observed between the free enzyme and the various substrates, and between the enzyme-substrate dimer and another substrate, as has been proved earlier.

In the case of liver phosphorylase, the correctness of reaction type Ib could not be unequivocally proved by processing the experimental data [18].

3. Determination of the Functional Groups of the Enzyme by Kinetic Methods

It appears from the previous section that phosphorylase possesses two identical or different functional groups for the binding of each single substrate. These groups have been determined by using both the synthesis reaction and the phosphorolysis reaction. In that procedure, the dependence on pH and temperature of the apparent Michaelis constants, the maximum reaction rates and the logarithmic values of the quotient of both former data were used. The dissociation constants (pK) were obtained from the dependence of the various functional groups on pH.

(i) Determination of Dissociation Constants in the Case of Synthesis [10]

The determination was carried out in the pH range 5–8, at 30°C. The Michaelis constants obtained for glucose-1-phosphate (K_a) and for the primary substance amylopectin (K_b), together with the corresponding maximum rates, are presented in Table VI/8.

Table VI/8

Dependence of K_a K_b, $V_{max,a}$ and $V_{max,b}$ on pH at 30°C [10]

pH	K_a (mM)	$V_{max\ a}$ (M/min.)	K_b (mM)	$V_{max,\ b}$ (M/min.)
5·0	26·90	$8{\cdot}6\times10^{-4}$	0·500	$3{\cdot}0\times10^{-4}$
5·3	16·60	$2{\cdot}4\times10^{-3}$	0·181	$1{\cdot}07\times10^{-3}$
5·6	10·70	$4{\cdot}7\times10^{-3}$	0·090	$2{\cdot}20\times10^{-3}$
6·0	4·57	$7{\cdot}5\times10^{-3}$	0·066	$4{\cdot}70\times10^{-3}$
6·6	4·00	$5{\cdot}2\times10^{-3}$	0·066	$4{\cdot}13\times10^{-3}$
7·0	4·70	$4{\cdot}1\times10^{-3}$	0·072	$2{\cdot}31\times10^{-3}$
7·3	5·24	$3{\cdot}1\times10^{-3}$	0·086	$2{\cdot}11\times10^{-3}$
7·6	5·05	$1{\cdot}4\times10^{-3}$	0·106	$1{\cdot}12\times10^{-4}$
8·1	5·30	$6{\cdot}7\times10^{-4}$	0·120	$4{\cdot}66\times10^{-4}$

On plotting the negative logarithmic values ($-\log K_a = pK_a$, and $-\log K_b = pK_b$) of the apparent Michaelis constants (expressing K_a and K_b in dimension of moles per litre (M)), against pH, the curves shown in Figs VI/12 and VI/13 were obtained. The points of intersection of the linear sections of these curves give the pK values of the functional groups participating in the reaction which takes place in the enzyme, in the enzyme-substrate complex and in the substrate. These values are 5·78 for glucose-1-phosphate, and 5·79 and 6·92 for amylopectin.

With high substrate concentrations, all the enzyme molecules are present in a form bound to the substrate. Thus, only the pK values of the functional group of the enzyme-substrate complex can be determined. The values of $\log V_{max,a}$ and $\log V_{max,b}$ are presented in Figs VI/12 and VI/13. The pK values obtained in this way are 5·48 and 6·90 for glucose-1-phosphate and 5·40 and 6·92 for amylopectin.

Conclusions as regards the ionization state of the functional groups can also be drawn from the pH-dependence of $\log V_{max,a}/K_a$ and $\log V_{max,b}/K_b$.

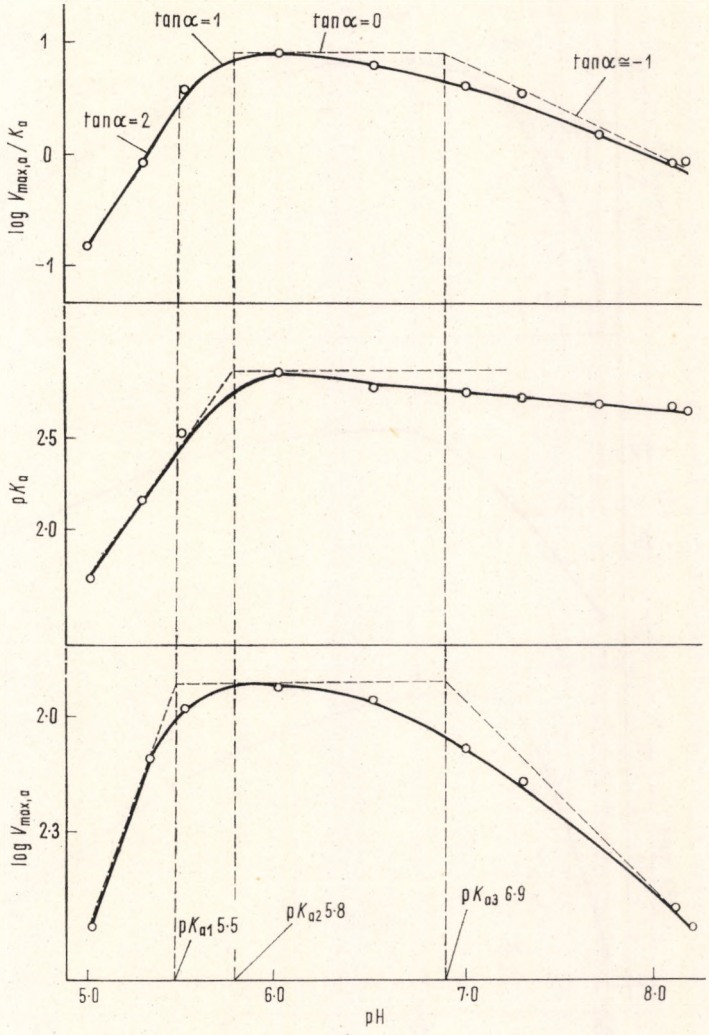


Fig. VI/12. Determination of dissociation constants in synthesis at 30°C, with glucose-1-phosphate [10]

The pK values along the curve are characteristic of the functional groups of both the substrate and the enzyme-substrate complex (Figs VI/12 and VI/13). The values of pK are 5·48, 5·78 and 6·90 with glucose-1-phosphate as substrate, and 5·40, 5·79 and 6·92 with amylopectin. It follows that the binding takes place on the group of pK 5·8 in the case of both substrates.

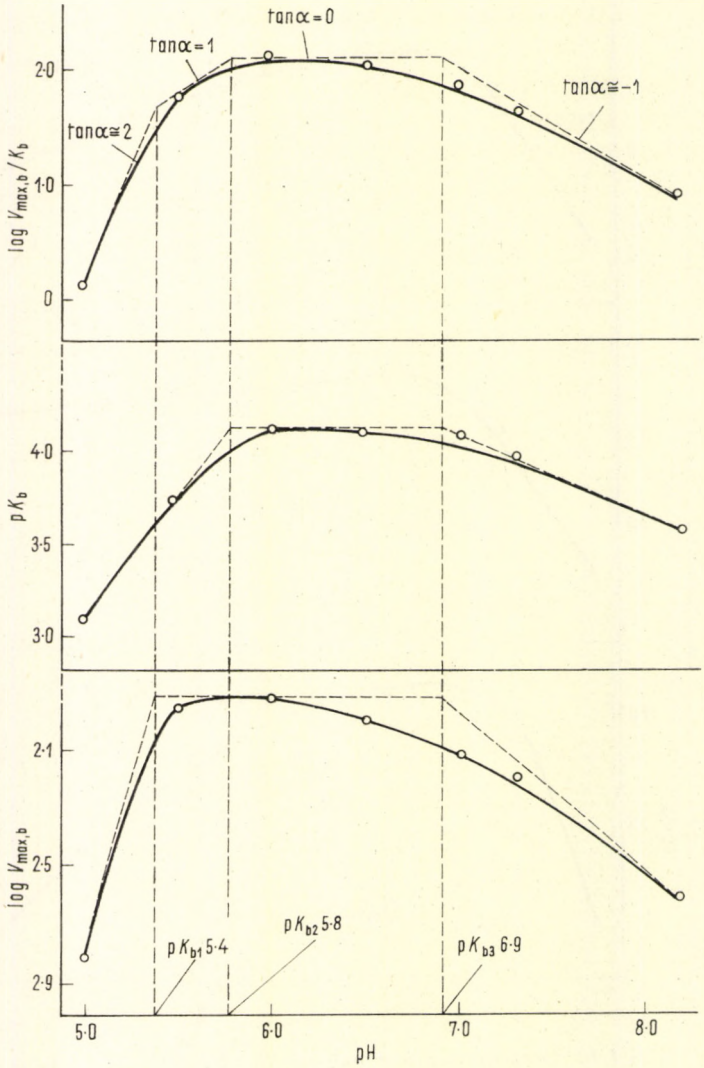


Fig. VI/13. Determination of dissociation constants in synthesis at 30°C, with amylopectin [10]

(ii) Determination of Dissociation Constants in Phosphorolysis [12]

This determination was carried out similarly in the pH range 5–8, at 30°C. The Michaelis constants obtained from the Lineweaver–Burk diagrams for inorganic phosphate (K_a) and for amylopectin (K_b), and the corresponding maximum reaction rates are given in Table VI/9.

Table VI/9

Dependence of K_a, K_b, and of $V_{max,a}$, $V_{max,b}$ on pH at 30°C [12]

pH	K_a (M)	$V_{max,a} \times 10^{-4}$ (M/min.)	K_b (M)	$V_{max,b} \times 10^{-4}$ (M/min)
5·0	9·14	15·9	10·32	12·2
5·5	370	19·6	17·8	15·5
6·0	520	17·4	42·5	25·0
6·5	329	9·3	30·7	12·2
7·0	358	6·7	22·05	12·8
7·5	391	6·0	8·07	7·2
8·0	510	0·325	—	—

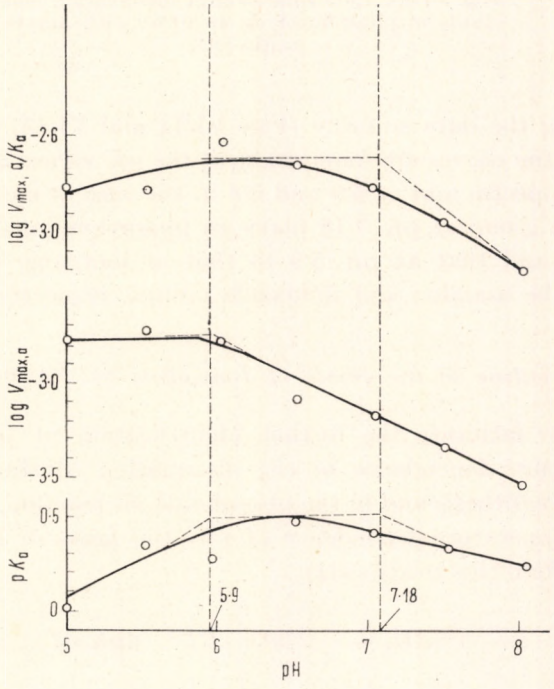


Fig. VI/14. Determination of dissociation constants in phosphorolysis at 30°C, with inorganic phosphate [12]

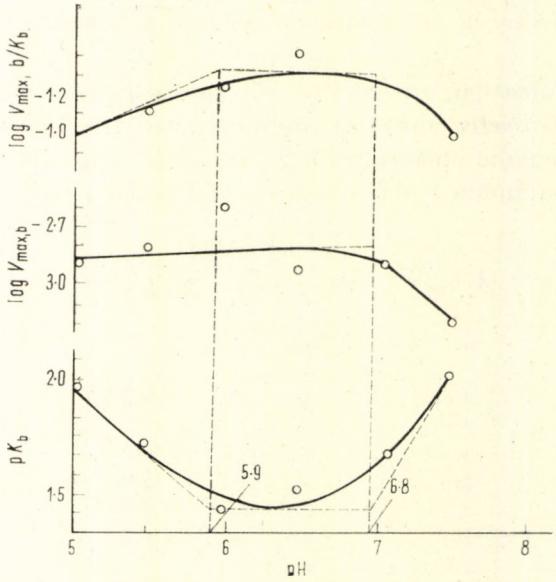


Fig. VI/15. Determination of dissociation constants in phosphorolysis at 30°C, with amylopectin [12]

On plotting the data suitably (Figs VI/14 and VI/15), the points of intersection of the curves are found to lie at the pK values 5·9 and 7·18 in the case of amylopectin and at 5·9 and 6·8 in the case of inorganic phosphate. Of these, the group at pK 7·18 plays an important role in the binding of amylopectin and that at pK 5·9 in that of inorganic phosphate. These groups may be α-amino and imidazole groups, respectively.

(iii) Determination of the Heats of Ionization of Functional Groups

In order to facilitate the further identification of functional groups, the temperature dependence of the dissociation constants of functional groups in the synthesis and in the phosphorolysis reaction were determined. From the temperature-dependence of pK, the heats of ionization can be determined fom the relationship

$$\Delta H_i = -2{\cdot}303 \cdot RT^2 - \mathrm{d}pK/\mathrm{d}T$$

where ΔH_i is the heat of ionization (cal/mole), $R = 1{\cdot}986$ cal/°C mole, $T = 295$°K (referring H_i to 25°C), dT = the temperature difference, and

dpK = the difference of the pK values measured at the two temperatures.

The Michaelis constants and maximum reaction rates were determined according to Section VI.3 *(i)* at 15 and 37°C also. On plotting these suitably, the corresponding pK values were obtained which, together with the data measured at 30°C, are given in Table VI/10.

Table VI/10

Dependence of pK on Temperature in Synthesis [10, 11]

Constants	15°C	30°C	37°C
pK_{a1}	5·60	5·48	5·40
pK_{a2}	6·02	5·78	5·66
pK_{a3}	7·00	6·90	6·80
pK_{b1}	5·46	5·40	5·34
pK_{b2}	5·56	5·79	5·84
pK_{b3}	7·08	6·92	6·69

The mean values of heat of ionization calculated by the above formula are as follows.

For glucose-1-phosphate:

$$pK_{a1} = 3500 \text{ cal/M}$$
$$pK_{a2} = 6700 \text{ cal/M}$$
$$pK_{a3} = 3100 \text{ cal/M}.$$

Of these constants, pK_{a2} is of particular interest because it plays a role in the binding of glucose-1-phosphate. The group is likely to be imidazole.

For amylopectin, the calculated heats of ionization are:

$$pK_{b1} = 3200 \text{ cal/M}$$
$$pK_{b2} = 5700 \text{ cal/M}$$
$$pK_{b3} = 10300 \text{ cal/M}.$$

In this latter case too the group corresponding to pK_{b2} plays a role in the binding of the substrate.

Heats of ionization have also been determined in phosphorolysis [12]. In that case, a group with a heat of ionization of 6700 cal/mole (probably an imidazole group) participates in the binding of amylopectin, and another group with a heat of ionization of 2980 cal/mole in the binding of inorganic

phosphate. This latter however cannot be identified with an α-amino group [4].

Only very few literature data can be found [9] for the dissociation of animal phosphorylases and of its use. On processing the published data, no conclusions could be drawn as regards the nature of the functional groups.

4. Thermodynamic Investigation of the Catalysis by Phosphorylase

From the rate constants of the formation, interconversion, dissociation and product formation of the enzyme-substrate complexes, the energetic conditions of the various partial processes of enzymatic catalysis can be precisely established on the basis of the temperature-dependence of these rate constants. However, the actual rate constants are known for only a small number of enzymes. Thus, for the time being the energetic conditions can only be established from the measured data by neglecting various factors [13].

In the case of potato phosphorylase, Arreguin *et al.* [1] were the first to attempt to evolve an energetic scheme. According to them, on starting with a simple enzymatic reaction scheme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + [\text{product}]$$

the relation of rate constants to each other can be expressed by the inequality $k_3 \ll k_1$ and k_2, in the case of potato phosphorylase. In the case of an inequality of this type, it is possible to determine the value of ΔE from the temperature dependence of the reciprocal of the Michaelis constant, and the heat of activation of the enzyme-substrate complex from the temperature-dependence of the logarithm of the maximum rate of reaction [13]. The heat of reaction is given by the temperature dependence of the equilibrium constants. According to Arreguin *et al.* [1], an energetic scheme such as that shown in Fig. VI/16 can be plotted from these data for the case of a synthesis reaction with glucose-1-phosphate as substrate.

These experiments were reproduced by Holló *et al.* with potato phosphorylase of high purity [11]. In addition, they determined also the above-mentioned constants for the 'primer' substrate and for the decomposition processes (Tables VI/11 and VI/12).

It can be seen from the data in these tables that the values obtained for the different processes are not identical. Therefore, the energetic schemes

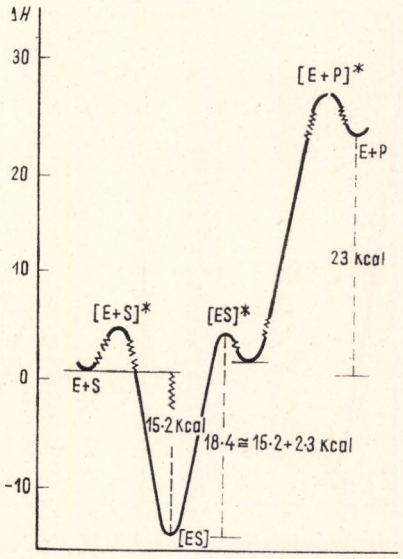


Fig. VI/16. Energetic scheme for glucose-1-phosphate, according to Arreguin *et al.* [*1*]

Table VI/11

Thermodynamic Data of Synthesis (kcal)

Constants	For Glucose-1-phosphate		For acceptor substrate [10]
	Arreguin *et al.* [1]	Holló *et al.* [10]	
ΔE	—15·2	8·0	—3·5
E_3	18·4	5·6	10·3
ΔH	23·0	—1·9	—1·9

Table VI/12

Thermodynamic Data of Decomposition (kcal) [12]

Constants	For inorganic phosphate	For acceptor substrate
ΔE	—3·1	5·4
E_3	16·8	3·8
ΔH	—1·9	—1·9

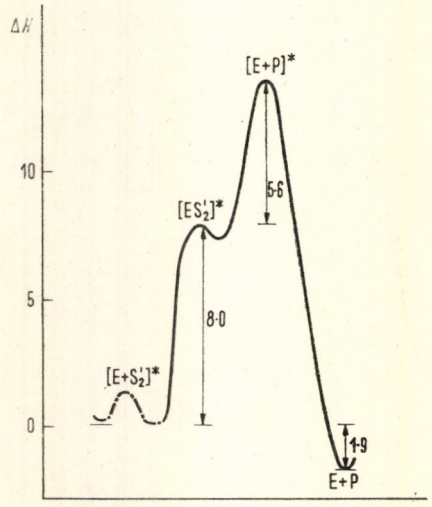


Fig. VI/17. Energetic scheme for glucose-1-phosphate according to Holló *et al.* [11]

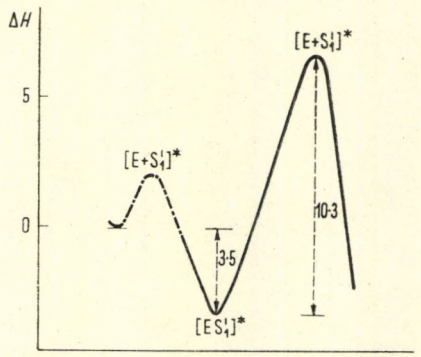


Fig. VI/18. Energetic scheme in synthesis, referring to amylopectin substrate [11]

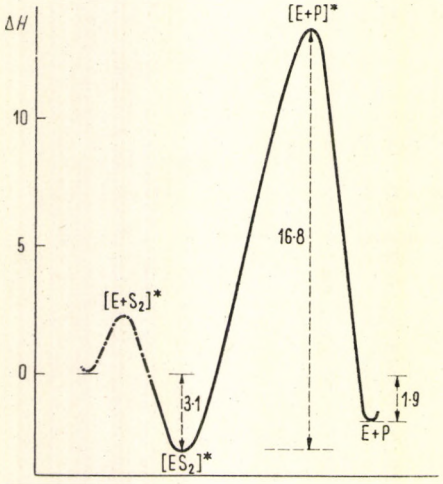


Fig. VI/19. Energetic scheme in phosphorolysis, referring to inorganic phosphate substrate [11]

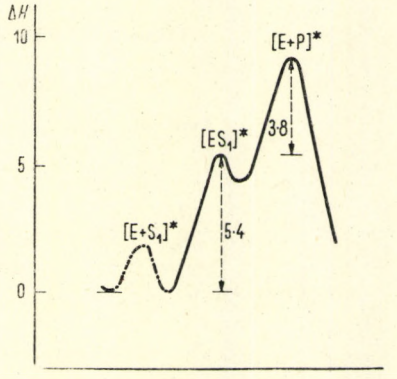


Fig. VI/20. Energetic scheme in phosphorolysis, referring to amylopectin substrate [11]

obtained for the processes of synthesis and decomposition, in each case for two substrates, are given separately (Figs VI/17–VI/20).

