The Specificity of Muscle Phosphorylase

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In 1940, Cori & Cori showed that glucose inhibited the activity of a purified cat-liver preparation possessing phosphorylase activity, whereas mannose, galactose, maltose, fructose and glucose-6-phosphate had no significant inhibitory effect. Further, from the effect of freshly prepared glucose solutions on the same preparation, they suggested that while α -glucose competitively inhibited the enzyme, β -glucose had little or no inhibitory effect. It seemed probable, therefore, that there was an α , β specificity of the enzyme for the sugar.

In 1943, Green & Cori obtained a crystalline phosphorylase preparation from rabbit muscle. Subsequently, Cori, Cori & Green (1943) showed that the synthesis of polysaccharide from α -glucose-1-phosphate with this enzyme was also competitively inhibited by glucose. In order to determine whether the crystalline enzyme also possessed an α , β specificity the effect of various glucosides, particularly α - and β -methylglucoside, has now been investigated. Moreover, if it is true that a competitive inhibitor of an enzyme combines with the same part of the enzyme surface as its natural substrate, it seemed possible that by determining the inhibitory effect of various sugars and related substances the stereoisomerism and nature of the groups involved in the formation of the enzyme-substrate complex might be elucidated.

METHODS AND MATERIALS

The preparation of the crystalline muscle phosphorylase followed closely the method described by Green & Cori (1943) with the modifications adopted by Campbell (1952). The latter paper also contains a full description of the methods adopted in the present experiments for following the course of the synthesis of polysaccharide from α -glucose-1-phosphate. The concentration of the test substance in the reaction mixture was usually 0.05 M. The inhibitory effect of the test substance was compared with that of a solution of glucose of similar concentration under identical conditions.

The α -glucose-1-phosphate was prepared enzymically from starch with potato phosphorylase according to the method of Hanes (1939).

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In the case of the commoner substances whose inhibitory properties were tested laboratory reagent samples were employed. Where special methods were used the following details are given.

1:2-isoPropylidene-D-glucose and 1:2:5:6-diisopropylidene-D-glucose. These substances were prepared according to the method of Bell (1935).

Gluconic acid. Calcium gluconate (1.12 g.) was dissolved in 10 ml. of water, and 0.4 g. of Na_2SO_4 (anhydrous) was added. The precipitated CaSO_4 was filtered off and the pH of the clear solution adjusted to 7.0.

Ascorbic acid. The ascorbic acid solution was brought to pH 6.7 by the addition of N-NaOH before use.

β -Methyl-D-glucoside. This substance (4 g.) was prepared from 20 g. of acetobromoglucose by the method of Koenigs & Knorr (1901). The product was crystallized from ethanol and an aqueous solution had $[\alpha]_D^{17} + 32.23^\circ \pm 0.01$.

α -Phenylglucoside. Tetraacetyl α -phenylglucoside was prepared from α -pentaacetyl glucose by the method of Montgomery, Richtmyer & Hudson (1942), and saponified by the technique of Zemplén & Pacsu (1929). The product was crystallized from a mixture of equal proportions of ethanol and ether; an aqueous solution had $[\alpha]_D^{20} + 172^\circ$.

β -Glucose-1-phosphoric acid (Ba salt). This substance was prepared from acetobromoglucose in 19% yield by the method of Reithel (1945). An aqueous solution of the purified Ba salt had $[\alpha]_D^{20} + 9.4^\circ$.

L-Talose and D-allose. Samples of these substances were very kindly supplied by Prof. T. Reichstein of Basle University.

RESULTS

The following substances were found to have no significant inhibitory effect on the synthesis of a polysaccharide by the crystalline muscle phosphorylase preparation at a concentration of 0.05 M: D-xylose, D-mannose, D-galactose, D-fructose, L-talose, D-allose, maltose, sucrose, *isopropylidene*-D-glucose, gluconic acid, sorbitol, inositol and ascorbic acid. In addition, *diisopropylidene*-D-glucose failed to show any inhibitory effect at a concentration of 0.02 M.

At a concentration of 0.05 M- α -methylglucoside significantly inhibited the synthesis of a polysaccharide from α -glucose-1-phosphate with the crystalline muscle phosphorylase preparation as compared with a control reaction mixture. The various samples of α -methylglucoside used were found to contain a little free reducing sugar as determined by the Hagedorn & Jensen (1923a, b)

estimation. On the assumption that the reducing sugar was glucose a similar amount of glucose was added to the control reaction mixture so that the reducing powers of the control and α -methylglucoside reaction mixtures would be the same.

