source of N^6-(aminoacyl)adenosine. There have been previous indications that yeast RNA contains amino acids which are relatively firmly bound. Ingram and Sullivan (1962) showed that yeast s-RNA contains amino acids, at a level of about one per molecule of s-RNA, which are quite distinct from the amino acids involved in the s-RNA–amino acid exchange. It is possible that the amino acid–RNA complexes described by these workers as well as by others (Ishihara, 1960; Jonsen *et al.*, 1959) are in actual fact the source of the N^6-(aminoacyl)adenosine group described in this paper. The small amount of the N^6-(aminoacyl)adenosines isolated in the present work is not necessarily a true representation of the levels found in nature, as undoubtedly during the isolation procedure some degradation and fractionation of this group occurred.

ACKNOWLEDGMENT

The author wishes to thank Dr. G. Tritsch for making the quantitative amino acid analysis and Dr. O. Roholt for help with the paper chromatography and identification of the amino acids. He also wishes to acknowledge the skilled technical assistance of Mr. J. Mozejko, Mr. L. Stasiuk and Mrs. H. Wilamowski. The author wishes to acknowledge the interest and encouragement of Dr. C. A. Nichol in this program.

REFERENCES

Bogdanov, A. A., Prokof'ev, M. A., Antonovich, E. G., Terganova, G. V., and Anisimova, V. M. (1962), *Biokhimiya 27*, 266.
Brink, J. J., and Schein, A. H. (1963), *J. Med. Chem. 6*, 563.
Carter, C. E., and Cohen, L. H. (1955), *J. Am. Chem. Soc. 77*, 499.
De Kleot, S. R., Schuurs, A. H., Koningsberger, V. V., and Overbeck, J. Th. G. (1960), *Koninkl. Ned. Akad. Wetenschap. Proc., Ser. C: 63*, 374.
Dunn, D. B. (1961), *Biochim. Biophys. Acta 46*, 198.
Habermann, V. (1959), *Biochim. Biophys. Acta 32*, 297.
Hall, R. H. (1962), *J. Biol. Chem. 237*, 2283.
Hall, R. H. (1963), *Biochim. Biophys. Acta 68*, 278.
Harris, G., and Wiseman, A. (1962), *Biochim. Biophys. Acta 55*, 374.
Holley, R. W., Apgar, J., Doctor, B. P., Farrow, J., Marini, M. A., and Merril, S. H. (1961), *J. Biol. Chem. 236*, 200.
Ingram, V. M., and Sullivan, E. (1962), *Biochim. Biophys. Acta 61*, 583.
Ishihara, H. (1960), *J. Biochem. (Tokyo) 47*, 196.
Jonsen, J., Laland, S., Smith-Kielland, I., and Sömme, R. (1959), *Acta Chem. Scand. 13*, 838.
Potter, J. L., and Dounce, A. (1956), *J. Am. Chem. Soc. 78*, 3078.
Preiss, J., Berg, P., Ofengand, E. J., Bergmann, F. H., and Dieckman, M. (1959), *Proc. Nat. Acad. Sci. U. S. 45*, 319.
Ralph, K. K., and Khorana, H. G. (1961), *J. Am. Chem. Soc. 83*, 2926.
Schuurs, A. H., and Koningsberger, V. V. (1960), *Biochim. Biophys. Acta 44*, 167.
Shabarova, Z. A., Polyakova, Z. P., and Prokof'ev, M. A. (1959), *J. Gen. Chem. (U.S.S.R.) Eng. Transl. 29*, 215.
Smith, M., Rammler, D. H., Goldberg, I. H., and Khorana, H. G. (1962), *J. Am. Chem. Soc. 84*, 430.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Wilken, D. R., and Hansen, R. G. (1961), *J. Biol. Chem. 236*, 1051.
Zachau, H. G., Acs, G., and Lipmann, F. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 885.

Studies on UDPG: α-1,4-Glucan α-4-Glucosyltransferase. VI. Specificity and Structural Requirements for the Activator of the *D* Form of the Dog Muscle Enzyme*

M. ROSELL-PEREZ† AND J. LARNER

From the Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland 6, Ohio

Received January 8, 1964

The present paper of this series deals with the specificity of the activator molecule and demonstrates some of the requirements that are necessary for activation. Of over thirty compounds tested, glucose-6-phosphate was the most potent activator. Important structural features of the molecule include the hydroxyl groups at carbons 2, 4, and 3, the phosphate attached at carbon 6, and a pyranose ring structure.