On the basis of these Figures, taking into account that the calculation was carried out with certain assumptions, and that the experimental data are insufficient, it did not appear to be advisable to combine all the data. This can only be done after the determination of the real rate constants which remains an unsolved problem.

5. Molecular Mechanism of the Action of Phosphorylase

It follows from the previous sections that quite a number of data are known of the structure, functional groups and effect of phosphorylase. However, only hypotheses are available as to the molecular mechanism of catalysis, and even these refer only to phosphorolysis.

In the opinion of Thoma and Koshland [22], a 'double rearrangement' takes place in phosphorolysis in that the glucopyranose unit recovers in fact its initial configuration. Since no phosphate exchange can be observed [3], the cited authors presume that the polyglucose chain is actually bound to the enzyme which contains a carboxylate anion and a proton donor amino group. The polyglucose chain is bound to the carboxyl group of the enzyme through the hemiacetal oxygen atom of the non-reducing glucose terminal unit (Fig. VI/21).

The role of pyridoxal-5-phosphate present in phosphorylase is taken into account by Nakamura [20]. In his opinion, spatially separated binding and activity centres exist in the enzymes. Between these centres, exchange

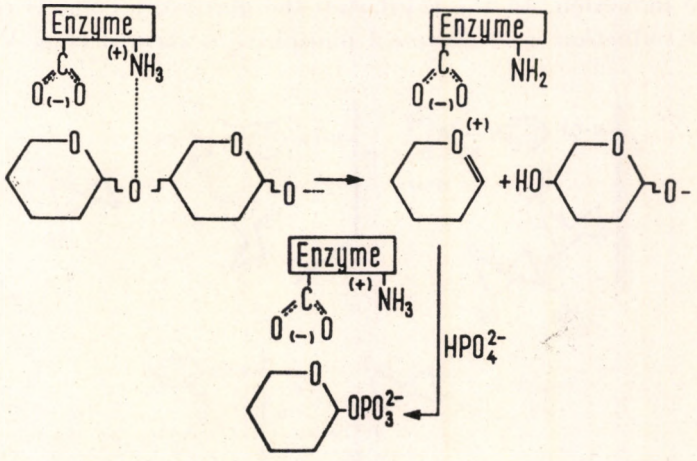


Fig. VI/21. Molecular mechanism of the effect of muscle phosphorylase according to Thoma and Koshland [22]

effects may occur by electron shifts. In protein molecules, the peptide moieties are attached to each other by hydrogen bridges [2, 6] (Fig. VI/22) through which the electron systems may undergo rearrangement. The substrate is bound to the centre through the group R_b with one of its key atoms linked (adsorbed) by a hydrogen bond. Changes in electron distribution start at the active group of the activity centre (R_a), e.g. by the dissociation

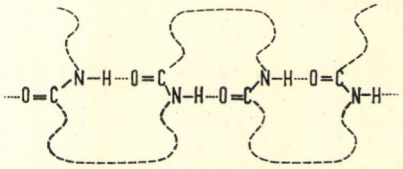


Fig. VI/22. The system peptide bondhydrogen bridge [20]

of the H-ion. Subsequently, this electron-distribution change is continued through the system peptide bond-hydrogen bridge to the binding group itself. From this latter it moves to the key substrate atom, inducing a change of electron distribution in the substrate molecule also, i.e. the substrate undergoes activation.

According to Nakamura, in the phosphorolysis carried out with muscle phosphorylase, pyridoxal-5-phosphate is linked to the binding group R_b. Hence, an electron shift occurs on its 3–O atom, causing an electron deficiency on the C atom of the carbonyl in the hemiacetal group of the non-reducing glucose terminal unit of the polysaccharide to be decomposed. In the second step, the phosphate ion is bound to the C-atom by a non-enzymatic induction. In the third step the glucosidic bond is cleaved by enzymatic induction, and glucose-1-phosphate is formed (Fig. VI/23).

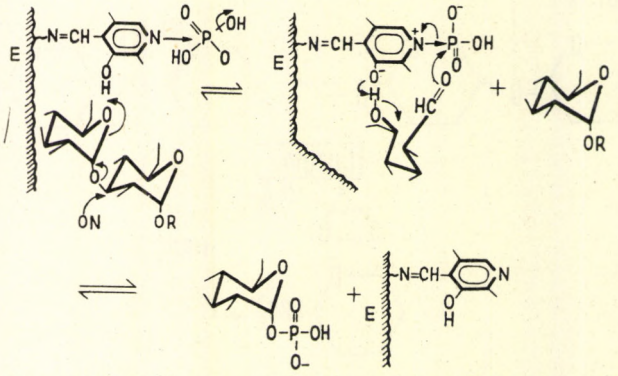


Fig. VI/23. Molecular mechanism of the effect of muscle phosphorylase according to Nakamura [20]

According to Song [21], the above-sketched Nakamura theory is not sufficient for the description of phosphorolysis proper. On the one hand, the particular role of pyridoxal-5-phosphate in the reaction is not proved by this hypothesis; on this basis, the products could be formed in the presence of any acid–base catalyst as well. On the other hand, the presumed electron shift can take place only in a stress state on the protein matrix, e.g. as a result of irradiation.

Song claims that his own hypothesis better explains the results obtained on cleaving the glucosidic bond with isotope ^{18}O, and also the experimental data observed with the 2-hydroxypyridine model substance. According to

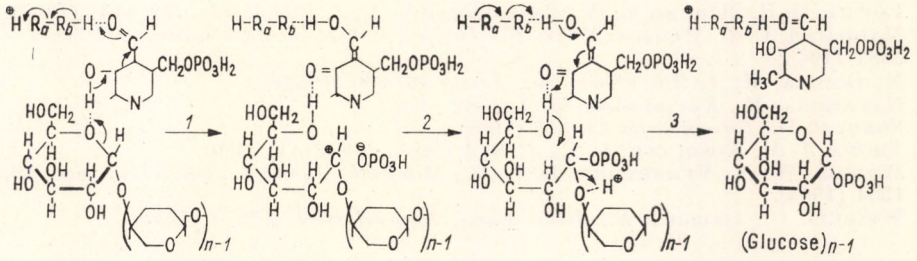


Fig. VI/24. Molecular mechanism of the effect of muscle phosphorylase according to Song [21]

the hypothesis, the hydroxy group of the inorganic phosphate is displaced by the nucleophilic attack of the *N* atom of pyridoxal-5-phosphate bound to the enzyme (Fig. VI/24). The conformation of the enzyme-substrate tetrameric complex formed can change in such a way that it may act as a general acid catalyst, inducing the formation of an sp^2 C-atom by hydrolysis at position 1. A similar mechanism of phosphorylation with pyridine has already been published [23]. As a second step, pyranose phosphate is formed by the phosphate bound electrostatically by a labile linkage, due to the nucleophilic attack of the carbonyl oxygen. Similar nucleophilic attacks of the carbonyl group are known in the general case of Lewis acids [8]. In connection with the discussed hypothesis, even Nakamura had certain reservations. Thus, the chemist must be careful to accept or discard any of the available hypotheses only as a result of actual experimental evidence.

REFERENCES

1. Arreguin, B., Miranda, S., Cetina, R., *Bol. inst. quim. univ. nal. auton. Méx. 15*, pp. 48–60 (1963).
2. Bücher, Th., *Adv. in Enzymol. 14*, 1 (1953).
3. Cohn, M., Cori, G. T., *J. Biol. Chem. 175*, 89 (1948).

4. COHN, R. J., EDSAL, J. T , *Proteins, Amino Acids and Peptides*, Reinhold Publishing Corp. New York 1963.
5. DALZIEL, K., *Acta Chem. Scand.11*, 1706 (1957).
6. EVANS, M. G., GERGELY, J., *Biochim. Biophys. Acta, 3*, 188 (1949).
7. FLORINI, J. R., WESTLING, C. S., *Biochem. Biophys. Acta 25*, 575 (1959).
8. GOULD, E. S., *Mechanism and Structure in Organic Chemistry*, Holt Rinchart, Inc. New York (1959), p. 550.
9. HELMREICH, E., CORI, C. F., *Proc. Natl Acad. Sci. U. S. 52*, 647 (1964).
10. HOLLÓ, J., LÁSZLÓ, E., HOSCHKE, Á., *Die Stärke 18*, 67 (1966).
11. HOLLÓ, J., LÁSZLÓ, E., HOSCHKE, Á., in the press.
12. HOLLÓ, J., LÁSZLÓ, E., JUHÁSZ, J., *Die Stärke, 19*, 285 (1967).
13. LAIDLER, K. J., *The Chemical Kinetics of Enzyme Action*, Oxford, 1958.
14. LÁSZLÓ, E., *Dissertation*, 1967. Budapest.
15. LÁSZLÓ, E., HOSCHKE, Á., HOLLÓ, J., *Lecture* presented at the 1967 Conference, Technical University Budapest.
16. LEE, Y. P., *Biochim. Biophys. Acta 43*, 18 (1960).
17. LOWRY, O. H., SCHULZ, D. W., PASSONNEAU V. J., *J. Biol. Chem. 239*, 1947 (1967).
18. MADDAIAH, V. T., MADSEN, N. B., *The Journal of Biological Chemistry, 241*, 3874—3881 (1966).
19. NAKAMURA, M., *J. Agr. Chem. Soc. Japan 26*, 260 (1952).
20. NAKAMURA, M., *Enzymologia 25*, 3 (1962—63).
21. SONG, P. D., *Enzymologia 28*, 313 (1965).
22. THOMA, J. A., KOSHLAND, D. E., *J. Biol. Chem. 235*, 2111 (1960).
23. WEHRLI, W. E., VERHEYDEN, D. L. M., MOFFATT, J. G., *J. Am. Chem. Soc. 86*, 1254 (1964).
24. WEIBULL, G., TISELIUS, A., *Arkiv. Kemi. Mineral. Geol. A19*, 1 (1945).

VII. METHODS APPLIED

In the present chapter, we shall deal only with the methods directly concerned with plant phosphorylase, such as the procedures for measuring activity; for determining the inorganic phosphate and glucose-1-phosphate contents (these are needed to follow the course of the reaction); for determining proteins required for establishing specific activity; for the investigation of enzyme purity; and for the preparation and purification of the donor and acceptor substrates and their derivatives.

1. Determination of the Inorganic Phosphate and Glucose-1-phosphate Contents

During the synthesis, the plant phosphorylase catalyzed enzyme reaction is followed in a relatively simple way. Enzyme activity and the initial rate of the reaction can be determined, under suitable experimental conditions, from the amount of inorganic phosphate liberated from the donor substrate (glucose-1-phosphate). In phosphorolysis, however, the case is not so simple because a great amount of inorganic phosphate is present in addition to the minute quantitites of glucose-1-phosphate formed. The calculation of exact kinetic data from either the decrease of the inorganic phosphate content or the amount of glucose-1-phosphate formed is rather questionable.

In phosphorylase research, different variants of the method based on the phosphomolybdenum complex have been employed by the majority of authors for the determination of the inorganic phosphate [49, 52, 53, 56, 64, 69, 87, 109, 156, 160], though a number of gravimetric [27, 28, 101, 130], and nephelometric [50, 79] methods, titration of the magnesium in $MgNH_4\ PO_4$ [31, 35], the strychnine [76], the uranium method [88, 133] and quite recently the polarographic determination of molybdenum [114, 139] are also available for the determination of orthophosphate.

The phosphomolybdenum methods are based on the formation of yellow phosphomolybdic acid $H_3[P(Mo_3O_{10})_4 \cdot n\ H_2O$.

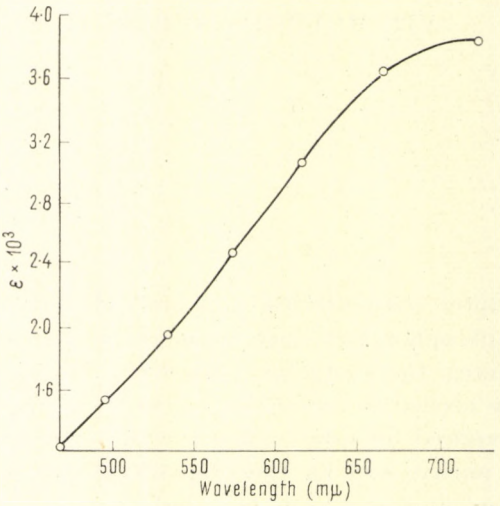


Fig. VII/1. Light absorption curve of phosphomolybdenum blue solution

However, since this reaction is not satisfactorily sensitive, the procedures employed are based rather on the measurement of the colour intensity of molybdenum blue formed by the reduction of phosphomolybdic acid. The reducing agents generally applied are: iron(II) salts in the presence of sulphite [15]; hydroquinone with sodium sulphite [6]; amino-naphthol-sulphonic acid and sodium bisulphite [32]; and monomethyl-*p*-amino-phenol sulphate in the presence of sodium sulphite and bisulphite [19, 134]. A method is also known where phosphomolybdic acid is reduced by stannous chloride, and the product extracted into ether [25, 131].

The colour intensity of molybdenum blue can be measured between 650–700 mμ where the maximum absorption (Fig. VII/1) of the phosphomolybdenum solution [3] exists.

Tungsten, vanadium, silicon, oxidizing agents and even strong reducing agents [3] interfere with this determination. The reduction of molybdic acid has a maximum rate at 0·2 N acid concentration. On increasing the acidity, this rate quickly diminishes. Phosphates accelerate the rate of reduction of molybdic acid, but the rate decreases with increasing acidity [7]. The rate of reduction of molybdic acid, the accelerating effect of phosphate and the end points of these reactions are all dependent on the molybdate concentration and on the concentration and nature of the applied reducing agents. Reduction does not go to completion because the catalyst is inactivated by the reduction products. Thus, the determination of

the exact end point and of the best reaction conditions are of great importance.

In the investigations referring to plant phosphorylase, the methods of Fiske–Subbarow, King, Lowry, Lopez and Butenko–Kirsch have been employed by various authors for the determination of inorganic phosphate.

(i) Method of Fiske and Subbarow [32]

This method has found the most general application in enzyme research.

Its principle is: phosphate is converted into phosphomolybdic acid in 0·5 M sulphuric acid, and subsequently reduced by 1-amino-2-naphthol-4-sulphonic acid.

Reagents:

A. 5 M H_2SO_4
B. 2·5% ammonium molybdate
C. Reducing solution prepared as follows.
D. 0·5 g recrystallized 1-amino-2-naphthol-4-sulphonic acid is dissolved in 15% $NaHSO_3$, and the solution diluted to 195 ml with 15% $NaHSO_3$. 5 ml of 20% Na_2SO_3 is added. The reagent is kept a refrigerator, in the dark (storable for about a week).

Procedure:

To a sample containing 0·1–12 μM phosphate, add 0·4 ml of solution *A*, 0·8 ml of solution *B* and 0·4 ml of solution *C*, mix and adjust the volume with distilled water to 10 ml. The solution is allowed to stand at room temperature for 10 minutes. Measure colour intensity at 660 mμ with a photometer. Accuracy of measurement: $\pm$0·005 μM.

Prepare the standard solution required to plot the calibration curve as follows. Dissolve 1·3613 g of analytically pure K_2HPO_4 in 1000 ml of distilled water. Use a 1 : 10 dilution of this stock solution (1 ml = 1 μM of phosphorous). After the addition of a few drops of chloroform, the stock solution can be stored in a refrigerator for longer periods.

In the case of biological substances, proteins are removed by a treatment with trichloroacetic acid (maximum final concentration 5–10%) or with perchloric acid (maximum final concentration 8%) [111]. When extremely labile organic phosphorus compounds are investigated, the procedure must be carried out very quickly, under cooling.

Determination of Total Phosphorus Content

Add 1 ml of 5 N sulphuric acid to the sample, and evaporate over an open flame. When the sample has become brown, allow to cool, add one drop of 2 N nitric acid, and heat continously until white fumes appear. If the residual liquid does not become

coulourless. repeat the last procedure. Excess nitric acid must be completely removed because its presence interferes with the subsequent colour development. The colourless sample is cooled, 1 ml water added, and the phosphorus content determined in the above way.

(ii) Method of Butenko and Kirsch [19]

This is a modification of the method of Fiske and Subbarow [32]. With the use of their suggested reducing agent and acid concentrations, Beer's law is obeyed over a wider phosphomolybdenum blue concentration range, (0·1–2·5 mg P_2O_5 in 50 ml). Iron, ammonium, calcium and potassium salts and even citric acid and silica do not interfere with the determination.

Reagents:

A. 5 N H_2SO_4
B. 2·5% ammonium molybdate solution
C. reducing solution prepared by dissolving 1 g metol (methylaminophenol · H_2SO_4), 20 g $Na_2SO_3 \cdot 7H_2O$ and 15 g $NaHSO_3$ in 500 ml distilled water
D. 2 N sodium acetate solution.

Procedure:

Add 1·5 ml of solution *A* to the solution to be analysed, which may contain not more than 0·15 mg of phosphorus. Mix, add 5 ml of solution *B* and 2·5 ml of solution *C*, mix again and allow the mixture thoroughly, to stand for 10 minutes at room temperature. After this adjust the volume with solution *D* to 25 ml, and measure the colour intensity at 660 mμ.

For the evaluation, a calibration curve must be plotted with a standard phosphorus solution prepared by dissolving 0·4393 g KH_2PO_4 in 1000 ml distilled water. Each ml of this solution contains 0·1 mg P.

(iii) Method of King [75]

This procedure is similar to that of Fiske and Subbarow, with the difference that the conversion of phosphorus into phosphomolybdenic acid is carried out in the presence of perchloric acid.

Reagents:

A. 60% perchloric acid
B. 5% ammonium molybdate solution
C. reducing solution: 0·5 g 1-amino-2-naphtholsulphonic acid, 30 g $NaHSO_3$ and 6 g Na_2SO_3 dissolved in 250 ml of distilled water. The solution is filtered and stored in a well-closed brown bottle. The reagent can be stored for about a week.

Procedure:

To a sample containing 0·1–10 μM of phosphate, add 1·2 ml of solution *A*, 1 ml of solution *B* and 0·5 ml of solution *C*. Adjust the volume with distilled water to 15 ml

mix thoroughly, allow to stand for 5 minutes, and measure the colour intensity with a photometer.

This method has been employed, with slight modifications, by Allen [1], Kuby *et al.* [82] and Nakamura [108] for the determination of the phosphorus content of organic compounds.

(iv) Method of Lowry and Lopez [95]

According to this method, phosphate is converted into phosphomolybdate in an acetate buffer at pH 4·0, and subsequently the complex formed reduced by ascorbic acid.

Reagents:

A. Acetate buffer of pH 4·0 (0·1 M acetic acid, 0·025 M sodium acetate)
B. 1% ascorbic acid solution
C. 1% ammonium molybdate solution in 0·025 M H_2SO_4.

Procedure:

Adjust the inorganic phosphate content of the sample to be analyzed to 0·015–0·1 mM (0·015–0·1 μM/ml) with acetate buffer of pH 4·0, add 0·1 ml of solution *B* and 0·1 ml of solution *C*. Readings are taken at 5 and 10 minutes after the addition of molybdate at 700mμ. (Any wavelength between 650 and 950 mμ is suitable). The two values observed are extrapolated to zero time. Simultaneous readings are made on a standard solution (containing 0·05 μmole P/ml) and a blank. The compositions of standard and blank should be as close as possible to that of the unknown.

The phosphomolybdenum blue colour reaction can be accelerated by raising the molybdate concentration (max. 1·5%) and the ascorbic acid concentration (the maximum final concentration may be 0·2–0·3%). The final pH of the reaction mixture may be varied between 3·5 and 4·2.