Fig. 1. Inhibition of polysaccharide synthesis by α -methylglucoside and glucose. Crystalline phosphorylase preparation incubated with: α -glucose-1-phosphate, 0.016M; cysteine-glycerophosphate buffer, 0.015M, pH 6.8; glycogen 1%. Δ , +glucose 0.009M (to compensate for glucose in α -methylglucoside solution); $\square$, + α -methylglucoside 0.05M; $\bullet$, +glucose 0.05M. At 30°.

Fig. 2. Failure of β -methylglucoside to inhibit polysaccharide synthesis. Crystalline phosphorylase preparation incubated with: α -glucose-1-phosphate, 0.016M; cysteine-glycerophosphate buffer, 0.015M, pH 6.8; glycogen 1%. Δ , in absence of added sugar; $\square$, in presence of β -methylglucoside 0.05M. At 30°.

Under similar conditions a 0.05M solution of β -methylglucoside showed no significant inhibitory effect. These results are shown graphically in Figs. 1 and 2. It will be observed in Fig. 1 that the inhibitory effect of α -methylglucoside is considerably less than that of glucose at a similar concentration. This inhibitory effect was not due to the partial breakdown of α -methylglucoside to glucose, since the reducing power of the reaction mixture did not increase during the course of the reaction. A similar inhibitory effect might also have been caused by a change in the pH during the course of the reaction. However, no significant change in the pH, as

Fig. 3. Effect of concentration of α -methylglucoside on inhibition of polysaccharide synthesis. Crystalline phosphorylase preparation incubated with: α -glucose-1-phosphate, 0.016M; cysteine-glycerophosphate buffer, 0.015M, pH 6.8. Δ , α -methylglucoside 0.1M; $\square$, α -methylglucoside 0.2M. The upper curve of each pair is a control containing glucose equivalent in amount to the reducing substance in the α -methylglucoside solution. At 30°.

Fig. 4. Effect of substrate concentration on inhibition of polysaccharide synthesis by α -methylglucoside. Crystalline phosphorylase preparation incubated with: α -glucose-1-phosphate, varying concentrations; cysteine-glycerophosphate buffer, 0.015M, pH 6.8; glycogen 1%. $\square$, in absence of α -methylglucoside; Δ , in presence of α -methylglucoside, 0.2M. At 30°.

measured with a glass electrode, took place. It was concluded, therefore, that α -methylglucoside had a direct inhibitory action on the enzyme.

The results shown graphically in Fig. 3 indicate that the inhibitory effect of α -methylglucoside is proportional to its concentration in the reaction mixture. Fig. 4 shows that an increase in the substrate concentration at a given concentration of α -methylglucoside leads to a decrease in the inhibition. This is the type of result which would be

Fig. 5. Effect of α -phenylglucoside on polysaccharide synthesis. Crystalline phosphorylase preparation incubated with: α -glucose-1-phosphate, 0.014 M; cysteine-glycerophosphate buffer, 0.015 M, pH 6.8; glycogen 1%. □, + glucose 0.25 mM (to compensate for glucose in α -phenylglucoside); Δ, + α -phenylglucoside 0.05 M. At 30°.

Fig. 6. Effect of β -glucose-1-phosphate on polysaccharide synthesis. Crystalline phosphorylase preparation incubated with: α -glucose-1-phosphate, 0.014 M; cysteine-glycerophosphate buffer, 0.015 M, pH 6.8; glycogen 1%. Δ, + glucose 2.0 mM (to compensate for glucose in β -glucose-1-phosphate); □, + β -glucose-1-phosphate 0.03 M. At 30°.

expected if α -methylglucoside were acting as a competitive inhibitor by competing with α -glucose-1-phosphate for the active groups of the enzyme. The maximum velocity of the reaction was determined for different concentrations of α -methylglucoside by the graphical method of Lineweaver & Burk (1934). Although the slope of the line obtained by plotting $1/V$ against $1/[S]$, where V = maximum velocity and S = the substrate concentration, did appear to depend on the inhibitor concen-

tration, it was not possible to show that the maximum velocity remained constant, which should have been the case if the inhibition had been competitive.

That α -phenylglucoside at a concentration of 0.05 M was also slightly inhibitory is shown in Fig. 5.

In order to check whether polysaccharide synthesis by phosphorylase from either α -methylglucoside or α -phenylglucoside was possible, these glucosides were incubated with the enzyme with the usual reaction mixture except for the absence of α -glucose-1-phosphate. Qualitative tests for the presence of amylose, by the addition of iodine solution, were carried out after 1 and 24 hr. incubation. In neither case was there any evidence of the synthesis of a blue-staining polysaccharide with iodine in the reaction mixture, so that it was concluded that neither α -methyl- nor α -phenylglucoside are suitable substrates for phosphorylase.