The enzyme UDPG: α-1,4-glucan α-4-glucosyltransferase (transferase)[1] catalyzes the transfer of the glucosyl residue from UDPG into glycogen. Leloir *et al.* (1959) reported that transferase from rat skeletal muscle was activated by glucose-6-phosphate, glucosamine-6-phosphate, galactose-6-phosphate, and fructose-6-phosphate. The last three compounds activated to a lesser extent than glucose-6-phosphate (Traut, 1962). Leloir and Goldemberg (1960) demonstrated that the enzyme prepared from liver was also activated by these sugar phosphates (Steiner *et al.*, 1961).

Two forms of the enzyme were prepared and partially purified from rat and rabbit skeletal muscle (Rosell-Perez and Larner, 1964a; Rosell-Perez *et al.*, 1962). They were differentiated from each other by the kinetics of the UDPG concentration dependence in the presence and absence of glucose-6-phosphate. In the case of the *D* (dependent) form of the enzyme, the activity without added glucose-6-phosphate was low or absent. In the presence of glucose-6-phosphate the *V* for UDPG was markedly increased. The K_m

* Papers I through V of this series were titled "Studies on UDPG-α-glucan Transglucosylase. This work was supported in part by a grant from the National Institute of Arthritis and Metabolic Diseases of the United States Public Health Service (A-2366) and by a research career award (K6-AM-985) from the National Institutes of Health.

† Present address: Catedra de Bioquimica, Facultad de Farmacia, Universidad de Barcelona, Pedralbes-Barcelona, Spain.

[1] Abbreviations used in this work: UDPG, uridine diphosphoglucose; Tris, tris(hydroxymethyl)aminomethane; transferase, UDPG-α-1,4-glucan transferase; TPN, nicotinamide adenine dinucleotide phosphate; DEAE, diethylaminoethyl.

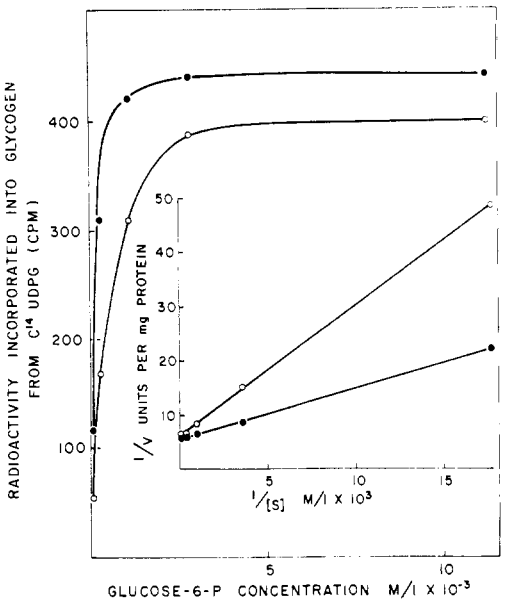

FIG. 1.—The direct and reciprocal plots of the glucose-6-phosphate concentration dependence of *D* form of dog muscle transferase. Lines between open circles represent glucose-6-phosphate alone, and those between the solid circles have 0.008 M $MgCl_2$. Assay conditions were standard. In the reciprocal plot the enzyme unit is that recommended by the Commission on Enzymes of the International Union of Biochemistry.