In the case of biological systems, proteins must be removed under given conditions prior to the determination. When non-labile phosphorus compounds are present, cold 5% trichloroacetic acid (0·3 N) and 3% perchloric acid (0·3 N) can be employed to remove proteins. In the presence of labile phosphorus compounds, a saturated solution of ammonium sulphate in a pH 4·0 acetate buffer can be applied. However, in this case, prior to the inorganic phosphate determination, the sample must be diluted at least five times with solution.

(v) Determination of the Glucose-1-phosphate and Inorganic Phosphate in the Presence of Each Other

Two methods are available for following enzyme phosphorolysis reactions: separation of the glucose-1-phosphate formed from the large

amounts of inorganic phosphate present, or measurement of the glucose-1--phosphate by an enzyme procedure.

Inorganic phosphate can be removed from the reaction mixture by several methods.

Calcium hydroxide was employed by Fiske and Subbarow [33] for the separation of inorganic phosphate. The neutralized sample was treated with one fifth its volume of 10% calcium chloride solution saturated with calcium hydroxide. The precipitate was allowed to stand for 10 minutes at room temperature, centrifuged, and washed with water containing a small volume of the calcium chloride reagent. Though the amount of organic phosphate can theoretically be determined in the supernatant liquid, the presence of the great quantities of salt may cause appreciable errors in the determination. Hence, organic phosphates formed in the enzyme reaction cannot be exactly measured in this way. The inorganic phosphates contents, however, can be precisely determined after dissolving the washed precipitate in hydrochloric acid.

Lipmann and Tuttle [94] applied calcium chloride and ethanol to remove inorganic phosphates.

Reagents:

A. 3·3% solution of anhydrous calcium chloride in 33% ethanol
B. Neutralization solution: A mixture of 100 ml of concentrated ammonia and 40 m of glacial acetic acid is made up to 1000 ml with water, and 100 ml of 0·4 M sodium bicarbonate solution is added.

Procedure:

The solution containing 1–4 μmole of phosphate is cooled and one drop of thymol blue added. If necessary the solution is neutralized and adjusted to pH 8 (to a greyish blue colour) with solution *B*. 2·5 ml of solution *A* is added, (the indicator turns yellow) and the contents are well mixed. When great amounts of precipitate are present, and particularly when the quantity of phosphate is very small, it is advisable to add dropwise 0·15 ml of 0·04 M sodium bicarbonate solution. The precipitate is quickly centrifuged, washed with 2 ml of solution *A*, again centrifuged and dissolved in hydrochloric acid. The inorganic phosphate content of this solution is determined quantitatively. As already mentioned, the determination of the organic phosphates in the supernatant solution is not advisable in view of the large errors expected.

Magnesia mixture can be employed for the precipitation of inorganic phosphates [89]. An excess of magnesia mixture is added to the sample neutralized with 10% ammonia solution. (The reagent is prepared as follows; 5·5 g of $MgCl_2 \cdot 6\ H_2O$ and 10 g NH_4Cl are dissolved in 50 ml of water, 10 ml of 15 N NH_4OH added, the mixture diluted to 100 ml with distilled water and filtered if necessary. One ml of this solution corresponds theoretically to 270 μmoles of phosphoric acid.) The solution is kept for 2–3 hours in a refrigerator, filtered, the filtrate neutralized with hydrochloric acid, and the organic phosphate determined as above.

(vi) Rapid Micromethod for the Measurement of Glucose-1-phosphate

Though the procedures based on the use of calcium chloride and calcium hydroxide [33], of calcium chloride and ethanol [94] and of magnesia mixture [89] are suitable for the separation of orthophosphate from organic phosphates, they have the common drawback that their sensitivity is rather low and that the high salt concentration (required for the formation of the precipitate) interferes with the determination of organic phosphates in the supernatant liquid.

Therefore, the observation of Sugino and Miyoshi [140] that at pH 1·0 inorganic phosphate can be precipitated with ammonium molybdate and triethylamine hydrochloride, was of great importance.

Holló *et al.* [57] used this reaction for the quantitative determination of the glucose-1-phosphate, formed in phosphorolysis.

The optimum ratio of reagents for the formation of the precipitate is: 1 mole of inorganic phosphate, 2 moles of ammonium molybdate and 3 moles of triethylamine hydrochloride.

Procedure:

To 1·0 ml of reaction mixture, 0·4 ml 6 N sulphuric acid is added (to adjust the pH and to suspend enzyme activity), and ammonium molybdate and triethylamine hydrochloride in the above proportions. The precipitate formed is centrifuged and 2×2 ml aliquots are pipetted from the supernatant solution. One of these aliquots serves as a blank test while the other is treated with 1·5 ml of 6 N sulphuric acid for 10 minutes at 100 °C in order to liberate the glucose-1-phosphate phosphate by hydrolysis. Subsequently, the phosphorus contents of the untreated (blank) and hydrolyzed tests are determined by photometry, using the method of Butenko and Kirsch [19].

(vii) Enzymatic Determination of the Glucose-1-phosphate

The most modern method [123] for the determination of glucose-1-phosphate formed during phosphorolysis is the enzymatic procedure of Keilich and Werner [71]. The principle of this method is as follows. Glucose-1-phosphate formed from soluble starch is converted with phosphoglucomutase into glucose-6-phosphate and then with glucose-6-phosphate dehydrogenase into gluconic acid-6-phosphate in the presence of nicotinamide-adenine-dinucleotide phosphate (NADP). NADP is present at the end of the reactions in a reduced form which shows maximum absorption at 366 mμ. The amount of reduced NADP which is ultimately equivalent to that of the glucose-1-phosphate formed can be determined by spectrophotometry.

Reagents:

Standard solution
0·1 ml 0·5 M $MgSO_4$
1·0 ml ethylenediaminotetraacetic acid (EDTA) solution (10 mg/ml)
1·0 ml glucose-1,6-diphosphate (1·2 mg of the K-salt/ml)
1·0 ml 0·5 M phosphate buffer (pH 7·0, according to Sörensen)
1·0 ml NADP solution (10 mg/ml)
1·0 ml of a solution of Zulkowsky's soluble starch (20 mg/ml)

Procedure:

Pipette, into a 3-ml, 1 cm thick cell 1 ml of standard solution, 0·02 ml of phosphoglucomutase (Boehringer) (0·02 mg), 0·02 ml of glucose-6-phosphate-dehydrogenase (Boehringer) (0·01 mg) and 1·95 ml of triethanolamine hydrochloride solution (pH 7·0, 0·05 M); incubate the mixture for 5 minutes at 25 °C, add 0·1 ml of a phosphorylase solution adequately diluted and measure the increase of the extinction continuously at 366 mμ.

2. *Methods for the Measurement of Activity*

In the case of α-glucan-phosphorylase, activity is measured by all author in a similar way.

The enzyme catalyzes a reversible bimolecular reaction in which the D-glucosyl radical of the donor substrate is transferred to the non-reducing end of the acceptor substrate [11, 20, 23, 52]. Then, in the later course of the reaction, further D-glucosyl radicals are linked to the acceptor in several consecutive steps. Meanwhile, inorganic phosphate radicals are liberated:

$$G\text{-}1\text{-}P + G\text{-}(G_n) - G \rightleftharpoons G - G(G_n) - G + P_{inorg}$$

The activity of phosphorylase is determined directly from the amount of inorganic phosphate liberated from glucose-1-phosphate during the synthesis, under conditions which ensure the occurrence of a first order reaction.

The various methods described differ from each other only in the experimental conditions (such as donor substrate concentration, acceptor substrate concentration, nature of donor and acceptor substrate, pH, reaction period, temperature, choice of the method for determining the inorganic phosphate liberated (cf. Section VII.1). However, this causes great difficulties in the comparison of the individual methods, since, under the various arbitrarily chosen conditions the reaction rates and the activity units calculated from them differ and are not evaluable.

(i) The Cori Method of Activity Measurement

Cori *et al.* [23] were the first to study the method of measurement of the activity of α-glucan-phosphorylase.

They chose the measurement conditions to ensure a first-order reaction.

In the case of crude or slightly purified enzyme solutions, activity was determined at pH 6·0 (where the effect of phosphoglucomutase contamination is a minimum) while for highly purified enzyme preparations a pH of 6·8 was applied.

Reagents:

A. 4% glycogen solution
B. 0·07 M sodium glycerophosphate (pH 6·0 or 6·8)
C. 0·06 M cysteine hydrochloride (freshly neutralized to pH 6·0 or 6·8)
D. 0·064 M crystalline dipotassium glucose-1-phosphate dihydrate (pH 6·0 or 6·8)
E. 0·064 M crystalline dipotassium glucose-1-phosphate dihydrate and 0·004 M adenosine-5-phosphate
F. 0·004 M adenosine-5-phosphate.

The enzyme solution is diluted, if necessary, with a 1 : 1 mixture of solutions (b) and (c).

Procedure [64]:

Dilute 0·1 ml enzyme with the suitable cysteine glycerophosphate buffer (not more than 25 times). To $2\times0{\cdot}4$ ml of the diluted enzyme solution, add 0·2 ml of solution *A* and pre-incubate at 30°C for 20 minutes. Then add 0·2 ml of reagent *E* to one of the samples and 0·2 ml of reagent *F* to the other one (in both cases heat the reagents to 30°C prior to addition). Allow the reaction mixtures to stand for 5, 10 and 15 minutes and pipette 0·2 ml aliquots into 7 ml of dilute sulfuric acid (10 ml 5 N H_2SO_4 + + 690 ml H_2O) in a Klett tube. (The acid stops the enzymatic reaction and the glucose-1-phosphate is not hydrolyzed to a measurable degree in 60 minutes at this acid concentration).

The amount of inorganic phosphate liberated during the enzyme reaction was determined by the Fiske–Subbarow method.

Enzyme units were calculated from the equation

$$K = \frac{1}{t} \log \frac{x_e}{x_e - x}$$

where K is the first-order rate constant, x_e the equilibrium percentage of inorganic phosphate, x the percentage of inorganic phosphate converted in time t minutes.

The value of x_e is pH dependent, being 78% at pH 6·8 and 86% at pH 6·0.

The enzyme unit (arbitrarily chosen by the authors mentioned) is 1000 times the calculated equilibrium constant K, multiplied by the required dilution factor in order to obtain enzyme units expressed per millilitre of the original enzyme solution.

The specific activity is equal to the total activity present in one millilitre of the original enzyme solution, divided by the protein content in milligrams.

(ii) Method of Green and Stumpf [49]

The first method for the measurement of the activity of plant phosphorylase was published by Green and Stumpf.

Reagents:

0·1 M glucose-1-phosphate (37·22 g/litre glucose-1-phosphate-$K_2 \cdot 2\ H_2O$) prepared by the method of McCready and Hassid [99]
0·5 M citric acid–sodium hydroxide buffer pH 6·0
5% solution of trichloroacetic acid.
5% solution of soluble starch

Procedure:

Prepare a reaction mixture from 0·2 ml of starch solution, 0·5 ml of buffer solution and a suitable amount of enzyme solution. If necessary, adjust the volume to 2·5 ml with distilled water, and incubate at 35°C. After equilibrium is attained, add 1·0 ml of glucose-1-phosphate solution, allow the mixture to stand for 10 minutes, and stop the enzyme reaction by adding 5 ml of trichloroacetic acid. Remove the precipitated protein by centrifugation, and determine the inorganic phosphate in the supernatant solution by the Allen method [1]. Use as blank a reaction mixture prepared from the reagents, but replacing the enzyme solution by an enzyme solution previously denatured by trichloroacetic acid.

Unit of activity: the amount of enzyme which is capable of liberating 0·1 mg of inorganic phosphate in 3 minutes under the above conditions.

(iii) Activity Measurement According to Weibull and Tiselius [156]

For the measurement of activity, these authors used the method of Green and Stumpf [49] with certain modifications. They suggested the following *procedure.*

Reaction temperature 38°C. The sample contains 0·005–0·05 millimoles of glucose-1-phosphate dissolved in 0·5 M maleic acid buffer solution, 15 mg of Zulkowsky's starch and about 0·9 ml of enzyme solution. The pH of the reaction mixture is 6·0 and changes during the liberation of the inorganic phosphate by not more than 0·02 units.

The reaction mixture (total volume 1·75 ml) is pipetted into a 25 ml volumetric flask which is thermostated.

The precipitation of proteins by trichloroacetic acid is not applied to stop the enzymatic reaction because on the one hand the possible hydrolysis of the residual glucose-1-phosphate must be prevented in the opinion of the authors, and, on the other hand, they observed that significant amounts of inorganic phosphate are absorbed by the precipitated protein (this was proved by adding known amounts of phosphate to the enzyme solution, and analyzing the reaction mixture after the precipitation of proteins).

Instead of denaturing by acid, the enzyme reaction was stopped by adding 2 ml of 0·15 N KOH. Under such conditions, glucose-1-phosphate remains stable. In these experiments, the enzyme solutions were so dilute that in the determination of phosphorus (by the King method) [75], the addition of molybdate caused only a slight opalescence. The resulting error must be corrected by the blank tests. The reaction time was about 10—60 minutes.

As unit phosphorylase activity, the first order rate constant is applied. This is directly proportional to enzyme concentration and is constant during the chosen reaction period.

(iv) Method of Hidy and Day for the Measurement of Activity [53]

Procedure:

Transfer 0·5 ml of enzyme solution, 0·5 ml of water, 1 ml of 0·5 M citrate buffer (pH 6·2) and 1 ml of a 2% solution of Zulkowsky's starch to a 50 ml volumetric flask, thermostat the mixture, and start the enzyme reaction by adding 1 ml of 0·1 M glucose-1-phosphate solution. After a reaction period of 10 minutes, denature the enzyme with 2·5 ml of a 10% solution of trichloroacetic acid, make up to 50 ml with distilled water, and determine the inorganic phosphate in an aliquot by the Fiske–Subbarow method [32].

Unit enzyme activity is the amount of enzyme which is capable of liberating 0·1 mg of inorganic phosphate under the above conditions.

(v) Method of Whelan and Bailey for the Determination of Enzyme Activity [161]

Two parallel measurements are carried out in this *procedure.*

1 ml of 0·2 M citrate buffer of pH 7·0, 0·5 ml of a 1% solution of soluble starch and 0·5 ml of distilled water are placed in each of two test tubes, and the reaction mixtures thermostated at 35°C. One of the mixtures is treated with 1 ml of 0·1 M glucose-1-phosphate solution preheated to 35°C, the mixture allowed to stand for 12 minutes, and a 1 ml sample withdrawn. The enzyme proteins are denatured by the addition of perchloric acid (10 ml of 12% perchloric acid), and the inorganic phosphate determined by the Allen method [1]. Similarly 1 ml of 0·1 M glucose-1-phosphate is added to the other sample, the mixture shaken, and the inorganic phosphate determined in a 1 ml aliquot serving as blank.

Unit enzyme activity is the amount of enzyme which is capable of liberating 0·1 mg of inorganic phosphate from glucose-1-phosphate in 3 min, under the above conditions.

(vi) The Lee Method [87] for the Measurement of Enzyme Activity

Reagents:

A. 1·0 M citrate buffer of pH 6·3
B. 0·1 M dipotassium glucose-1-phosphate dihydrate
C. 1·52% solution of amylopectin
D. 5% trichloroacetic acid

Procedure:

Incubate a mixture of 0·5 ml of solution *C*, 0·1 ml of solution *A*, 0·1 ml of solution *B* and 0·3 ml of a suitably purified enzyme solution at 30°C for 5 minutes. The pH of the reaction mixture is 6·3. The reaction is stopped by adding 0·5 ml of solution *D*, then the pH adjusted to about 4 by adding 2 ml of a 0·1 M sodium acetate solution.

The inorganic phosphorus formed is determined by the Lowry and Lopez method [95].

The above reaction mixture is employed as a blank, with the difference that glucose-1-phosphate is added to the reaction mixture after the denaturing of the enzyme by trichloroacetic acid. Under these conditions, the reaction is first order with respect to glucose-1-phosphate.

Unit enzyme, just as in the definition of Cori, is 1000 times the rate constant (k) of the first-order reaction.

To obtain the specific activity (expressed as enzyme units per mg protein), the protein content of the crude enzyme solution was determined by the biuret method of Gornall [48], while in the case of purified enzyme fractions a spectrophotometric technique was employed (Beckman spectrophotometer, 1 cm cells, at 280 mμ, in solutions of the concentration order mg/ml; $I_0/I = 1{\cdot}29$).

Holló *et al.* [56] similarly applied the Lee method, with slight modifications, for the determination of enzyme activity. At the end of the reaction, instead of precipitation by trichloroacetic acid, denaturing is effected by a 5-minute heat treatment in a 100°C water bath to inactivate the enzyme (rather than trichloroacetic acid precipitation), and the Fiske–Subbarow method modified by Butenko and Kirsch is used for the determination of the inorganic phosphate liberated [19].

vii) Method of Kamogawa et al. *[69] for the Measurement of Activity*

The procedure is as follows. A reaction mixture containing 0·2 ml of 0·5 M citrate buffer of pH 6·0, 0·2 ml of an 5% solution of soluble starch, 0·05 ml of a suitably diluted enzyme solution (in a buffer of 0·005 M tris-HCl of pH 7·5), 0·1 ml of glucose-1-phosphate (0·1 M) and 0·45 ml of distilled water is incubated at 30 °C for 10 minutes, (this time is measured after the addition of glucose-1-phosphate). The reaction is then stopped by adding 0·5 ml of 5% trichloroacetic acid. The protein precipitate is removed by centrifuging. The liberated inorganic phosphate in the supernatant solution is determined by the Fiske–Subbarow method [32].

Unit enzyme activity is the amount of enzyme capable of liberating μmole of inorganic phosphorus (P_i) in 1 minute under the above conditions. Protein content is determined by the Lowry *et al.* method [96].

(viii) Method of Keilich and Werner [71]

Keilich and Werner [71] measure activity units from the amount of glucose-1-phosphate formed from soluble starch during phosphorolysis (the procedure is given in detail in Section VII.1 *(vii)*.

The amount of glucose-1-phosphate can be calculated from the changes

in extinction measured at 366 mμ (E) from the relationship

$$\frac{3 \times \Delta E/\text{min}}{3 \cdot 28} = \mu\text{M of glucose-1-phosphate/3 ml solution.}$$

By their definition, unit phosphorylase activity is the amount of enzyme capable of liberating 1 μM of glucose-1-phosphate in 1 minute, under defined conditions (pH 7·0; 25°C; $4{\cdot}5 \times 10^{-3}$ M anhydroglucose; $1{\cdot}66 \times 10^{-2}$ M phosphate).

3. Determination of Protein Content

The exact knowledge of the protein content of the enzyme is indispensable for the determination of specific activity and, during the preparation of the enzyme, of the degree of purification of the enzyme. In general, the protein content of crude enzyme preparations is determined by the biuret method [48], by the Folin method [37] or by the ninhydrin method [84, 106] while for purified enzyme preparations the ultraviolet absorption method is applied [155]. In any case, a calibration curve must be plotted with a standard sample or the total nitrogen content must be established by the Kjeldahl method.

(i) Biuret Method

One of the methods for the determination of the protein content in crude extracts is that of Gornwall *et al.* [48], essentially a modification of the earlier methods of Robinson and Hogden [128] and Weichselbaum [157].

This method is based on the principle that in alkaline medium, a purple complex is formed by compounds containing two or more peptide bonds in the presence of a copper salt.

Biuret reagent:

Dissolve 1·5 g of $CuSO_4 \cdot 5\ H_2O$ and 6·0 g of Rochelle salt ($NaKC_4H_4O_6 \cdot 4\ H_2O$) in 500 ml of distilled water; add, with continuous stirring, 300 ml of 10% sodium hydroxide solution, and adjust the volume to 1000 ml with distilled water. This reagent keeps for an unlimited time in a paraffin-coated bottle. (The eventual formation of sediments can be prevented by the addition of 0·1% potassium iodide solution)

Procedure:

1 ml of the protein solution (of a concentration of 1–10 mg/ml of protein) is added, with vigorous stirring, to 4·0 ml of biuret reagent, the mixture allowed to stand 30 minutes at room temperature (20–25°C), and then subjected to photometry at 540–560 mμ. A mixture of 4 ml of biuret reagent and 1 ml of distilled water or 1 ml of an adequate buffer mixture serves as blank.