The effect of β -glucose-1-phosphate is shown in Fig. 6. At a concentration of 0.03 M no significant inhibitory effect was observed.

DISCUSSION

The fact that the α isomer of methylglucoside inhibits phosphorylase, whereas the β isomer does not, confirms the presence of an α , β specificity for phosphorylase, a fact that was originally suggested by Cori & Cori (1940) as a result of their experiments with a freshly prepared solution of glucose. This α , β specificity is further confirmed by the results obtained with α -phenylglucoside and β -glucose-1-phosphoric acid. In the latter case it seems that not only is the β isomer an unsuitable substrate for phosphorylase, but that it also fails to act as a competitive inhibitor.

The inability to demonstrate rigorously that α -methylglucoside competitively inhibits phosphorylase is probably due to the complex nature of the reaction mixture. Cori *et al.* (1943) have shown that while cysteine increases the activity of phosphorylase, sodium glycerophosphate acts as an inhibitor and since both these substances were present in the reaction mixture used in the present experiments, the effect of any other inhibitor must be complicated. It would seem desirable in any further work to arrange the conditions so that there is only one inhibitor present in the reaction mixture, e.g. by the use of a solution of KCl instead of sodium glycerophosphate (Green & Cori, 1943).

It is believed that a competitive inhibitor combines with the same active group of an enzyme as does its natural substrate, but with the formation of an inactive, instead of an active, enzyme complex. As far as is known, the only substance that will form an active enzyme complex with phosphorylase, that is one leading to polysaccharide synthesis, is α -D-glucose-1-phosphate. However, other gluco-

sides are able to unite with the active centres of phosphorylase, as is shown in these experiments by the fact that polysaccharide synthesis from α -D-glucose-1-phosphate is inhibited when the aglycone consists of hydroxyl (α -D-glucose), methyl or phenyl radicals, affinity for the enzyme decreasing in the order given. It is assumed that the enzyme complex formed with these glucosides is inactive since it was not possible to demonstrate the synthesis of a blue-staining polysaccharide with iodine when the enzyme was incubated with the glucosides in the absence of α -glucose-1-phosphate. If the aglycone be glucose (maltose) or fructose (sucrose) no inhibition occurs. Possibly the size of the aglycone is important in this gradual loss of affinity.

The effect of ascorbic acid was tested, for Gill & Lehmann showed in 1939 that this substance inhibited the formation of Robison ester from glycogen with a crude rabbit-muscle extract showing phosphorylase activity. However, no evidence was obtained from the present experiments that ascorbic acid had any significant effect on the rate of polysaccharide synthesis from α -glucose-1-phosphate with a crystalline muscle phosphorylase preparation.

Apart from the glucosides, of the substances tested only glucose inhibited phosphorylase. Since sorbitol and inositol were without effect a pyranose structure appears to be necessary for the substance to be inhibitory. The failure of D-xylose indicates the necessity for a primary alcohol group on C₍₅₎, and that of D-mannose, D-galactose, D-allose and D-talose that the configuration of the groups attached to C₍₂₎, C₍₃₎ and C₍₄₎ must be as in glucose (cf. Gottschalk, 1947, 1950). The importance of the configuration of the groups attached to C₍₅₎ in glucose could be tested if β -L-idose were available, for this sugar has exactly the same configuration as α -D-glucose except on C₍₅₎ where the position of the attached group is reversed. The effect of substituting a methoxyl for a hydroxyl group in glucose has already been demonstrated by the lack of inhibitory effect of 3-methyl glucose (Campbell, 1952).

The failure of D-fructose to inhibit phosphorylase can be interpreted in a number of ways. First, any furanose form of this sugar which may be present in solution would understandably not act as an inhibitor as its ring C atoms would be planar and not somewhat kinked as in a pyranose structure. Secondly, on the basis that C₍₁₎ of D-glucopyranose should be compared with C₍₁₎ of D-fructopyranose the configuration of the groups attached to the C atom is different in the two molecules. Thirdly, there is a primary alcohol group on C₍₅₎ of glucose but not on C₍₆₎ of fructose. Finally, Pigman & Goepp (1948) suggest that it is the β isomer of D-fructose which predominates.