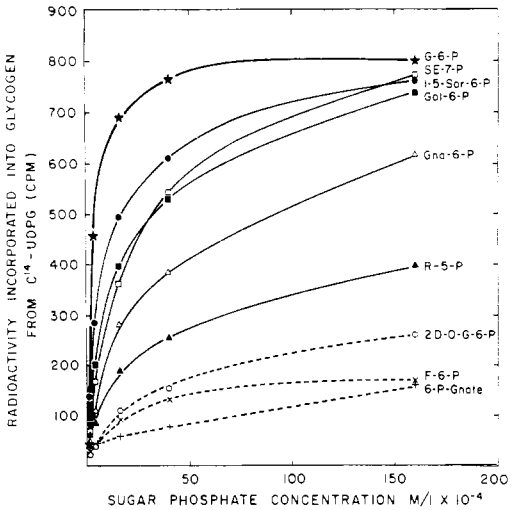

FIG. 2.—The action of glucose-6-phosphate and other sugar phosphate derivatives on the *D* form of dog muscle transferase. Assay conditions were standard. Abbreviations used are SE-7-P, sedoheptulose-7-phosphate; 1,5-Sor-6-P, 1,5-sorbitan-6-phosphate; Gal-6-P, galactose-6-phosphate; Gna-6-P, glucosamine-6-phosphate; R-5-P, ribose-5-phosphate; 2D-O-G-6-P, 2-deoxyglucose-6-phosphate; F-6-P, fructose-6-phosphate; 6-P-Gnate, 6-phosphogluconate; 2P-G-A, 2-phosphoglycerate; P-P, inorganic pyrophosphate; Me-P, methyl phosphate.

was decreased either slightly in the case of the rat enzyme, or more noticeably in the case of the rabbit enzyme. With the *I* (independent) form, activity was present in the absence of glucose-6-phosphate. In the presence of glucose-6-phosphate, the K_m for UDPG was decreased, but *V* was unchanged.

In an attempt to establish the structural requirements of the activator molecule, an extensive study of a number of phosphorylated compounds was undertaken. These were tested on the enzyme from dog muscle, since it is a ready source of the *D* form of the enzyme that is totally dependent on glucose-6-phosphate for activity (Rosell-Perez and Larner, 1964b). In order to obtain data to compare directly with glucose-6-phosphate, activators were tested at several concentrations and a coefficient of activation was calculated according to the formula:

$$CA = \frac{V_a}{V_g} \times \frac{K_{m_g}}{K_{m_a}}$$

where V_a and V_g denote the maximal velocities given by the activator and glucose-6-phosphate, respectively; K_{m_g} and K_{m_a} denote the concentrations of glucose-6-phosphate and activator that give half-maximal activation (Sols and Crane, 1954).

Several ions and phosphorylated compounds were found to be inhibitory. The inhibition with inorganic phosphate and sulfate was of a competitive type and surmounted by increasing the concentration of glucose-6-phosphate. Glucose was found to be inhibitory. The inhibition was noncompetitive with glucose-6-phosphate.

MATERIALS

Analytical.—The method for measuring transferase activity was as previously described (Villar-Palasi and Larner, 1961). Curves were constructed relating enzyme activity to activator concentration at saturating levels of UDPG (4.5×10^{-3} M), and from these K_m and *V* were calculated. In order to obtain comparative values of *V*, a curve with glucose-6-phosphate was constructed in each experiment. The assay conditions used were the same as those described in paper IV of this series (Rosell-Perez and Larner, 1964a).

Chemicals.—In addition to the chemicals described in previous papers of this series, the following compounds were obtained from the following sources: D-erythrose-5-phosphate (dimethylacetal, dicyclohexylammonium salt), L-α-glycerophosphate (dicyclohexylammonium salt), β-glycerophosphate (Na salt), dihydroxyacetonephosphate (dicyclohexylammonium salt), DL-glyceraldehyde-3-phosphate (diethylacetal, Ba salt), hydroxypyruvic acid phosphate (dimethylketal, tricyclohexylammonium salt), 3-D-phosphoglyceric acid (tricyclohexylammonium salt), 2-D-phosphoglyceric acid (Ba salt), glycolaldehyde phosphate (diethylacetal, dicyclohexylammonium salt), D-gluconic-6-phosphate (Ba salt), D-gluconate (K-salt), D-glucuronate (Na salt), and D-fructose-1,6-diphosphate (tricyclohexylammonium salt) were all purchased from California Corp. for Biochemical Research; UDPG (Na salt), ATP (Na salt), AMP (Na salt), 2-deoxy-D-glucose-6-phosphate (Na salt), D-fructose-1-phosphate (cyclohexylammonium salt), D-galactose-6-phosphate (Ba salt), D-mannose-6-phosphate (Ba salt), and α-D-galactose-1-phosphate (dipotassium salt) were purchased from Sigma Chemical Co.; D-glucosamine-6-phosphate (Ba salt), D-glucose-1-phosphate (K salt), D-fructose-6-phosphate (Ba salt), creatine-phosphate, and ADP (Na salt) were purchased from Nutritional Biochemicals Corp.; D-ribose-5-phosphate (Ba salt) was obtained from Schwarz BioResearch Incorp., and monomethylphosphate was purchased from K & K Laboratories, Inc. The following were received as gifts: D-1,5-Sorbitan-6-phosphate (1,5-anhydrodulcitol-6-phosphate) (dicyclohexylammonium salt; estimated as 90% pure) from Dr. A. Sols; myoinositol-2-phosphate from Dr. Th. Posternak; D-xylose-6-phosphate from Dr. H. Z. Sable; glucosamine-6-phosphate from Dr. S. Roseman; and D-altroheptulose-7-phosphate (estimated as 78% pure) from Dr. B. L. Horecker.