The protein concentration of the sample is determined from using a calibration curve, this is generally plotted using pure serum protein the nitrogen content of which has been previously determined by the Kjeldahl method. Great amounts of lipids interfere with this determination. The method cannot be used in the presence of ammonium salts, though this interfering effect can be eliminated by the technique of Robinson and Hogden [128].

(ii) Determination of Protein Content by the Folin–Ciocalteu Reagent [37]

This method has been published by Lowry *et al.* [96]. The final colour is due, on one hand, to the biuret reaction and, on the other, to the reaction between the phosphomolybdenum-phosphotungstate reagent and the tyrosine and tryptophane present in the enzyme protein.

Reagents:

A. a 2% solution of sodium carbonate in 0·1 N sodium hydroxide
B. a 0·5% solution of $CuSO_4 \cdot 5\ H_2O$ in 1% sodium tartrate solution
C. an alkaline copper solution prepared from 50 ml of solution *A* and 1 ml of solution *B* (it can be used only on the day of preparation)
D. carbonate-containing copper solution, prepared from 50 ml of 2% sodium carbonate solution and 1 ml of solution *B* (it can be used only on the day of preparation)
E. Folin–Ciocalteu reagent [37], diluted to a concentration of 1 N with respect to acid.

Preparation of the Folin Reagent:

Dissolve 100 g of $Na_2WO_4 \cdot 2\ H_2O$ and 25 g of $Na_2MoO_4 \cdot 2\ H_2O$ in 700 ml of distilled water, add 50 ml of 85% phosphoric acid and 100 ml of concentrated hydrochloric acid. Reflux for 10 hours, add a solution of 150 g lithium sulphate in 50 ml of distilled water and a few drops of bromine water, and boil for 15 minutes (in order to remove excess bromine). Cool, adjust the volume to 1000 ml with distilled water and filter. Determine the acid content of the reagent by alkalimetric titration in the presence of phenolphthalein as indicator.

Standard protein: human serum in a 100–1000-fold dilution (about 70–700 μg/ml). The nitrogen content must be checked by the Kjeldahl method.

Procedure:

To a 0·2 ml sample (which may contain 5–100 μg of protein), add 1 ml of reagent *C*, shake the mixture thoroughly, and allow it to stand for 10 minutes. Add 0·1 ml of diluted Folin reagent, allow to stand for 30 minutes, and subject the mixture to photometry at 750 mμ. In the case of higher nitrogen contents, photometry must be carried out at 500 mμ.

In the case of poorly soluble proteins (particularly after precipitation with trichloroacetic acid or perchloric acid), the protein precipitate (5–100 μg) must be allowed to stand in 0·1 ml 1 N sodium hydroxide (for at least half an hour), 1 ml of solution *D* is added (without alkali), the mixture is allowed to stand for 10 minutes, and treated with 0·1 ml of diluted Folin reagent.

In all cases, a calibration curve must be plotted, and the nitrogen content determined by the Kjeldahl method. Other phenol derivatives, besides tryp-

tophane and tyrosine, and guanine and xanthine also, react with the Folin reagent to yield coloured products. The colour formation is not disturbed by the presence of inorganic salts (generally up to a final concentration of 0·5%), of 5% ethanol, 5% ether and 0·5% acetone (final concentrations). However, ammoniumsulphate (in concentrations over 0·15%), glycine ($>$ 0·5%) and hydrazine ($>$ 0·5%) strongly interfere with the colour reaction.

This method offers the advantage of being 10–12 times more sensitive than the spectrophotometric measurement carried out at 280 mμ, and 100 times more sensitive than the biuret method.

Its disadvantage is that the colour formation is markedly different with the various proteins, and that it is not strictly proportional to the protein concentration.

(iii) Protein Determination by the Ninhydrin Method [67, 84, 106]

The principle of this method is the reaction of ninhydrin with the terminal α-amino groups of proteins and with the ε-amino group of lysine, resulting in colour formation.

Reagents:

A. 0·2 M citrate buffer of pH 5·0, prepared as follows. Dissolve 2·101 g of citric acid monohydrate in 20 ml of 1 N sodium hydroxide, make up the volume to 50 ml with distilled water. Prior to use, dilute the buffer with distilled water in a ratio of 1 : 1.
B. Methylcellosolve (ethyleneglycol monomethyl ether)
C a 1 : 1 mixture of n-propanol and distilled water
D. Ninhydrin reagent prepared as follows. Dissolve 40 mg of $SnCl \cdot 2\ H_2O$ in 25 ml of citrate buffer, and add a solution of 0·4 mg of ninhydrin in 12·5 ml methylcellosolve.

Procedure:

Transfer 0·1 ml of protein solution (containing 1–20 μg of nitrogen) to a glass-stoppered bottle, add 0·5 ml of ninhydrin reagent, mix, and keep the sealed bottle for 20 minutes in boiling water. Add 2 ml of reagent C, mix again, and centrifuge. Subject the supernatant solution to photometry at 570 mμ. Use a mixture of 0·1 ml of distilled water with the above reagents as blank.

For the evaluation, a calibration curve must be plotted with a known amino acid or protein.

(iv) Determination of Protein Content by ultraviolet Absorption

Most proteins show an absorption peak in the ultraviolet. This is attributed mainly to the tyrosine–tryptophane content. In the opinion of Warburg and Christian [155], the tyrosine–tryptophane content varies in

the different enzymes within rather narrow limits. Thus, the absorption peak at 280 mμ lends itself to the quick and precise determination of the enzyme protein concentration.

The only disadvantage of this method is that nucleic acids, similarly present in enzyme preparations, also have a strong ultraviolet absorption at 280 mμ, (though the peak maximum appears at 260 mμ).

In the method of Warburg and Christian [155], the optical density of the suitably diluted protein solution is measured at 280 and 260 mμ, then the R value is established from the quotient of the two data. The constant F relating to R is determined by means of the Table prepared by them for various concentrations of protein and nucleic acid

$$F = \frac{2.303}{\beta_{280}} \times \frac{\%\text{protein}}{100}$$

and the value obtained is substituted into the equation:

$$\text{protein concentration (mg/ml)} = F \times \frac{1}{l} \times D_{280}$$

where l is the cell length in cm, and D the optical density.

The method leads to very large errors at nucleic acid concentrations over 20%.

According to Kalckar [68], the protein content of enzymes can be determined by means of the following equation:

$$\text{protein content (mg/ml)} = 1{\cdot}45\ D_{280} - 0{\cdot}74\ D_{260}$$

where D_{280} and D_{260} are the optical densities of the enzyme solution measured at 280 and 260 mμ, respectively.

Large errors may arise from the use of this method because the various proteins and nucleic acids do not show the same absorption, and because other substances also (such as purine, pyrimidine nucleotides) have appreciable absorptions in the range 260–280 mμ.

However, the ultraviolet absorption method is very widespread, mainly for the determination of the protein content of purified enzyme solutions. In the case of pure enzyme solutions, the concentration of enzyme protein can be established from the total nitrogen content determined by the Kjeldahl method. Thus, on measuring the molar absorption values, the concentration of the enzyme protein of an identical type can be measured quickly and in a small volume, with the use of the ultraviolet absorption values (at 280 mμ). Another advantage offered by this method is that inorganic salts (mainly ammonium sulphate) do not interfere with the measurement.

4. Investigation of the Purity of Enzymes

The use of well-defined, preferably crystalline enzymes is indispensable in research in the kinetics and the mechanisms of enzymatic synthesis and phosphorolysis and in the more and more intensive investigations of the tertiary structure and functional groups of enzymes. This is however insufficient. It is also necessary to develop new analytical methods which are capable of detecting enzyme contaminants present in very minute amounts. In the case of phosphorylase, these contaminants may be other enzymes or various oligo- or polysaccharides as primary contaminants.

In phosphorylase the most detrimental enzyme contaminant, which can be removed only by very difficult procedures, is amylase. On attempting to prove the presence of amylase in phosphorylase by the conventional methods, very great difficulties are encountered.

(i) Investigation of Amylase Contamination

The detection of α-amylase as contaminant is relatively the simplest *procedure.*

5 ml of an enzyme solution is mixed with 2 ml of a 0·5% solution of soluble starch (Merck), and 0·01 N calcium chloride; 3 drops of toluene are added and the solution incubated at 25°C for 48 hours. Then 0·01 N iodine solution is added dropwise. If the solution turns deep blue, the preparation is free of α-amylase. If however the solution turns yellow or a transitional purple, red, or brown, α-amylase is present as contaminant [148].

The viscosimetric method of Husemann *et al.* [62] has a sensitivity exceeding that of the previously described procedure. According to Husemann *et al.*, the aqueous solution of hydroxyethylamylose undergoes retrogradation only very slowly, and thus, the solution retains its initial viscosity for a long time. Consequently, on adding an enzyme sample to a solution of hydroxyethylamylose, extremely small amounts of amylase can be detected in a very simple way because minute degrees of hydrolysis are very sensitively reflected in changes in viscosity.

The Husemann method has been employed by Holló *et al.* [56] for the detection of contaminants in enzymes as follows.

In the first step, hydroxyethylamylose was prepared [63].

Procedure:

8·1 g of amylose was dissolved in 225 ml of 1 N sodium hydroxide, with continuous stirring under nitrogen. Then 7·9 g of ethylene oxide was added to the strongly viscous solution during 2 hours, with continuous stirring, still under a nitrogen atmosphere. The reaction mixture was allowed to stand for 12 hours, subjected to dialysis against distilled water, the neutral solution evaporated in vacuo, and the residue dried with acetone (degree of substitution: 0·15).

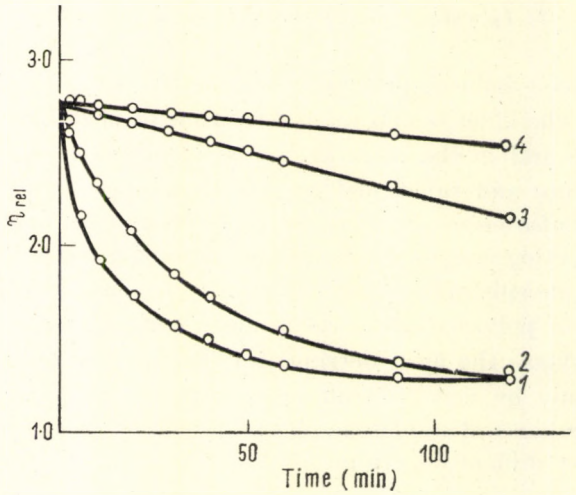


Fig. VII/2. Changes of values η_{rel} in potato juice and in potato phosphorylase solutions of various purity against time [58]. *1:* Potato juice; *2:* prefractionated enzyme with $(NH_4)_2SO_4$; *3:* purified enzyme; *4:* blank

The second step was the determination of amylase activity by viscosimetry.

A suitable amount of enzyme solution (i.e. 0·2 ml of potato juice, or of the enzyme fractionated with ammonium sulphate, or 1·0 ml of the purified fractions) was added to 4 ml of a 2% solution of hydroxyethylamylose. If necessary, the volume of the reaction mixture was adjusted with distilled water to 5·0 ml. After thermostating at 25°C, the decrease of viscosity was measured in the samples by means of an Ostwald viscosimeter.

As blank, a reaction mixture prepared under similar conditions was used, but before use the enzyme solution was inactivated by keeping it in a 100°C water bath for 10 minutes. Eventual protein precipitates were removed by centrifuging. Unit activity of amylase is:

$$\frac{(\eta_0 - \eta_{60})_{sample}}{(\eta_0 - \eta_{60})_{blank}} - 1$$

where η_{60} is the relative viscosity of the enzyme solution and of the blank after a reaction period of 60 minutes, and η_0 the same value extrapolated to zero time.

Changes in η_{rel} plotted against time for potato phosphorylase preparations of various purity are shown in Fig. VII/2. Much to our regret, this method did not prove as sensitive as expected from the use of viscosimetry, because, as the data in Fig. VII/2 indicate, the blank test disclosed no constant viscosity, either.

The problems of the above method (mainly the retrogradation of hydroxyethylamylose) are completely eliminated by using the photometric iodine titration [127].

The measurement is based on the principle that on treating amylose spirals of different degrees of polymerization with iodine, the uptake of iodine by the spirals does not take place in a simultaneous process [59]. The formation of the iodine complexes of amylose molecules of higher degrees of polymerization occurs at lower iodine concentrations, or more precisely, at lower equilibrium free iodine concentrations, than the complex formation from amyloses of lower degrees of polymerization. Thus, conclusions can be drawn regarding the distribution of degrees of polymerization of amylose molecules present in the solution from the measured extinction values at a fixed wavelength plotted against the amounts of iodine added. For this titration, an automated photometric titrator is employed, and the titration must be carried out at 650 mμ.

In order to establish the degree of contamination of phosphorylase by amylase traces, samples of various degrees of conversion have been investigated by László [86]. For the titration, the dosing syringe was filled with 0·005 N iodine in potassium iodide, then the samples were diluted so as to attain an amylose concentration of 2 mg in the 100 ml solution transferred into the measuring cell.

With the cell in the photometer, extinction values zero and infinity were adjusted (0 and 100% absorption) under continuous stirring, the addition of iodine was started, and the sigma-shaped curve was recorded automatically.

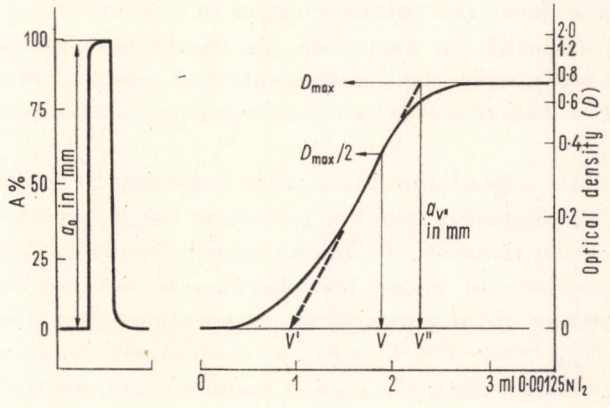


Fig. VII/3. Theoretical sigma curve for the evaluation of photometric iodine titration

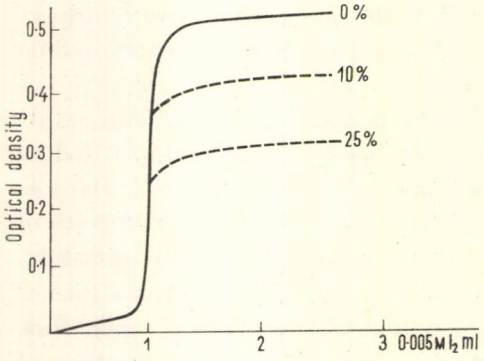


Fig. VII/4. Photometric iodine titration [86] of contaminated potato phosphorylases of different degres of conversion (prefractionated with ammonium sulphate)

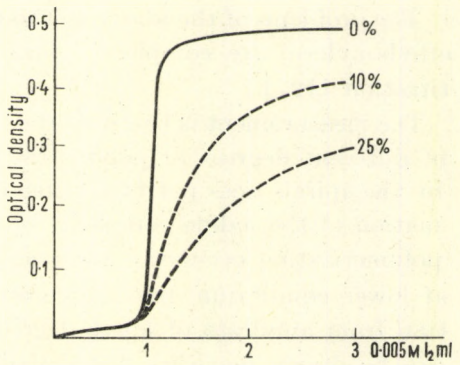


Fig. VII/5. Photometric iodine titration [86] of amylase-free potato phosphorylases of different degrees of conversion (fractionated by chromatography on a DEAE-cellulose column)

Four parameters (D_{max}, V′, V″ and V) can be read from the sigma curve (Fig. VII/3).

Of these parameters, V′ is the abscissa projection of the ascending linear section of the sigma curve, while V″ is the point of intersection of the ascending straight line and the horizontal line corresponding to D_{max}, projected onto the abscissa. Consequently, the values of V′ and V″ indicate the amounts of iodine solution required for the commencement of the intensive colouration by iodine and for saturation, respectively. V is the volume of titrant corresponding to $D_{max}/2$, and is the iodine equivalent to the semi-saturation of the investigated sample.

Figure VII/4 shows the titration curves of a potato phosphorylase preparation contaminated by α-amylase (on the basis of samples withdrawn at different times), while the titration curves of another potato phosphorylase preparation free of α-amylase contamination can be seen in Fig. VII/5 [86].

Phosphorolytic degradation takes place according to multichain mechanisms [161]. Consequently, the low molecular weight products appear only at the end of the titration. It follows clearly from Figs. VII/4 and VII/5 that the prefractionated potato phosphorylase is contaminated by amylase, because in the case of the pure enzyme preparations the values of V (corresponding to $D_{max}/2$) are the same in the samples withdrawn at different intervals (Fig. VII/5) while in the case of samples contaminated by α-amylase, V increases (Fig. VII/4); i.e. the iodine amount required for semi-saturation rises with the increase of conversion [86].

Phosphatase contamination in phosphorylase enzyme preparations can be detected by glycerophosphate [4, 109], and contamination by Q-enzyme by the measurement of the degree of branching via oxidation with periodate [55].

5. Preparation of Donor Substrate

The production of a donor substrate of high purity is indispensable for the kinetic investigation of enzyme catalyzed processes.

Two processes are available for the production of glucose-1-phosphate. Although the enzymatic method can be carried out more simply and with higher yields, it has the disadvantage that the substrate produced contains 'primer' contaminants which may interfere with the kinetic investigations. Primer-free donor substrate can be prepared by organic procedures, but these are extremely complicated, and the yields are very poor.

(i) Enzymatic Synthesis of Glucose-1-phosphate

The enzymatic methods of production are based on the following principle: the various carbohydrates undergo phosphorylation to α-D-glucose-1-phosphate by the action of orthophosphate, in the presence of the enzyme phosphorylase, according to the scheme:

$$\text{starch} + \text{orthophosphate} \overset{\text{phosphorylase}}{\rightleftharpoons} \alpha\text{-D-glucose-1-phosphate}.$$

The various procedures differ from each other in the type of carbohydrate employed (starch, glycogen etc.), in the purity of the applied enzyme (plant extract, purified potato phosphorylase, crystalline muscle phosphorylase), and in the yield of glucose-1-phosphate. In 1938, Kiessling [77] first prepared the dipotassium salt of glucose-1-phosphate from glycogen using rabbit muscle phosphorylase (yield 10–12%).

Hanes [51], Sumner and Somers [142] applied soluble starch, and bean and potato phosphorylases for the preparation of glucose-1-phosphate. The enzyme was produced as a cyanide extract of potato. The enzymatic reaction was stopped with 0·1 N iodine solution, and the residual dextrins in the reaction mixture decomposed by a 2% solution of pancreatin. Inorganic phosphate was precipitated as the barium salt. Subsequently, the barium salt of glucose-1-phosphate was precipitated from the solution by adding 95% ethanol, and the precipitate converted to the potassium salt. The yield was about 20%, and the purity of the product nearly 85%.

The method does not give higher yields when the procedure suggested by Bernfeld is applied [8]. This author used potato phosphorylase purified partially by ammonium sulphate instead of cyanide.

The best practical method so far (with fair yield and a high purity product) was published by McCready and Hassid [99]. Starch (potato, cereal, cassava or maize starch) is phosphorylated to α-D-glucose-1-phosphate which is then separated from unreacted starch, from inorganic phosphate, and from all the contaminants introduced by the potato extract. At the pH applied (6·5), equilibrium is attained (4 : 1) between the orthophosphate and the α-D-glucose-1-phosphate. Orthophosphate is precipitated as magnesium ammonium phosphate, and the pure dipotassium dihydrate salt of glucose-1-phosphate recovered from the supernatant solution by cation exchange followed by anion exchange.

Reagents:

A. Phosphate buffer (pH 6·7), prepared by dissolving 35 g (0·2 mole) of K_2HPO_4 and 27 g (0·2 mole) of KH_2PO_4 in 500 ml of distilled water,

B. Starch solution, prepared by vigorously stirring a suspension of 20 g of starch (generally soluble starch) in 500 ml of distilled water on a hot water bath until a solution is obtained, and cooling the liquid at room temperature.