It has been shown that the substitution of the hydroxyl groups on C₍₂₎, C₍₃₎, or C₍₄₎ by methoxyl or tosyloxy groups renders a β -glucoside resistant to the action of many β -glucosidases (Helferich & Grünler, 1937; Pigman & Richtmyer, 1942). However, it is possible to make substitutions by replacement of OH by H, F, Br or Cl at C₍₆₎ of β -glucosides without rendering the glucoside resistant to hydrolysis by β -glucosidases (Helferich, Grünler & Gnüchtel, 1937). It might be thought that the same consideration would apply in the case of substitutions on C₍₆₎ of glucose and its ability to combine with phosphorylase. Experiments designed to check this possibility would be of great interest, although Cori & Cori (1940) have shown that glucose-6-phosphate did not inhibit a purified cat liver phosphorylase preparation.

It seems, therefore, that the only substances which are able to combine with the active centres of muscle phosphorylase are α -D-glucopyranose and certain glucosides, with the possible exceptions already mentioned. In no circumstances can the aglycone of an inhibitory glucoside be another sugar molecule, except of course in the case of glycogen and similar polysaccharides which are degraded by phosphorylase.

SUMMARY

1. The following substances have been shown to have no significant inhibitory effect on the synthesis of a polysaccharide from α -glucose-1-phosphoric acid with a crystalline muscle phosphorylase preparation under the experimental conditions used: D-xylose, D-mannose, D-galactose, D-fructose, L-talose, D-allose, maltose, sucrose, *isopropylidene*-D-glucose, *diisopropylidene*-D-glucose, gluconic acid, sorbitol, inositol, ascorbic acid, β -glucose-1-phosphoric acid and β -methylglucoside.

2. Under identical experimental conditions the following substances significantly inhibited the reaction: D-glucose, α -methylglucoside and α -phenylglucoside.

3. The significance of these results in the elucidation of the specificity of phosphorylase is discussed.

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Studies on the Biochemistry of Zinc

3. FURTHER INVESTIGATIONS ON THE INFLUENCE OF ZINC ON METABOLISM

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Changes in the liver and bones and in the metabolism of nitrogen, phosphorus and sulphur in rats maintained on a stock diet supplemented with zinc have already been reported (Sadasivan, 1951*a*, *b*). It was also observed that on a high-fat, low-protein diet, zinc supplements caused a lowering in fat content of rat livers. In this paper, results are presented of investigations on the metabolism of nitrogen, phosphorus and sulphur and the urinary excretion of uric acid and total creatinine (creatinine and creatinine) in rats fed on a high-fat, low-protein diet supplemented with 0.5 and 1.0% of zinc oxide. Results of experiments on the influence of the element on the assimilation of fat from the intestines and on the activity of the phosphatases and lipase in the system are also included.

EXPERIMENTAL

Metabolism experiments. The details of the metabolism experiments have already been described in the previous paper in this series. In the present experiments, the high-fat, low-protein diet described in Part 1 was fed to rats. The daily food intake was recorded and calculated for each 3-day period. The collection of urine, faeces, etc., and their analyses were carried out in the same manner as described in Part 2 (Sadasivan, 1951*b*). The first three groups of young rats weighed about 40–50 g. each and were about a month

old, while groups 4 and 5 were older ones weighing between 90 and 100 g. and 2–2.5 months old. Younger and older rats were compared because preliminary observations suggested that younger rats are probably more susceptible to the effects of zinc than older ones.

For the experiment on fat metabolism, each group consisted of six rats equally distributed between the sexes and litters. Both the groups were fed the high-fat diet, the second group receiving, in addition, ZnO at 0.5% level. The faeces were collected for each 3-day period over the experimental period of 15 days and the fat content of the dried faeces was determined by extraction with ether in a Soxhlet extractor. For the determination of fat in the liver, the animals were killed at the end of the experiment. The liver was removed, freed of blood by pressing lightly against blotting paper and ground with a small amount of acetone in a mortar. Fat was then extracted in a Soxhlet extractor with acetone.

Determination of phosphatase activity. Two groups of six rats, each weighing between 60 and 80 g., were fed the stock diet described in Part 1. The second group received, in addition, ZnO at 1.0% level of diet. At the end of 15 days, the animals were killed and liver, small intestines, kidneys and femurs were removed for the determination of phosphatase activity. The tissues were freed of blood by washing once with distilled water and femurs cleaned of adhering flesh. Portions of the tissues and the whole of the femurs were prepared for determination of activity according to the method of Bodansky (1937) and of Martland & Robison (1929). Activity was measured in sample portions of the material suspended in 0.033M-veronal-acetate buffer adjusted to a pH of 9.1. Sodium β -glycerophosphate at concentrations of 1.0% served as substrate and the mixture

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