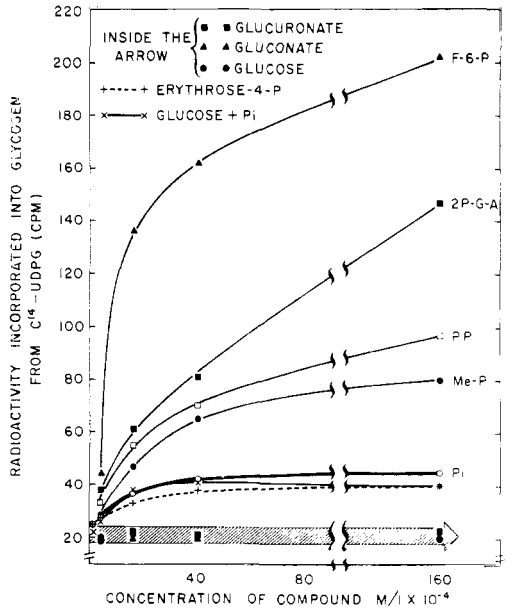

FIG. 3.—The action of different substances on the *D* form of dog muscle transferase. The vertical scale was increased 5-fold as compared to that in Fig. 2. The width of the arrow represents the range of radioactivity incorporated into glycogen in several experiments with no glucose-6-phosphate added to the test mixtures. Abbreviations are as explained in Fig. 2.

Other gifts were D-glucoheptulose-7-phosphate, D-allose-6-phosphate, α-methyl-D-glucoside-6-phosphate (containing 5% glucose-6-phosphate and estimated as 90% pure), and α-L-sorbose-1-phosphate which were received from Dr. R. K. Crane.

The barium salts were dissolved in acid (HCl) and the barium was precipitated with sodium sulfate. The other soluble compounds were neutralized first to *p*H 7.5–7.8. The acetal or ketal derivatives of glycolaldehydephosphate, hydroxypyruvic acid phosphate, D-erythrose-5-phosphate, and DL-glyceraldehyde-3-phosphate were used directly and also were rerun after acid hydrolysis. For hydrolysis, solutions were brought to *p*H 1.5 with HCl, allowed to stand at 31° for 24 hours, and then neutralized to *p*H 7.5–7.8. No difference was noted before and after acid hydrolysis.

EXPERIMENTAL

Activation of the Enzyme by Glucose-6-phosphate.—The particulate enzyme from dog muscle depends on glucose-6-phosphate for activity in an absolute manner (Rosell-Perez and Larner, 1964b). Figure 1 shows the direct and reciprocal plot of the glucose-6-phosphate concentration dependence. Half-maximal activation at saturating concentrations of UDPG was found at a concentration of 5×10^{-4} M glucose-6-phosphate. With Mg^{2+} present (0.008 M), half-maximal activation by glucose-6-phosphate was obtained at a concentration of 2×10^{-4} M.

Activation by Other Sugar Phosphates.—It was of interest to know whether other sugar phosphate derivatives would activate the enzyme from dog muscle. Although, of over thirty compounds tested, glucose-6-phosphate was the most effective activator found, a considerable number of the other compounds also activated the enzyme. Of these, 1,5-sorbitan-6-phosphate was the most effective. In decreasing order were galactose-6-phosphate, glucosamine-6-phosphate, allose-6-phosphate, sedoheptulose-7-phosphate, and