C. *Preparation of the enzyme:* About 300 g peeled, washed potatoes are treated for 2 minutes with 300 ml of distilled water in a blandor. The pulp is transferred to a nylon bag, and pressed out by hand; the starch grains which separate out when the liquid stands for 5 minutes are removed. The yield is about 500 ml of a 1 : 1 potato juice.

D. *Ion exchangers:* a strongly acidic cation exchanger of high capacity (Dowex 50, Amberlite IR 112 or Permutit Q) is treated with 5% hydrochloric acid, then washed with distilled water to neutrality; and an anion exchanger of medium basicity and high capacity (Permutit A, Amberlite IR 4B or Permutit S), is treated with 5% sodium hydroxide and washed with distilled water to neutrality.

Procedure:

Throughly mix 500 ml of potato juice, 500 ml of starch solution and 500 ml of phosphate buffer, add 0·25 g of phenylmercuric nitrate, keep the reaction mixture a room temperature for 16 hours, then heat it rapidly to 95 °C (when the enzyme is inactivated, and the proteins are coagulated). After cooling, filter the solution, and add 86 g (0·4 M) $MgAc_2 \cdot 4\,H_2O$ to the filtrate. When this salt has dissolved, adjust the pH to 8·5 with a 14% solution of ammonium hydroxide. Remove the inorganic phosphate precipitate by filtration, and subject the solution to ion exchange on a Dowex 50 cation exchanger (bed volume 60 × 5 cm, flow rate 5 bed volumes per hour), and elute with 600 ml of distilled water. The initial pH of the eluate is 3·5. When glucose-1-phosphate appears, the pH of the solution decreases to 1·8. Subsequently, the solution is subjected to anion exchange on Permutit A (bed volume 45 × 2·5 cm; ow rate 10 bed volumes per hour).

After the absorption of glucose-1-phosphoric acid, the bed is washed with 2 litres of distilled water (flow rate: 10 bed volumes per hour), in order to remove starch, sugar and other contaminants. Then the α-D-glucose-1-phosphate is eluted with 5% potassium hydroxide (Table VII/1).

3 volumes of methanol are added to the first 200 ml portion of the eluate (the fractions containing glucose-1-phosphate), with slow stirring. Dipotassium α-D-glucose-1-phosphate dihydrate crystallizes spontaneously. After cooling, the ester crystals are

fired, and redissolved in 100 ml of distilled water. Prior to recrystallization, 1 ml of 1% potassium hydroxide solution and 3 volumes of methanol are added. The crystals formed are filtered and dried in vacuum over calcium chloride. Yield 41%.

Table VII/1

Elution of Glucose-1-phosphate Absorbed by Anion Exchanger Permutit A, with 5% KOH as Eluting Agent [99]

Fractions (of 50 ml)	pH	Glucose-1-phosphate in the eluate (% of the transferred amount)
1	5·7	4·2
2	5·6	57·6
3	12·0	25·8
4	12·2	4·0
5	12·6	1·0
6	13·0	0·3

(ii) Chemical Synthesis of Glucose-1-phosphate

The preparation of the donor substrate (glucose-1-phosphate) by chemical synthesis is necessary mainly for research into the detailed mechanism of the enzymatic reaction (high purity product free of primer and protein contaminants is required), and for the kinetic investigation of the inhibition reaction of the donor substrate and of the homologous derivatives.

The first chemical method for the preparation of glucose-1-phosphate was published by Cori *et al.* [21, 22]. In their process, α-D-acetobromo-glucose is reacted with trisilver phosphate, and the product consisting mainly of triester is subjected to a partial hydrolysis by acid. The monoester formed is subsequently deacetylated.

This method is rather cumbersome (operations in reduced light to follow the progress of the hydrolysis), and the yield is very poor.

The Posternak method [124] starts similarly from acetobromo glucose. However, silver diphenyl phosphate is employed for the production of the phosphate ester, then the phenyl groups are hydrogenated at room temperature and atmospheric pressure in the presence of a platinum oxide catalyst, while the acetyl groups are saponified.

The detailed *procedure* is as follows:

Dissolve 0·6 g of acetobromo glucose in 2 ml of anhydrous benzene, add 0·52 g of finely powdered dry silver diphenyl phosphate [125] and reflux the mixture for 30 minutes. Add a further 0·25 g of silver diphenyl phosphate, continue the reflux for another 30 minutes, centrifuge, and wash the silver salts with anhydrous benzene;

distil the combined benzene fractions at reduced pressure, dissolve the benzene-free residue in 8 ml of anhydrous ethanol, and subject the filtered solution to hydrogenation at room temperature and atmospheric pressure, in the presence of 100 mg of platinum oxide as catalyst.

At the end of the reaction, remove the catalyst and add 1 M sodium hydroxide dropwise to the pure solution, with continuous stirring, until a permanent red tint is obtained in the presence of phenolphthalein; this indicates the completion of deacetylation.

Ethanol is removed, 500 ml of saturated aqueous barium acetate solution added, and the insoluble barium salt separated by centrifugation and washed with distilled water. To the combined aqueous washings and supernatant solution, add three volumes of anhydrous ethanol, separate the precipitated barium salt of glucose-1-phosphate by centrifugation, wash it with ethanol and with ether, and dry it.

Dissolve the barium salt obtained (350 mg) in hot water, filter and add 155 mg of potassium sulphate. During this treatment, barium is exchanged and precipitated as barium sulphate. Remove the precipitate by filtration, and add to the supernatant solution 1·7 volumes of anhydrous ethanol. On keeping the system at 0°, dipotassium glucose-1-phosphate dihydrate separates out in the form of fine needle crystals. Yield 37%.

The yield referred to glucose can be improved by employing the one-step preparation of acetobromoglucose suggested by Kőrösy and Bárczay–Martos [81].

The recently evolved McDonald method [100] also lends itself to the preparation of a product which is of high purity from the aspect of preparative organic chemistry. This method has the advantage of being simple and resulting in fair yields. The initial glucose derivative is β-D-pentaacetyl glucose.

Procedure:

β-D-pentaacetyl glucose is prepared by the Fischer method [30] (yield 74%, referred to glucose). When β-D-pentaacetyl glucose is treated with crystalline phosphoric acid, a Walden inversion takes place. Details of the method are as follows.

Allow 5·7 g of crystalline phosphoric acid and 5 g of β-D-pentaacetyl glucose to react at 50°C in a high vacuum (2 torr). The acetic acid formed during the reaction distils off, and the solid acetate gradually dissolves. The evolution of gas is complete after about 2 hours. Dissolve the homogeneous melt formed in 30 ml of anhydrous tetrahydrofuran, pour the solution into 240 ml 0° 1 N lithium hydroxide and allow the mixture to stand overnight at room temperature (the acetyl groups undergo saponification). Remove the precipitated lithium phosphate by filtration, pour the residual basic solution onto a Dowex 50 W cation exchanger column (2 × 20 cm) and elute the column with 200 ml of distilled water.

Now add 10 ml freshly distilled cyclohexylamine to the aqueous solution, evaporate it to dryness in vacuum, and stir the residual white crystalline substance with 200 ml of isopropanol at room temperature (cyclohexylammonium acetate is extracted). Centrifuge the reaction mixture, wash the crystalline product consecutively with 30 ml of isopropanol and 30 ml of ether, then dry it in vacuum over calcium chloride (yield 3·38 g). Dissolve the product in 3 ml of water and recrystallize after the addition of 75 ml of hot anhydrous ethanol. Filter the white crystals which precipitate during cooling, wash them with ether, and dry. Yield 1·87 g.

Finally, convert dicyclohexylammonium glucose-1-phosphate monohydrate into the dipotassium salt. Pour a solution of the product in 50 ml of water onto a Dowex 50W cation exchanger column, elute with water, and adjust the pH of the eluate to 8·5

with 0·5 N potassium hydroxide. Evaporate the alkaline solution to 40 ml in vacuum, add 2 volumes of 0° anhydrous ethanol, and crystallize the liquid at 0°. Yield of the recrystallization 96%.

(iii) Purification of Glucose-1-phosphate

In the opinion of Kamogawa *et al.* [69], it is simpler and more practical to remove the primer contamination from the donor substrate prepared by the enzymatic method, prior to its use, than to use glucose-1-phosphate synthesized chemically. It was proved experimentally that incorrect conclusions may be drawn as a result of the presence of various primer contaminants [49, 110]. Three different methods were used by Kamogawa *et al.* for the purification of glucose-1-phosphate.

1. Treatment with Glucoamylase. 0·3 g of dipotassium glucose-1-phosphate (Sigma Chem. Co.) was dissolved in 20 ml of 0·005 M tris-hydrochloric acid buffer (pH 7·5), the pH of the solution was adjusted to 7·4 with hydrochloric acid, and 10 mg of crystalline glucoamylase added (Endomyces sp. was crystallized according to Fukui and Nikuni [43]). After incubation of the mixture for 2 hours at 40 °C, the reaction was stopped by adding 1/20 volume of 1 N potassium hydroxide. The system was then boiled for 10 minutes and 4 volumes of ethanol added to precipitate glucose-1-phosphate which was consecutively washed with ethanol and ether, and dried in vacuum.

2. Treatment with Charcoal. 1 g of dipotassium glucose-1-phosphate was dissolved in 10 ml of distilled water, the solution poured onto a 2·5 × 10 cm column packed with a

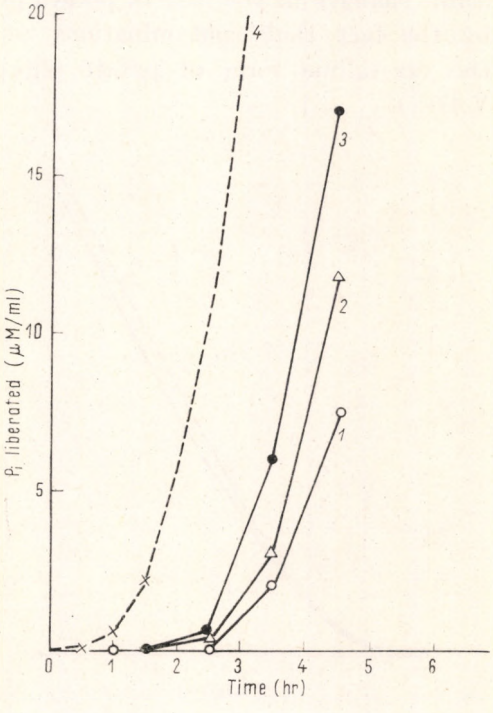


Fig. VII/6. Synthesis reaction of rabbit muscle phosphorylase without any acceptor substrate, in the presence of starter-free and of untreated glucose-1-phosphate [69]. *1:* Charcoal-treated; *2:* Dowex 1-treated; *3:* glucoamylase-treated; *4:* untreated

Experimental conditions:

0·2 ml of glycerophosphate buffer (0·1 M, pH 6·8)
0·01 ml AMP (0·1 M, pH 7·0)
0·05 ml cysteine (0·3 M, pH 7·0)
0·84 mg recrystallized rabbit muscle phosphorylase
0·4 ml glucose-1-phosphate (0·1 M in distilled water)

Total volume: 1 ml. The reaction mixture was incubated at 30°C. Samples of 0·1 ml were withdrawn and the inorganic phosphate liberated was measured in these samples

mixture of 5 g Norit 'SX-II' and 5 g celite '535'. The absorbed glucose-1-phosphate was eluted with distilled water. The pH of the combined donor substrate fractions was adjusted to 12 with 1 N potassium hydroxide, then glucose-1-phosphate precipitated with 4 volumes of ethanol, and treated as described above.

3. *Treatment with Dowex-1.* A solution of 1 g of dipotassium glucose-1-phosphate in 50 ml of water was allowed to pass through a Dowex-1 cation exchange column (2·8 × 5·0 cm, chloride form), then the column washed consecutively with 200 ml of distilled water and 200 ml of 0·01 N hydrochloric acid which contained 0·01 M potassium chloride.

Glucose-1-phosphate was eluted with 200 ml of 0·02 N hydrochloric acid plus 0·02 M potassium chloride as eluting agent. The pH of the combined fractions which contain glucose-1-phosphate was adjusted to 12 with 1 N potassium hydroxide. The solution was concentrated to a volume of 20 ml (in a rotary evaporator) at 40°C at reduced pressure, 4 volumes of ethanol were added, and the crystals of dipotassium glucose-1-phosphate treated as described above.

Figures VII/6 and VII/7 show the time dependence of the liberation of inorganic phosphate in the course of the synthesis reaction catalyzed by rabbit muscle phosphorylase and potato phosphorylase. No acceptor substrates were employed in these cases. Glucose-1-phosphate was purified by the above procedures. It can be seen in Fig. VII/6 that the lag period appreciably increased in the presence of rabbit muscle phosphorylase when glucose-1-phosphate in purified form was applied. As Fig. VII/7 discloses, the removal of primer contaminants from the donor caused only insignificant changes in the case of potato phosphorylase. This can be explained by the fact that contaminations of the primer are present even in the crystalline form of potato phosphorylase (cf. in detail in Section V.1*(vi)*).

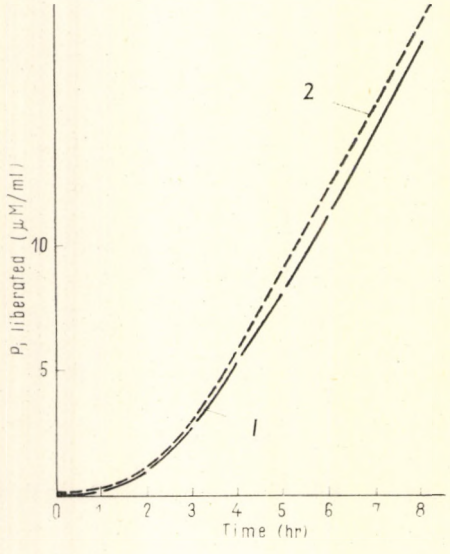


Fig. VII/7. Synthesis reaction of potato phosphorylase, without any acceptor substrate, in the presence of starter-free and of untreated glucose-1-phosphate [69]. *1:* Glucoamylase, charcoal or Dowex 1-treated; *2:* untreated

Experimental conditions:

0·2 ml citrate buffer (0·5 M, pH 6·0);
0·147 mg repeatedly crystallized potato phosphorylase;
0·4 ml glucose-1-phosphate (0·1 M in distilled water).

Total volume: 1 ml. The reaction mixture was incubated at 30°C, and the liberated inorganic phosphate was measured in 0·1 ml samples

(iv) Preparation of Donor Substrate Analogues

From preparative aspects, the McDonald method [100] was chosen by Holló *et al.* [60] as most suitable for the production of the derivatives of glucose-1-phosphate (cf. in detail in Section VII.5 *(ii)*). They found that the 2-deoxy derivative cannot be prepared by either the Posternak method [124, 125] or the McDonald procedure [100], presumably due to the lability of this compound. However, 6-deoxy- and 3-O-methylglucose-1-phosphate could be produced by the McDonald method.

(a) Preparation of 6-Deoxy-glucose-1-phosphate [86]

Preparation of 6-Deoxy-glucose, 1,2-5,6-diisopropylidene glucose was prepared from glucose and then converted into 1,2-O-isopropylidene-glucose. This protected glucose was tosylated on carbon atom 6 and the product reduced with lithium aluminium hydride. On removing the protecting groups from the protected 6-deoxy-glucose, the desired 6-deoxy-glucose is obtained [9, 13, 70, 136].

In this synthesis, diffiiculties were encountered in the production of the benzylidene derivative in that the literature yield could not be attained. However, when reduction with lithium aluminium hydride [70] is carried out, in anhydrous tetrahydrofuran, instead of in ether, as described in the literature, the reaction takes place more quickly and the yield is higher [86].

Data for the 6-deoxyglucose obtained are: m.p. 141–142°C; rotatory power 5 minutes after dissolution $[\alpha]_D^{20} = +72{\cdot}69$, after 3 hours $[\alpha]_D^{20} = +24{\cdot}85$ (c 0·3, H_2O), in good agreement with literature data. Yield 6%, referred to initial glucose.

Preparation of 6-Deoxy-β-D-glucose-tetraacetate [86]. A mixture of 2·5 g of 6-deoxy-glucose and 1·31 g of anhydrous sodium acetate in 10·1 ml of acetic anhydride is stirred on a boiling water bath for 2 hours after complete dissolution. After cooling, the solution is poured into 110 ml of ice-water with stirring. After the separation of a white precipitate stirring is continued for a further 2 hours, then the precipitate filtered off and suspended in 110 ml of water, and allowed to stand in a refrigerator overnight. The precipitate is filtered and dried. Yield 4·5 g (89·2%), m.p. 125–138°C. After three recrystallizations from absolute ethanol, the m.p. remains constant. Yield 3·29 g (65·2%), mp.147–148°C.

Preparation of Dicyclohexylammonium 6-Deoxy-α-D-glucose-1-phosphate Monohydrate [86]. 2·13 g of 6-deoxy-β-D-glucose tetraacetate is reacted with 2·85 g of crystalline phosphoric acid at a pressure of 2 torr. The reaction starts at 56°C, as indicated by a vigorous gas evolution. The reaction mixture is kept at 57°C until the gas evolution ceases, then dissolved in 15 ml of tetrahydrofuran and the solution poured into 120 ml of ice-cold 1 N lithium hydroxide solution; it is allowed to stand overnight, and the precipitated lithium phosphate separated by filtration. The filtrate is passed through a Varion KS cation exchange column. The acid solution obtained is collected

in 5 ml of freshly distilled cyclohexylamine. The column is washed with water until free of acid. The aqueous solution is evaporated to dryness in vacuum, and the solid residue washed with 100 ml and then with 2 × 15 ml of isopropanol to remove cyclohexylammonium acetate. The residual white solid is washed with ether and dried. Yield 0·74 g (25·2%). It is dissolved in 4 ml of hot water, 40 ml of hot ethanol is added, and the solution allowed to crystallize in a refrigerator. Yield 0·58 g, decomposition point: 198°C.

Preparation of 6-Deoxy-α-D-glucose-1-phosphate [86]. A solution of 0·58 g of dicyclohexylammonium salt in 15 ml of distilled water is subjected to ion exchange on a column of Varion KS. The pH of the acid solution is adjusted to 8·5 with 0·5 N potassium hydroxide. The volume is reduced to 10 ml in vacuum, and absolute ethanol slowly added, cooling with ice, until the liquid becomes turbid. The liquid is allowed to stand in a refrigerator, when the crystalline phosphate ester is precipitated. After filtration, it is washed consecutively with ethanol and ether, and dried in vacuum. Yield 0·406 g, m.p. 173–175°C (decomposition), $[\alpha]_D^{20} = +110°$ (c 2, H_2O).

On the basis of the organic phosphate content, the product is of 90·3% purity.

(b) Preparation of 3-O-Methyl-glucose-1-phosphate [86]

3-O-methyl-gluscose was prepared according to literature methods [46, 172]. Methylation was carried out by dimethyl sulphate in the presence of sodium hydroxide.

Preparation of 3-O-Methyl-β-D-glucose-tetraacetate [86]. A mixture of 23·8 g of 3-O-methyl-glucose, 10·5 g of anhydrous sodium acetate and 82 ml of acetic anhydride is stirred in a 250 ml round-bottomed flask on a boiling water bath for 2 hours, subsequent to complete dissolution. The mixture is then poured into 1000 ml of ice-water with continuous stirring. An oily product separates which is extracted with 3 × 100 ml of chloroform. The combined chloroform extracts are shaken with 3 × 50 ml of a 10% solution of sodium hydrogen carbonate, then washed with water to neutrality. After clarification and drying, the liquid is evaporated to a thick syrup under reduced pressure, ether is poured onto it, and the mixture allowed to crystallize in a refrigerator. The separated crystals are filtered and dried. Yield 14·65 g (33%), m. p. 91–94 °C. On recrystallization from anhydrous ethanol, yield 12 g, m.p. 98–99°C.

Preparation Dicyclohexylammonium 3-O-Methyl-α-D-glucose-1-phosphate [86], 4·63 g of 3-O-methyl-β-D-glucose tetraacetate is reacted with 5·7 g of crystalline phosphoric acid at 55 °C for 3 hours at a pressure of 3 torr. The reaction mixture is taken up in 30 ml of tetrahydrofuran, and poured into 240 ml of ice-cold 1 N lithium hydroxide. The system is stood overnight at room temperature, the precipitated lithium phosphate is filtered off, and the solution treated with cation exchanger Varion KS to remove the cations.

The acid solution is collected in 10 ml of freshly distilled cyclohexylamine. The ion exchanger column is washed with water to neutrality. The aqueous solution is evaporated to dryness in vacuum. According to the McDonald method [100], the sugar phosphate must be separated from cyclohexylammonium acetate with isopropanol. However, the reaction mixture is soluble in isopropanol. However, the reaction mixture is soluble in isopropanol. Cyclohexylammonium acetate can be extracted with chloroform but all attempts to crystallize the dicyclohexylammonium salt of the sugar phosphate failed.

(c) *Preparation of Barium 3-O-Methyl-glucose-1-phosphate [86].* The aqueous solution of the oil obtained is poured onto Varion KS ion exchanger. The liquid passing through the ion exchanger is neutralized with a saturated solution of barium hydroxide, clarified, evaporated in vacuum, and the barium salt precipitated with ethanol, washed consecutively with ethanol and ether, and dried. Yield 1·27 g (24·21%), $[\alpha_D^{20}] = +90_0$ (c 2·0, H_2O)

On the basis of its organic phosphate content, this barium salt is of 46% purity. Accordingly, the actual yield is 11%.

6. Preparation of Various Acceptor Substrates

In the enzyme reactions, in addition to the glucose-1-phosphate, an acceptor substrate ('primer' or 'starter') is also indispensable. These 'starters' may be maltooligosaccharides, amylose, amylopectin and glycogen.

In the case of plant phosphorylases, the starters are prepared from the reserve nutrient of plants, the starch, by fractionation, hydrolysis etc.

(i) Fractionation of Starch

Starch contains two different types of polysaccharide: a linear one (amylose) and another of branched nature (amylopectin). In 1903–4 Maquenne and Roux [97, 98] succeeded in separating these two fractions from each other. Their method was based on the retrogradation of amylose. Since then various procedures, such as aqueous extraction [44, 102], electrophoresis [91, 132], selective adsorption [115, 144, 145], enzymatic method [93], selective precipitation with an organic polar solvent [2, 85, 135, 163, 170], fractionation with an inorganic salt [16, 17, 107] and separation by chromatography [149, 150] have been employed for the separation of polysaccharides of different type from each other. Of the above methods, those of Lansky *et al.* [85], Schoch [135], and Bus *et al.* [16–18] have proved to be quite suitable.

(a) Method of Lansky *et al.* [85]

Primary Separation. Suspend 300 g of starch in 15 litres of distilled water and 1 litre a Pentasol (a mixture of synthetic pentanol isomers) in a 25 litre Pyrex flask. In order to adjust the pH to 6·2–6·3, add 8·2 g of anhydrous potassium dihydrogen phosphate and 1·8 g of anhydrous dipotassium hydrogen phosphate to the suspension. Equip the flask with a reflux condenser and a high-speed stirrer. At first stir the suspension in the cold, then heat it on a water bath to 92°C, and and gently reflux for 3 hours. Subsequently, allow it to cool overnight at room temperature, with continuous stirring, and refrigerate for a further 24 hours. The linear complex fraction (amylose) crystallizes as minute spherules or as needle clusters. Better crystal formation is obtained when the vessel is jacketed by a heat insulator, and thus, the cooling procedure takes place more slowly.

Amylose can readily be separated from the cooled mixture by means of a Sharples supercentrifuge (flow rate 250–300 ml/min, speed 50 000 rpm). With repeated supercentrifugation of the supernatant solution, it is possible to separate completely the linear fraction (amylose) from the branched fraction (amylopectin); the latter can be precipitated by adding an equal volume of methanol to the supernatant solution freed from traces of amylose. The precipitate is kept overnight in a refrigerator, then comminuted for 2–3 minutes in a Warring Blandor, and dehydrated with further amounts of methanol. After filtration on a Buchner funnel, it is dried at 50°C. Amylopectin prepared by this procedure contains 0–3% of amylose, depending on the variety of starch.

Recrystallization of Amylose. For the second recrystallization it is advisable to use n-butanol in place of Pentasol. Amylose separated by supercentrifugation is added,

in a 25 litre round-bottomed Pyrex Flask, to a mixture of 15 litres of distilled water and 1 litre of n-butanol, with continuous vigorous stirring and heating (hot water bath). Amylose is completely dissolved after 20–30 minutes of this treatment. The hot solution is allowed to pass through a Sharples supercentrifuge, in order to eliminate the residual contaminants (cellular substances, proteins) from the first recrystallization. Centrifugation must be carried out quickly since at too low a temperature amylose may also be precipitated. If this is the case, the mixture must be re-heated and centrifuged. The pure supernatant solution which contains the amylose fraction free of contaminants, is placed again in a hot water bath, butanol added, and if necessary, cooled overnight with continuous stirring. It is of advantage to cool amylose in a refrigerator for a further 24 hours. Then the recrystallized amylose is separated from the mother liquor by supercentrifugation. If necessary, recrystallization is repeated in a way identical to that described above.

(b) Modified Schoch Method [60]

100 g of potato starch is mixed with 500 ml of distilled water, then processed in 3500 ml of boiling distilled water, with continuous stirring. Subsequently, the suspension is heated in an autoclave at 1·4 atm pressure for 2 hours, the processed viscous mass poured into a 6-litre round-bottomed flask equipped with an insulation jacket, cooled to about 85–80°C, with continuous stirring, 200 g of n-butanol is added, and the mixture cooled to room temperature, with continuous stirring, 200 g of n-butanol is added, and the mixture cooled to room temperature, with continuous stirring. The precipitated butanol–amylose complex is separated by centrifugation and washed twice with 8% butanol.

The amylose complex is suspended in 2000 ml of distilled water and heated to about 90°C, with continuous stirring. After complete dissolution, the liquid is filtered through a glass filter G-1, the clear amylose solution re-heated to 90°C, 100 g of n-butanol is added, the mixture cooled as described above, and the amylose-butanol complex separated from the mother liquor by centrifugation. The product is washed twice with 8% butanol, yield 212 g of butanol–amylose complex of 4·6% dry matter content.

Amylopectin is precipitated from the supernatant solution of the first fractionation by 70–80% ethanol in a 1 : 3 proportion. The amylopectin solution must be poured slowly into the ethanol. The rubber-like flakes of amylopectin are washed with anhydrous ethanol, comminuted in anhydrous ethanol, and dried in vacuum.

(c) Method of Bus *et al.* [16–18]

The possibility of the fractionation of starch with a solution of magnesium sulphate was investigated by Bus, Muetgeert and Hiemstra [16–18]. They employed a liquid–liquid phase separation [36] of amylose and amylopectin.

Their method for the fractionation of potato starch is as follows. Starch is dissolved in water in the presence of magnesium sulphate by heating to 160°C. The solution produced contains 10% starch and 13% magnesium sulphate. The hot solution is cooled to 80°C, and the precipitated amylose removed by centrifugation. Cooling is continued to 20°C, with the precipitation of amylopectin. Since, however, technical difficulties are encountered on separating amylose at 80°C, the procedure was modified in that the hot starch solution is cooled to only 90°C and then water is injected to adjust the salt concentration to 10%. Under such conditions, amylose can be separated at 20°C, and amylopectin precipitates only when the salt concentration of the supernatant solution is readjusted to 13%.

(ii) Subfractionation of Amylose

In the course of the synthesis of polymers, in general the molecular weight varies within rather broad limits. That amylose behaves in this way has been proved by a number of authors who prepared polymers of

various molecular weights by the subfractionation of amylose by various methods.

The first subfractionated amyloses were prepared by Meyer *et al.* [103] and Kerr [72] from aqueous leachings. However, this fractionation was rather rough, and the fractions were contaminated by amylopectin. Later, Kerr [73] subfractionated maize amylose dissolved in ethylenediamine, by a stepwise addition of diethyl ether. Similar results were obtained when isopropanol was added to a solution of amylose in 0·5% potassium hydroxide solution [138], and when amylose was precipitated from a 15% aqueous pyridine solution at various temperatures [38].

Recently, an excellent subfractionation method was published by Everett and Foster [29] for potato amylose. These authors started from amylose freshly prepared by the dimethylsulphoxide method of Killion and Foster [74]. By gradually adding ethanol to this amylose at a constant temperature, they succeeded in isolating seven different subfractions (Table VII/2) of molecular weights varying from $1{\cdot}5\times10^5$ to $2{\cdot}2\times10^6$.

Table VII/2

Results of the Subfractionation of Amylose, according to the Investigations of Everett and Foster [29]

Fractions	Precipitating agent (%)	$\frac{MgI_2}{100\text{ mg amylose}}$	$M_w\times10^{-5}$*	Radius of gyration (Å)*
AF IA	20	19·2	22·2	935
AF IB	12	19·3	13·5	724
AF II	12	—	10·5	656
AF III	13	20·0	8·47	610
AF IV	16	17·6	5·52	543
AF V	16	18·3	2·70	425
AF VI	14	—	1·46	334

* Molecular weights and radii of gyration were determined by light scattering in dimethyl sulphoxide.

Holló and Szejtli [60] carried out the subfractionation of aqueous and of pyridine solutions of potato amylose by gradual addition of iodine solution. Iodine in the solution is in equilibrium with the amylose–iodine complex. However, the large molecules combine with iodine to form more stable complexes which are partially precipitated. The precipitate is removed by centrifugation. On the further addition of iodine, the fractions of lower molecular weight also precipitate as iodine complexes. In this way, five different amylose fractions could be isolated from an aqueous solution

Table VII/3

Subfractionation of Amylose as Amylose–Iodine complex [60]

Number of fraction	Weight of fraction (mg)	% of total	Viscosity (ml/g)	Degree of polymerization (DP_w)
Unfractionated amylose	2498	100·0	149	1520
1	423	19·1	178	1840
2	444	20·0	154	1570
3	450	20·2	141	1430
4	452	20·3	135	1360
5	452	20·3	58	550

of potato amylose (Table VII/3). The molecular weight of these fractions varied from 3×10^5 to 9×10^4, while their degrees of polymerization ranged from 1840 to 550. From the pyridine solution, four fractions of higher degrees of polymerization were isolated, with molecular weights from $4{\cdot}8\times10^5$ to $2{\cdot}9\times10^5$. Their mean degrees of polymerization varied from 3010 to 1780 (Table VII/4).

Table VII/4

Subfractionation of Amylose Dissolved in Pyridine, as Amylose–iodine Complex [60]

Number of fraction	Weight of fraction (mg)	% of total	Viscosity ml/g)	Degree of polymerization (DP_w)
Initial amylose	3128	100·0	236	2490
1	750	24·0	282	3010
2	767	24·6	215	2250
3	688	22·0	188	1950
4	782	25·0	173	1780

Procedure:

60 g of butanol complex is dissolved in 1100 ml of distilled water (the dry matter content of the complex is 4·1%), the solution concentrated in vacuum, and the butanol removed. After cooling, 20 g of sodium chloride is added, and also 5·0 ml of 0·1 N iodine solution dropwise with continuous stirring. After one hour of stirring at 30°C, the precipitated iodine complex is removed by centrifugation. This fractionation procedure is then repeated four times with 4×50 ml portions of 0·1 N iodine solution. The iodine complexes are decomposed either by addition of thiosulphate or by boiling.

(iii) Preparation of Maltooligosaccharides

The preparation and purification of the various maltooligosaccharides is of great importance because they are indispensable for the study of the 'starter' specificity of the enzyme phosphorylase, of the mechanism of synthesis and decomposition catalyzed by this enzyme, and of a number of other kinetic problems.

Three possibilities are available for the synthesis of oligo- and megalosaccharides: (a) enzymatic hydrolysis (b) transfer, and (c) chemical hydrolysis.

(a) Enzymatic Hydrolysis

The principle of this method is a treatment of various plant and animal starches with α-amylase. As a result, maltose [154], maltotriose [116, 159], maltotetraose [116, 159] and branched oligosaccharides [61, 113, 154, 162] are formed. The structure of these latter depends on the origin of the maltose employed for decomposition [61, 104, 113, 154, 162]. The best method for preparing maltose is that of Pazur [118], based on a treatment of starch with β-amylase. An elegant method was published by Wallenfels [152] for the preparation of maltotriose of chromatographic purity. In essence, this procedure consists in the decomposition of pullulan (a polysaccharide synthesized extracellularly from glucose by the strain Pullularia) by the enzyme pullulanase isolated from *Aerobacter aerogenes*.

It has been observed by a number of authors that in the course of the controlled enzyme hydrolysis of starch and amylose carried out with various amylases, linear oligosaccharides are formed in appreciable amounts (*DP* 4–8) in the early and middle stages of hydrolysis [26, 116, 129, 159]. The changes in the amount and molecular weight of oligosaccharides during amylolysis proves that the effect of amylases is far from random [41]. By means of the controlled enzyme decomposition of starch, the problem of producing an oligosaccharide mixture containing compounds of a definite range of chain length can be adequately solved. This method may offer some advantages over acid hydrolysis when a separation of pure saccharides by fractionation with chromatography (the longest and the most difficult section of the preparation procedure) of the mixture obtained is planned.

(b) Enzymatic Transfer

For transglucosidation, two different mechanisms are presumed [65, 80] as shown by Eqs (1) and (2).

$$\begin{aligned} &E + MD \rightleftharpoons [E \cdot M{-}D] \rightleftharpoons [E{-}M \cdot D] \rightleftharpoons EM + D \\ &EM + A \rightleftharpoons [E{-}M \cdot A] \rightleftharpoons [E \cdot M - A] \rightleftharpoons E + MA \end{aligned} \qquad (1)$$

The mechanism of Eq. (1) probably includes the transfer to the enzyme, of a part (M), of the suitable donor substrate (D), leading to the formation of a glucosyl-enzyme compound. After the liberation of the donor part of the original molecule, M is transferred to the acceptor (A) molecule. This results in a double displacement reaction [80].

According to the other, alternative mechanism (Eq. (2)), the formation of a termolecular complex is followed by the direct transfer of M from the donor substrate (D) to the acceptor (A) molecule [65].

$$E+M-D+A \rightleftharpoons [E \cdot M-D \cdot A \rightleftharpoons E \cdot M-A \cdot D] \rightleftharpoons E+MA+D \quad (2)$$

It appears from Eqs (1) and (2) that the enzymes have a broad acceptor specificity which makes possible the formation of a wide variety of modified starch dextrins during catalysis. The natures of *Bac. macerans* amylase and of the potato D-enzyme prove the extent of this transfer. By coupling the Schardinger dextrins to isomaltose and panose [141], branched oligosaccharides containing 4–7 glucose residues have been prepared. *Bac. macerans* amylase is capable of forming maltodextrins with a homologizing action [40, 112], as illustrated in Fig. VII/8.

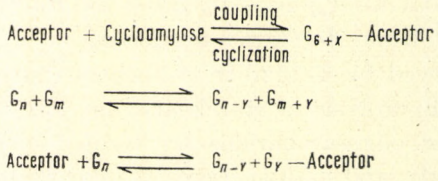


Fig. VII/8. Some reactions of *Bac. macerans* β-amylase and of D-enzyme. Any of these reactions may take place in the presence of D-enzyme, while the last two reactions may occur in the presence of *Bac. macerans* β-amylase. The value of x depends on the dimensions of the cycloamylose. The permissible limit of y is uncertain, depending on the nature of the enzyme

Other D or disproportionating enzymes are also capable of homologizing maltodextrin saccharides by transferring a portion of one dextrin to another or to glucose [120–122, 153]. Since D-enzyme and *Bac. macerans* amylase possess this broad acceptor specificity, a whole series of maltodextrins could be produced in this way.

Other enzymes participating in starch metabolism are similarly capable of synthesizing maltodextrin saccharides from 'activated' glucose; e.g.

phosphorylase [14, 34], uridine-diphosphoglucose-glycogen-transglucosidase (UDGGT) [47].

One important use of the 'coupling' enzymes is the preparation of isotopically labelled maltooligosaccharides. With enzymes of this type, the position of the labelling is precisely known [5, 34, 117].

One of the advantages offered by the transfer method is that with the aid of enzymes modified starch fragments of precisely known structure can be prepared, the formation of side-products can be suppressed to a minimum level, and the fractionation of products is very simple. In addition, since the energy content of the glycosidic bonds is in essence retained during the reaction, higher yields are obtained than with the hydrolysis method. The free energy change for transglucosidation is only a few hundred calories as contrasted to several thousand calories for hydrolysis. Furthermore, in the hydrolysis reaction, limit dextrins are formed with the use of the glucosidic bonds, and these limit dextrins interfere with the enzyme effect.

(c) Chemical Methods

For the production of starch oligosaccharides, acid hydrolysis [146, 165 171], reversion [45, 119, 137], pyrodextrinization and dehydration [45], isomerization [92] and various other chemical syntheses are applied [12].

Chemical methods are in general restricted to the synthesis of disaccharides. The greatest drawbacks of the chemical syntheses are the formation of an appreciable amount of undesired side products, the difficulties in carrying out the synthesis and purification, and the low yields. Consequently, chemical synthesis cannot be considered as a preparative method.

Of the methods suggested up to the present, controlled acid hydrolysis, combined with chromatographic fractionation, has found greatest use for the preparation of maltodextrins.

For the time being, commercial amylose is the most suitable starting material. Hydrolysis must be performed in a sufficiently diluted solution, in order to reduce the acid-catalyzed reversion to a minimum level [147].

$\alpha(1 \rightarrow 6)$ bonds are present in amylopectin and glycogen. These bonds are cleaved in the acid hydrolysis and, thus, only a small amount of branched product is formed [171]. These conclusions can easily be proved by the analysis of natural probability calculations of acid hydrolysis. According to a number of authors [66, 171], probability analysis indicates the yield which is actually a function of chain length and degree of hydrolysis.

After presuming that all the bonds are of the same strength with regard to acid hydrolysis, a number of authors attempted to forecast the distri-

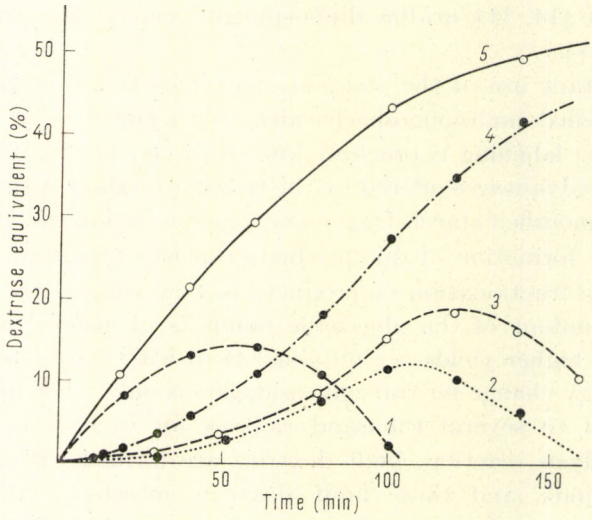


Fig. VII/9. Distribution of maltooligosaccharide products in the course of hydrolysis by hydrochloric acid, plotted against time [86]. *1:* Dextrins; *2:* maltotriose; *3:* maltose; *4:* glucose; *5:* total reducing value

bution of the product concentration on the basis of probability calculations [54, 78, 83]. It was found, however, that terminal glucose units are split off more rapidly [42, 143], and thus, product distribution can only be determined empirically, by way of experiments.

László [86] hydrolyzed potato starch with 0·2 N hydrochloric acid. The analysis of samples withdrawn at different times proved that the total reducing power and the glucose content increase in a logarithmic manner. The concentration of maltooligosaccharides as intermediates attains a maximum level, then decreases, due to the continuation of the hydrolytic process. Under the above experimental conditions, maximum maltose production was reached after 120 minutes of hydrolysis, that of maltotriose after 90 minutes and that of maltotetraose–hexaose after 70–50 minutes (Fig. VII/9).

According to Whistler *et al.* [164, 167], the commercial starch syrup of 42 dextrose equivalents contains 7% maltotetraose, 8% maltopentaose and 6% maltohexaose. Descending paper chromatography, where the repeated development favourably improves elution, is a method which can be performed very simply. However, a drawback is that it is suitable only for analytical purposes. As a running agent, the following mixtures can be used:

6 : 4 : 3 = n-butanol : pyridine : water [10, 151]
10 : 4 : 3 = ethyl acetate : pyridine : water [10, 151]
40 : 12 : 16 : 1 = n-butanol : ethanol : water : ammonia [10], and
3 : 2 : 1 = n-butanol : ethanol : water [166].

Ammoniacal silver nitrate or aniline phthalate solutions are suitable [151] as developing agent.

Adsorption column chromatography on carbon columns [158, 168], on cellulose powder columns [146, 169] and on diatomaceous earth columns [90] is employed for preparative purposes.

The use of carbon columns offers the advantage that the packing has a high capacity [105], while its drawback is that the column must be repacked freshly after each run. Whistler, however, has succeeded in separating maltooligosaccharides of 2–5 members from each other [166] by this method.

For the time being the most suitable procedure is chromatography on cellulose powder columns, though these columns have a capacity lower than that of carbon columns [90]. Pulverized cellulose has an exceptional developing power [146], and partition chromatography lends itself to a great extent to the separation of isomers of identical degrees of polymerization. However, cellulose fibres are destroyed by acidic and alkaline solvents, and the use of the latter must be avoided if the repeated employment of the columns is desirable [90].

A 3–10 cm diameter, 50 cm column, jacketed and packed with Whatman cellulose powder (Fig. VII/10) was applied by Thoma *et al.* [146] for the separation of maltooligosaccharides. Cellulose powder suspended in hot water (50 g per litre) was allowed to flow into the column at a rate of 50–75 ml/minute, then the column compacted at a pressure of 0·5 atm. During the procedure, a circulation of hot water (53°C) was maintained in the jacket.

When packing was finished, the column was washed with the base solvent (water : ethanol : butanol) until the height of the column did not show any further decrease.

(For perfectly homogeneous packing, it is essential that the base solvent and the eluting liquid be completely free of gases, and that no changes should occur in the temperature of the column.) The above-mentioned authors transferred 1·0–2·5 g of hydrolysate onto the column, in the form of a solution in 2–3 ml of hot water. To this solution, an eluting solvent was added until two distinct phases were formed. During this procedure the flow rate was 1 drop per 3 seconds. After the transfer of the hydrolysate, the column was completely packed with cellulose powder, and elution was commenced. (Flow rate 1 ml/minute, fractions of 25 ml were collected.) Mixtures of water–ethanol–butanol of various ratio served as eluting agents. The

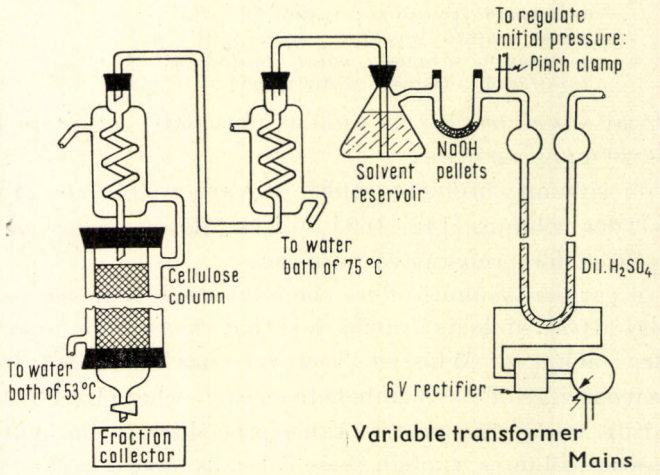


Fig. VII/10. Scheme of apparatus used for high-temperature column chromatography by Thoma *et al.* [146]. A fritted glass disc sealed into the lower part of the column supports the powdered cellulose. The rate of solvent flow is controlled by varying the voltage supplied to the electrolysis cell at the right. During solvent flow the pressure may be greater or less than atmospheric pressure

water content of the eluting solution was increased by 2% for each subsequent fraction up to a degree of polymerization of 5, and by 1% for homologues above this level. The elution curve is shown in Fig. VII/11. The eluates were

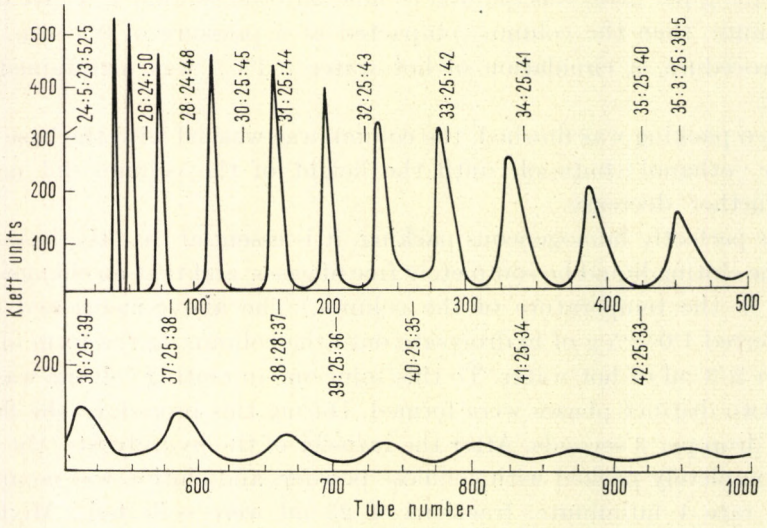


Fig. VII/11. Elution diagram of maltooligosaccharides. Eluting agent: water–ethanol–butanol [146]

analyzed with anthrone. At the end of the elution, the separated maltooligosaccharide fractions were evaporated in vacuum to a volume of about 50 ml. The aqueous phases were twice extracted with butanol and ethyl ether, then evaporated to dryness in vacuum.

On raising the temperature, polymerization products of degrees of polymerization up to 18 were obtained (in average amounts of 5–100 mg).

At present, experiments to separate maltooligosaccharides by chromatography on cellulose columns combined with gel filtration are in progress in our laboratory [58].

(iv) Preparation of Cycloamyloses

Cycloamyloses are of great importance in a study of the detailed mechanism of the enzyme reaction. In the case of phosphorylase, many authors have reported inhibition experiments carried out by cycloamyloses and have published valuable conlcusions.

A standard method for the production of cycloamyloses is the selective precipitation of products formed from starch treated with *Bac. macerans* amylase [39]. The yield can be improved to a great extent by continuous incubation. The best method for the separation of cycloamyloses has been evolved by Cramer [24] (cf. Table VII/5). In this, the variation in cavity diameter by precipitation with size-selective organic molecules is utilized. Advantages of this procedure over earlier methods are the convenience of technique and the completeness of precipitation.

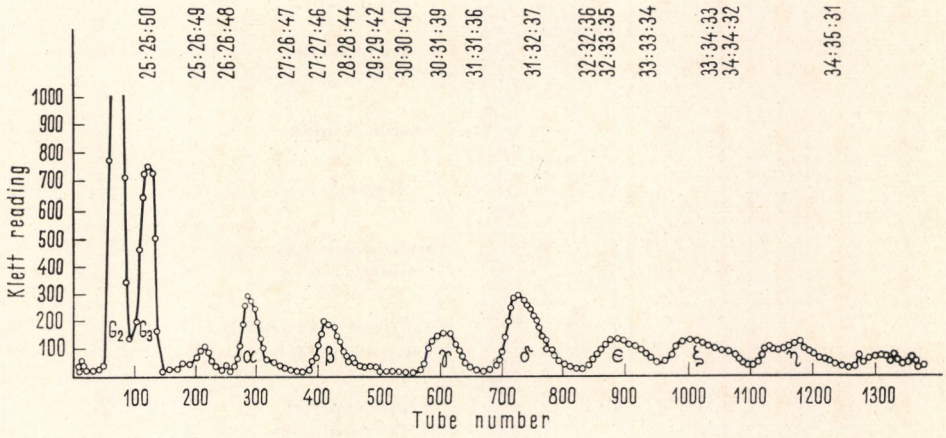


Fig. VII/12. Elution diagram of cycloamyloses by chromatography on cellulose column [126]. Eluting agent: water–ethanol–butanol. Peaks denoted by G_2 and G_3 are of maltose and maltotriose, respectively. Peaks denoted by Greek letters show various cycloaymloses

Table VII/5

Fractionation Scheme for Cyclohexa-, Cyclohepta- and Cyclooctaamylose by Cramer [24]

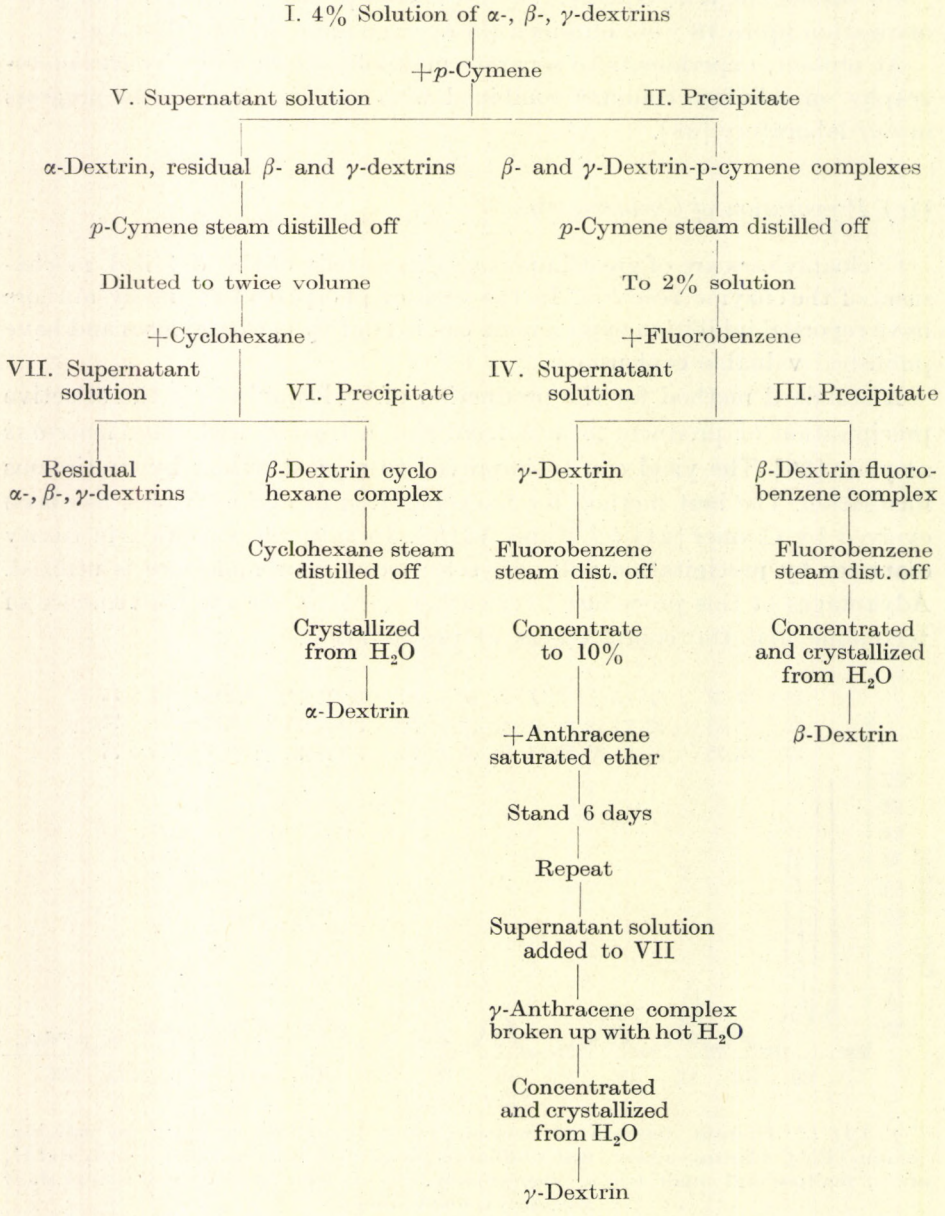
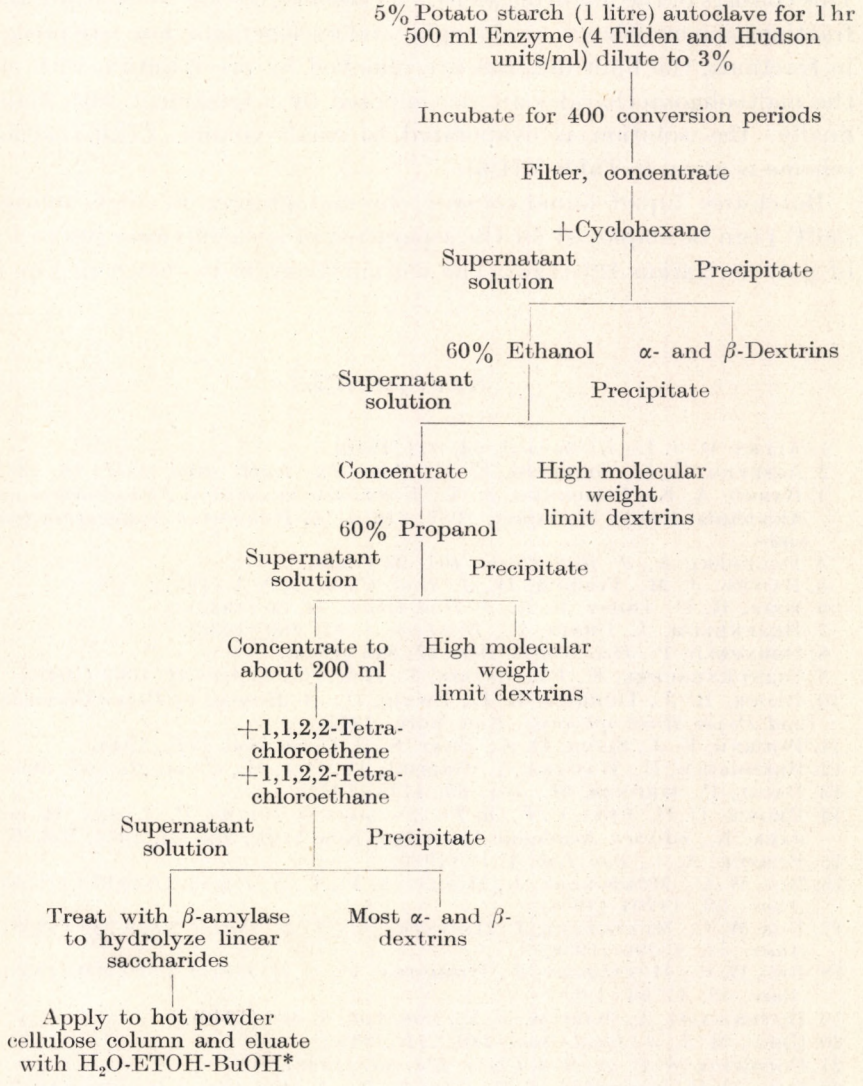


Table VII/6

Fractionation Scheme for Isolation of Larger Cycloamyloses by Pulley and French [126]



* see Fig. VII/12 for elution pattern

Higher homologues (of degrees of polymerization over 9) are formed only in minute amounts in the course of treatments with *Bac. macerans* amylase. Thus, they cannot be obtained by this method.

However, there is another method available for their preparation [126]. This consists of the following steps. The homologues are concentrated; during fractionation cyclohexa-, cyclohepta- and cyclooctaamylose are precipitated in fractions; the limit dextrins are removed by precipitation with ethanol; the maltooligosaccharides are decomposed by a treatment with β-amylase; finally, the solution is evaporated to small volume. (The fractionation scheme is given in Table VII/6).

Batchwise liquid–liquid column chromatography at high temperature (50°C) can be employed for the separation of cycloamyloses (up to a degree of polymerization 12) [126]. The elution diagram is shown in Fig. VII/12

REFERENCES

1. Allen, R. J. L., *Biochem. J. 34*, 858 (1940).
2. Alsberg, C. L., Griffing, E. P., *Proc. Soc. Exptl. Biol. Med. 23*, 728 (1926).
3. Babko, A. K., Pilipenko, A. T., *Kolorimetriás analízis* (Colorimetric analysis). Akadémiai Kiadó, Budapest, 1953. 216 p. (in Hungarian, translation from Russian).
4. Badenski, A., *J. Biol. Chem. 101*, 93 (1933).
5. Bailey, J. M., French, D., *J. Biol. Chem. 226*, 1 (1957).
6. Bell, R. D., Doisy, E. A., *J. Biol. Chem. 44*, 55 (1920).
7. Berenblum, J., Chain, E., *Biochem. J. 32*, 286 (1938).
8. Bernfeld, P., *Helv. Chim. Acta 27*, 843 (1944).
9. Blindenbacher, F., Reichstein, T., *Helv. Chim. Acta 31*, 1669 (1948).
10. Block, R. J., Durrum, E. L., Zweig, G., *A Manual of Paper Chromatography and Paper Electrophoresis*. New York, 1953.
11. Bourne, E. J., Sitch, D. A., Peat, S., *J. Chem. Soc. 1949*, 1548.
12. Bredereck, H., Wagner, A., Geissel, D., Ott, H., *Chem. Ber. 95*, 3064 (1962).
13. Bridl, P., Grüner, H., *Ber. 65*, 1428 (1932).
14. Brown, D. H., Cori, C. F., in *The Enzymes* by Boyer, P., Lardy, H. and Myrbäck, K., editors. Academic Press Inc. New York, 2nd ed. 1961. Vol. 5, p. 207.
15. Bursuk, A. J., *Zav. Lab. 8*, 12 (1939).
16. Bus, W. C., Muetgeert, J., Hiemstra, P., *U. S. Patent* 2,829,988 (1958); *Chem. Abstr. 52*, 14204 (1958).
17. Bus, W. C., Muetgeert, J., Hiemstra, P., *U. S. Patent* 2,829,989 (1958); *Chem. Abstr. 52*, 13296 (1958).
18. Bus, W. C., Muetgeert, J., Hiemstra, P., *U. S. Patent* 2,829,990 (1958); *Chem. Abstr. 52*, 17768 (1958).
19. Butenko, G. A., Kirsch, H. V., *Zav. Lab. 9*, 555 (1940).
20. Chou, M. J., *J. Biol. Chem. 180*, 771 (1949).
21. Colowick, S. P., *J. Biol. Chem. 124*, 557 (1938).
22. Cori, C. F., Colowick, S. P., Cori, G. T., *J. Biol. Chem. 121*, 465 (1937).
23. Cori, C. F., Cori, G. T., Green, A. A., *J. Biol. Chem. 151*, 39 (1943).
24. Cramer, F., Henglein, F. M., *Chem. Ber. 91*, 308 (1958)
25 Denigés, G., *Compt. rend. 171*, 902 (1920).
26. Dube, S. K., Nordin, P., *Arch. Biochem. Biophys. 94*, 121 (1961).
27. Elek, A., *J. Am. Chem. Soc. 50*, 1213 (1928).

28. Embden, G., *Hoppe-Selyer's Z. physiol. Chem. 113*, 138 (1921).
29. Everett, W. W., Foster, J. F., *J. Am. Chem. Soc. 81*, 3459 (1959).
30. Fischer, E., *Ber. 49*, 584 (1916).
31. Fiske, C. H., *J. Biol. Chem. 46*, 285 (1921).
32. Fiske, C. H., Subbarow, J. S., *J. Biol. Chem. 66*, 375 (1925).
33. Fiske, C. H., Subbarow, J. S., *J. Biol. Chem. 81*, 629 (1929).
34. Fitting, C., Putman, E. W., *J. Biol. Chem. 199*, 573 (1952).
35. Flaschka, H., Holasch, A., *Hoppe-Selyer's Z. physiol. Chem. 289*, 279 (1952).
36. Flory, P. J., *Principles of Polymer Chemistry*. Cornell Univ. Press, Ithaca, N. Y. 1953 p. 344.
37. Folin, O., Ciocalteu, V., *J. Biol. Chem. 73*, 627 (1927).
38. Foster, J. F., Paschall, E. F., *J. Am. Chem. Soc. 75*, 1181 (1953).
39. French, D., Levine, M. L., Pazur, J. H., Norberg, E., *J. Am. Chem. Soc. 71*, 353 (1949).
40. French, D., *Adv. Carbohydrate Chem. 12*, 189 (1957).
41. French, D., *Bull. Soc. Chim. Biol. 42*, 1677 (1960).
42. Freudenberg, K., Kuhn, W., Dürr, W., Bolz, F., Steinbrunn, G., *Chem. Ber. 63*, 1510 (1930).
43. Fukui, T., Nikuni, Z., *Abstr. 7th Internat. Congr. of Biochem. Tokyo, Aug. 19–25*, 770 (1967).
44. Gatin-Gruzewska, Z., *Compt. rend. 146*, 540 (1908).
45. Georg, A., Pictet, A., *Helv. Chim. Acta 9*, 612 (1926).
46. Glenn, W. L., Myers, G. S., Grant, G. A., *J. Chem. Soc. 1951*, 2568.
47. Goldberg, S. H., *Biochim. Biophys. Acta 56*, 357 (1962).
48. Gornall, A. G., Bardawill, C. J., David, M. M., *J. Biol. Chem. 177*, 751 (1949).
49. Green, D. E., Stumpf, P. K., *J. Biol. Chem. 142*, 355 (1942).
50. Gusev, S. I., *Trudy Komissii Anal. Khim.*, Akad. Nauk S.S.S.R *3*, 215 (1951); *Chem. Abstr. 47*, 2642b (1953).
51. Hanes, C. S., *Proc. Roy. Soc. B128*, 421 (1939-40).
52. Hanes, C. S., *Proc. Roy. Soc. 129*, 174 (1940).
53. Hidy, P. H., Day, H. G., *J. Biol. Chem. 160*, 273 (1945).
54. Holló, J., Fejes, P., Szejtli, J., *Fette, Seifen, Anstrichmittel 57*, 923 (1955).
55. Holló, J., László, E., Gantner, G. S., Hoschke, Á., Szejtli, J., *Die Nahrung 7*, 35 (1963).
56. Holló, J., László, E., Hoschke, Á., *Die Stärke 16*, 243 (1964).
57. Holló, J., László, E., Hoschke, Á., Unpublished results.
58. Holló, J., László, E., Juhász, J., *Die Stärke 19*, 246 (1967).
59. Holló, J., Szejtli, J., *Die Stärke 10*, 248 (1958).
60. Holló, J., Szejtli, J., *Die Stärke 14*, 75 (1962); *Kolloid J.* (Russian) *20*, 227 (1958).
61. Hughes, R. C., Smith, E. E., Whelan, W. J., *Biochem. J. 88*, 63 (1963).
62. Husemann, E., Lippert, R., Pfannemüller, B., *Makromol. Chem. 26*, 214 (1958).
63. Husemann, E., Resz, R., *J. Polymer Sci. 19*, 389 (1956).
64. Illingworth, B., Cori, G. T., *Biochem. Preparations 3* (1953).
65. Jermyn, M. A., *Australian J. Biol. Sci. 15*, 233, 248 (1962).
66. Jones, R. W., Dimler, R. J., Rist, C. E., *J. Am. Chem. Soc. 77*, 1659 (1955).
67. Kabat, E. A., Glusman, M., Kuaub, V., *Am. J. Med. 4*, 653 (1948).
68. Kalckar, H. M., *J. Biol. Chem. 167*, 461 (1947).
69. Kamogawa, A., Fukui, T., Nikuni, Z., *Biochemistry 63*, 361 (1968).
70. Karrer, P., Boettcher, A., *Helv. Chim. Acta 36*, 570 (1953).
71. Keilich, G., Werner, R., *Biochem. Z. 342*, 217 (1967).
72. Kerr, R. W., *Arch. Biochem. 7*, 377 (1945).
73. Kerr, R. W., *J. Am. Chem. Soc. 67*, 2268 (1945).
74. Killion, P. J., Foster, J. F., *J. Polymer Sci. 46*, 65 (1960).
75. King, E. J., *Biochem. J. 26*, 292 (1932).
76. Kirk, E., *J. Biol. Chem. 106*, 191 (1934).
77. Kiessling, W., *Biochem. Z. 298*, 421 (1938).
78. Klages, F., *Chem. Ber. 65*, 368 (1932).
79. Kleinmann, H., *Biochem. Z. 174*, 43 (1926).
80. Koshland, D. E. Jr., in *The Enzymes*, Boyer, P., Lardy, H., Myrbäck, K., editors, Academic Press Inc. 2nd ed. Vol. 1, p. 305.

81. Kőrösy, F., Bárczay-Martos, M., *Nature 165*, 369 (1950).
82. Kuby, S. A., Noda, L., Lardy, H. A., *J. Biol. Chem. 209*, 191 (1954).
83. Kuhn, W., *Chem. Ber. 63*, 1503 (1930).
84. Kunkel, H. G., Ward, S. M., *J. Biol. Chem. 176*, 367 (1948).
85. Lansky, S., Kooi, M., Schoch, T. J., *J. Am. Chem. Soc. 71*, 4066 (1949).
86. László, E.: *Thesis* for the title of Cand. Chem., Budapest, 1968 (in Hungarian)
87. Lee, Y. P., *Biochim. Biophys. Acta 43*, 18 (1960).
88. Leiboff, S. L., *J. Biol. Chem. 79*, 611 (1928).
89. Leloir, L. F., Cardini, C. E., in *Methods in Enzymology*, Colowick, S. P., and Kaplan, N. O., editors, Academic Press, New York, 1957. Vol. 3, p. 850.
90. Lemieux, R. U., in *Methods in Carbohydrate Chemistry*, Whistler, R. L., Wolfrom, M. L. editors, Academic Press Inc. New York 1962, Vol. 1, p. 45,
91. Lewis, B. A., Smith, F., *J. Am. Chem. Soc. 79*, 3929 (1957).
92. Lindberg, B., *Acta Chem. Scand. 3*, 1355 (1949).
93. Ling, A. R., Nanni, D. R., *J. Chem. Soc. 123*, 2666 (1923).
94. Lipmann, F., Tuttle, L. C., *J. Biol. Chem. 153*, 571 (1944).
95. Lowry, O. H., Lopez, J. A., *J. Biol. Chem. 162*, 421 (1946).
96. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem. 193*, 265 (1951).
97. Maquenne, L., *Compt. rend. 138*, 213, 375 (1904).
98. Maquenne, L., Roux, E., *Compt. rend. 140*, 1303 (1905).
99. McCready, R. M., Hassid, W. Z., *J. Am. Chem. Soc. 66*, 560 (1944).
100. McDonald, D. L., *J. Org. Chem. 27*, 1107 (1962).
101. McKenzie, A. J., Dean, L. A., *Anal. Chem. 20*, 539 (1948).
102. Meyer, K. H., Brentano, W., Bernfeld, P., *Helv. Chim. Acta 23*, 845 (1940).
103. Meyer, K. H., Bernfeld, P., Wolff, E., *Helv. Chim. Acta 23*, 854 (1940).
104. Meyer, K. H., Gonon, W. F., *Helv. Chim. Acta 34*, 308 (1951).
105. Miller, G. L., *Anal. Biochem. 1*, 133 (1960).
106. Moore, S., Stein, W. H., *J. Biol. Chem. 176*, 367 (1948).
107. Muetgeert, J., *Adv. Carbohydrate Chem. 16*, 299 (1961).
108. Nakamura, G. R., *Anal. Chem. 24*, 1372 (1952).
109. Nakamura, M., *J. Agr. Chem. Soc. (Japan) 26*, 260 (1952); *Chem. Abrts. 46*, 11266a (1952).
110. Nakamura, M., *Nature 171*, 795 (1953).
111. Neuberg, C., Strauss, E., Lipkin, L. E., *Arch. Biochem. 4*, 101 (1944).
112. Norberg, E., French, D., *J. Am. Chem. Soc. 72*, 1202 (1950).
113. Nordin, P., French, D., *J. Am. Chem. Soc. 80*, 1445 (1958).
114. Novak, J. V. A., *Sbornik Nezinárod. Polarog. Sjezdu Praza, 1st Congr. 1951*, Part III.; *Chem. Abstr. 47*, 10408i (1953).
115. Pacsu, E., *J. Am. Chem. Soc. 63*, 1168 (1941).
116. Pazur, J. H., *J. Biol. Chem. 205*, 75 (1953).
117. Pazur, J. H., *J. Am. Chem. Soc. 77*, 1015 (1955).
118. Pazur, J. H., in *Methods in Carbohydrate Chemistry*, Whistler, R. L., Wolfrom, M. L., editors, Academic Press, New York 1962, Vol. 1, p. 335.
119. Peat, S., Whelan, W. J., Edwards, T. E., Owen, O., *J. Chem. Soc. 1958*, 586.
120. Peat, S., Whelan, W. J., Jones, G., *J. Chem. Soc. 1957*, 2490.
121. Peat, S., Whelan, W. J., Kroll, G. W. F., *J. Chem. Soc. 1956*, 53.
122. Peat, S., Whelan, W. J., Rees, W. R., *J. Chem. Soc. 1956*, 44.
123. Pfannemüller, B., *Die Stärke 20*, 351 (1968).
124. Posternak, T., *J. Am. Chem. Soc. 72*, 4824 (1950).
125. Posternak, T., *J. Biol. Chem. 180*, 1260ő (1950).
126. Pulley, A. O., French, D., *Biochem. Biophys. Res. Commun. 5*, 11 (1961).
127. Richter, M., Szejtli, J., *Die Stärke 18*, 95 (1966).
128. Robinson, H. W., Hogden, G. G., *J. Biol. Chem. 135*, 707 (1940).
129. Robyt, J. F., French, D., *Arch. Biochem. Biophys. 100*, 451 (1963).
130. Roche, M. J., *Bull. soc. chim. biol. 10*, 1061 (1928).
131. Roskin, L. D., *Zav. lab. 5*, 267 (1936).
132. Samec, M., Mayer, A., *Kolloidchem. Beih. 13*, 272 (1921).
133. Sato, A., *J. Biol. Chem. 35*, 473 (1928).
134. Schell, C., *Z. anal. Chem. 105*, 256 (1936).

135. SCHOCH, T. J., *J. Am. Chem. Soc. 64*, 2957 (1942). *Cereal Chem. 18*, 121 (1941).
136. SCHMIDT, O. T., in *Methods in Carbohydrate Chemistry*, WHISTLER, R. L., WOLFROM, M. L., editors, Academic Press, New York 1963, Vol. 2, p. 318.
137. SOWDEN, J. C., SPRIGGS, A. S., *J. Am. Chem. Soc. 78*, 2503 (1956).
138. SPEISER, R., WHITTENBERGER, R. T., *J. Chem. Phys. 13*, 349 (1953).
139. STERN, A., *Ind. Eng. Chem., Anal. Ed. 14*, 74 (1942).
140. SUGINO, Y., MIYOSHI, Y., *J. Biol. Chem. 239*, 2360 (1964).
141. SUMMER, R., FRENCH, D., *J. Biol. Chem. 222*, 469 (1956).
142. SUMNER, J. B., SOMERS, G. F., *Arch. Biochem. 4*, 11 (1944).
143. SZEJTLI, J., *Dissertation*, Budapest, 1959.
144. TANRET, C., *Compt. rend. 158*, 1353 (1914).
145. TANRET, C., *Bull. soc. chim. 17*, 83 (1915).
146. THOMA, J. A., WRIGHT, H. B., FRENCH, D., *Arch. Biochem. Biophys. 85*, 452 (1959).
147. THOMPSON, A., WOLFROM, M. L., QUINN, E. J., *J. Am. Chem. Soc. 75*, 3003 (1953).
148. TIPPLES, K. H., TKACHUCK, R., *Cereal Chem. 42*, 111 (1965).
149. ULMANN, M., *Makromol. Chem. 9*, 76 (1952).
150. ULMANN, M., *Die Stärke 14*, 175 (1962).
151. VÁMOS, E., *Kromatográfia*, Budapest 1959, (in Hungarian)
152. WALLENFELS, K., personal communication.
153. WALKER, G. J., WHELAN, W. J., *Biochem. J. 67*, 548 (1957).
154. WALKER, G. J., WHELAN, W. J., *Biochem. J. 76*, 257, 264 (1960).
155. WARBURG, O., CHRISTIAN, W., *Biochem. Z. 310*, 384 (1941).
156. WEIBULL, G., TISELIUS, A., *Arkiv Kemi, Mineral, Geol. 19A*, 1 (1945).
157. WEICHSELBAUM, T. E., *Am. J. Clin. Pathol. Suppl. 10*, 40 (1946).
158. WHELAN, W. J., BAILEY, J. M., ROBERTS, P. J. P., *J. Chem. Soc. 1953*, 1293.
159. WHELAN, W. J., BAILEY, J. M., ROBERTS, P. J. P., *J. Chem. Soc. 1953*, 1298.
160. WHELAN, W. J., BAILEY, J. M., *Biochem. J. 58*, 560 (1954).
161. WHELAN, W. J., in *Handbuch der Pflanzenphysiologie*, RUHLAND, W. R. editor, Springer Verlag, Berlin. 1958. Vol. 6, p. 197.
162. WHELAN, W. J., *Die Stärke 12*, 358 (1960).
163. WHISTLER, R. L., *J. Am. Chem. Soc. 76*, 1671 (1954).
164. WHISTLER, R. L., *J. Am. Chem. Soc. 77*, 1017 (1955).
165. WHISTLER, R. L., *J. Am. Chem. Soc. 77*, 5761 (1955).
166. WHISTLER, R. L., BEMILLER, J. N., in *Methods in Carbohydrate Chemistry*, WHISTLER, R. L., WOLFROM, M. L., editors, Academic Press, New York 1962. Vol. 1, p. 42.
167. WHISTLER, R. L., BEMILLER, J. N., in *Methods in Carbohydrate Chemistry*, WHISTLER, R. L., WOLFROM, M. L., editors, Academic Press, New York 1962. Vol. 1, p. 47.
168. WHISTLER, R. L., HICKSON, J. L., *Anal. Chem. 27*, 1514 (1955).
169. WHISTLER, R. L., HILBERT, G. E., *J. Am. Chem. Soc. 67*, 1161 (1945).
170. WIEGEL, E., *Z. Physik. Chem. A 188*, 137 (1941); *Kolloid-Z. 102*, 145 (1943).
171. WOLFROM, M. L., LASETTREE, E. N., O'NELL, A. N., *J. Am. Chem. Soc. 73*, 595 (1951).
172. WOOD, H. B., DIEHL, H. W., FLETHER, H. G., *J. Am. Chem. Soc. 79*, 1986 (1959)

LIST OF SYMBOLS

a	initial concentration of G-1-P (of Arreguin *et al.*)
a	thermodynamic activity
A	equilibrium amount of inorganic phosphate
A%	light adsorption %
A_p	amylopectin
AMP	adenosine-5-monophosphate
ATP	adenosine-5-triphosphate
dpK	difference of the pK values measured at the temperatures
dT	temperature difference
D_{20}	diffusion constant
D_{260}	optical density at 260 mμ
D_{280}	optical density at 280 mμ
DEAE-cellulose	diethylaminoethyl-cellulose
D-enzyme	disproportional enzyme of plant
DP	degree of polymerization
DP_n	number-average degree of polymerization
DP_w	weight-average degree of polymerization
E	symbol of enzyme
[E]	enzyme concentration
E_3	activation energy
ΔE	change of activation energy
ΔE	changes of extinction
EDTA	ethylenediaminotetraacetic acid
ES	symbol of enzyme-substrate complex
[ES]	enzyme-substrate complex
[ES]*	activated enzyme-substrate complex
[ES_1]*	activated [ES_1]
[ES_2]*	activated [ES_2]
[ES_2']*	activated [ES_2']
[E + P]*	activated [E + P]
[E + S]*	activated [E + S]
[E + S_1]*	activated [E + S_1]
[E + S_1']*	activated [E + S_1']
[E + S_2]*	activated [E + S_2]
[E + S_2']*	activated [E + S_2']
f/f_0	frictional ratio
G-1-P	glucose-1-phosphate
G-1-P · K_2 · $2H_2O$	dipotassium glucose-1-phosphate dihydrate

G-OX	donor substrate
G_n	initial, 'primary' molecule
ΔH	change in heat content (enthalpy)
ΔH^*	activation enthalpy
ΔH_i	heat of ionization (cal/mole)
H-OR	acceptor substrate
$[I]$	inhibitor concentration
I/I_0	ratio of light intensity
IAA	iodoacetamide
J_p	integral weight ratio to P degree of polymerization
k	rate constant
k_1	rate constant in the formation of ES
k_2	rate constant in the dissociation of ES
k_3	rate constant in the formation of product
K	equilibrium constant
°K	absolute temperature
K_1-K_8	equilibrium constants (of Florini and Vestling)
K_1	dissociation constant for phosphoric acid
K_2	dissociation constant for G-1-P acid
K_a	Michaelis constant for G-1-P or inorganic phosphate
K_b	Michaelis constant for amylopectin
K_i	inhibitor constant
K_m	Michaelis constant
K'_m	apparent Michaelis constant (of Pfanemüller)
l	cuvette length in cm
mA	milliampere
mμ	wave length
M	mole
M	mole/litre
M.W.	molecular weight
n	number of glucose units in the amylose formed
n	number of molecules of substrate
NADP	nicotinamide-adenine-dinucleotide phosphate
NEMI	N-ethylmaleic imide
*p*CMB	*p*-chloromercuribenzoate
pH_{50}	pH-range at half velocity
pK	$-\log K$
pK_a	$-\log K_a$
pK_b	$-\log K_b$
P	symbol of product
P_i	inorganic phosphate
P_{org}	organic phosphate
PLP	pyridoxal-5′-phosphate
PR enzyme	phosphorylase phosphatase
Q_{10}	temperature coefficient
Q-enzyme	branching enzyme of plant
rpm	revolution per minute
R	1·986 cal/°C mole gas constant

$ROPO_3H_2$	glucose-1-phosphoric acid
S	symbol of substrates
$[S]$	substrate concentration
$[S_1]$	amylopectin or glycogen substrate
$[S'_1]$	amylopectin or glycogen substrate with a molecular weight lower by one glucose unit
$[S_2]$	inorganic phosphate substrate
$[S'_2]$	glucose-1-phosphate substrate
S_{20}	sedimentation constant
t	reaction time (minute)
tan α	slope
T	295 °K absolute temperature
TN	turnover number
tris	tris-(hydroxymethyl)aminomethane
v_0	initial velocity
v_i	initial velocity of inhibited reaction
V_{20}	partial specific volume
V_{max}	maximal rate
V'_{max}	apparent maximum rate (of Pfanemüller)
$V_{max,a}$	maximal rate for donor substrates
$V_{max,b}$	maximal rate for acceptor substrates
x	the amount of products liberated during the reaction
x	the concentration of G-1-P reacted up to time t (of Arreguin *et al.*)
x_e	equilibrium amounts of products
$\frac{x}{n}$	the concentration of amylose formed during t time (of Arrenguin *et al.*)
ε	extinction value
$\Delta\varepsilon/\varepsilon$	dissymmetry factor (of Kuhn)
λ_{max}	maximal wavelength of adsorption curves
η_0	the same value extrapolated to zero time
η_{60}	relative viscosity after a reaction period of 60 minutes
η_{rel}	relative viscosity of hydroxyethylamylose solution
η_{spec}	specific viscosity
Φ_0, Φ_1, Φ_2, Φ_{12}	Dalziel constants in phosphorolysis
Φ'_0, Φ'_1, Φ'_2, Φ'_{12}	Dalziel constants in synthesis

AUTHOR INDEX

C

D

E

F

G

H

L

M

N

O

P

Q

R

S

T

U

SUBJECT INDEX

Previous volumes published in the Series

RECENT DEVELOPMENTS IN THE CHEMISTRY OF NATURAL CARBON COMPOUNDS

Vol. 1.

FODOR, G.:
New Methods and Recent Developments of the Stereochemistry of Ephedrine, Pyrrolizidine, Granatane and Tropane Alkaloids

NÁDOR, K.:
Relationships Between the Structure and Pharmacological Activity of Tropeines

TORGOV, I. V.:
Achievements in the Total Synthesis of Natural Steroids

In English — 319 pages — 155 figures — 46 tables — Numerous tables of chemical formulae — 17×25 cm — Cloth

Vol. 2.

SHEMYAKIN, M. M. and OVCHINNIKOV, YU. A.:
The Chemistry of Natural Depsipeptides

OGNYANOV, I. and IVANOV, D.:
Constituents of the Bulgarian Zdravets Oil

SZÁNTAY, CS.:
Structure and Synthesis of Ipecac Alkaloids

In English — 129 pages — 3 figures — 8 tables — 17×25 cm — Cloth

Distributors
KULTURA
Budapest 62. P.O.B. 149