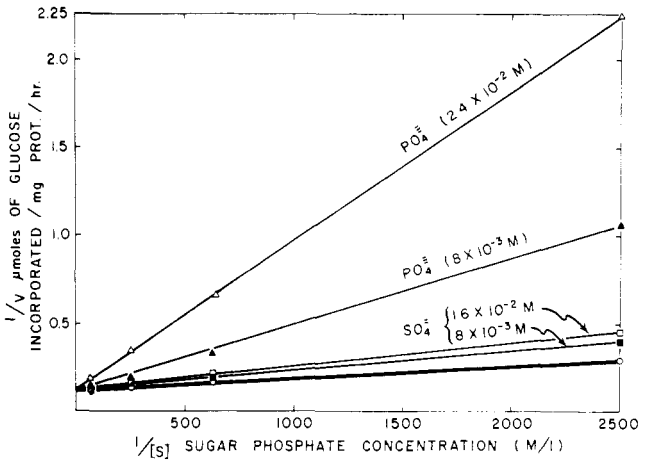

FIG. 4.—The inhibition of the *D* form of dog muscle transferase by inorganic sulfate and phosphate and its reversal with glucose-6-phosphate. The thick line between the open circles represents the activity curve with glucose-6-phosphate alone.

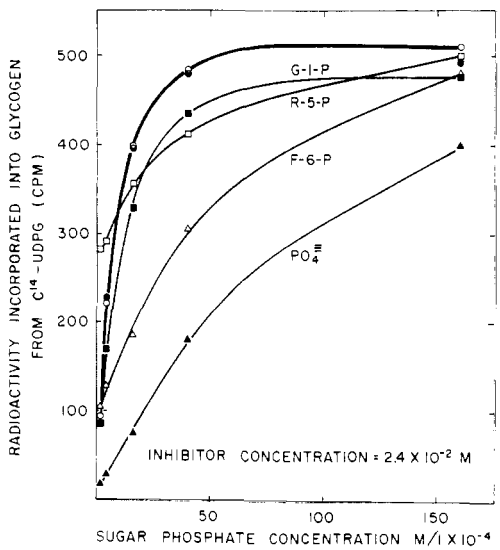

FIG. 5.—Activity concentration curves with different inhibitors. The thick line between the open circles represents the activity in the presence of increasing concentrations of glucose-6-phosphate. The other lines represent the same activities with the concentration of inorganic phosphate or phosphate derivative stated in the figure. The black circles on the glucose-6-phosphate curve indicate the activity with 6-phosphogluconate added at the same concentration as the other compounds. Abbreviations are as defined in Fig. 2.

ribose-5-phosphate (Table I). A substantial activation, but at a still lower level, was obtained with 2-deoxy-glucose-6-phosphate, mannose-6-phosphate, and fructose-6-phosphate. Slight activation was detected with various two- and one-carbon phosphate derivatives. Erythrose-4-phosphate was the only four-carbon phosphate tested, and it did not activate. Figure 2 shows the characteristic family of curves obtained with several of the most active compounds, whereas Figure 3 shows some of the less active compounds. The K_m, relative V, and coefficient of activation (CA) for all the compounds tested are presented in Table I. In this table the figure "0" denotes that no activation was detected. The values obtained with these compounds fell in the range of the shadowed arrow of Figure 3, which indicates the variation of the very small number of counts incorporated into glycogen

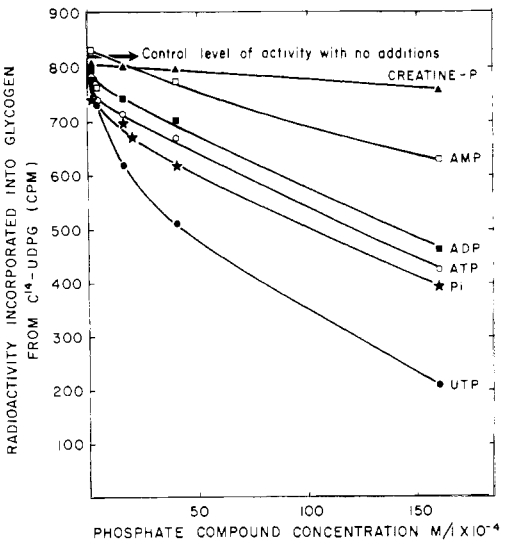

FIG. 6.—The inhibition of the *D* form of dog muscle transferase by nucleotides. The arrow shows the activity with no additions other than glucose-6-phosphate (0.01 M). Also shown is the inhibition by inorganic phosphate.

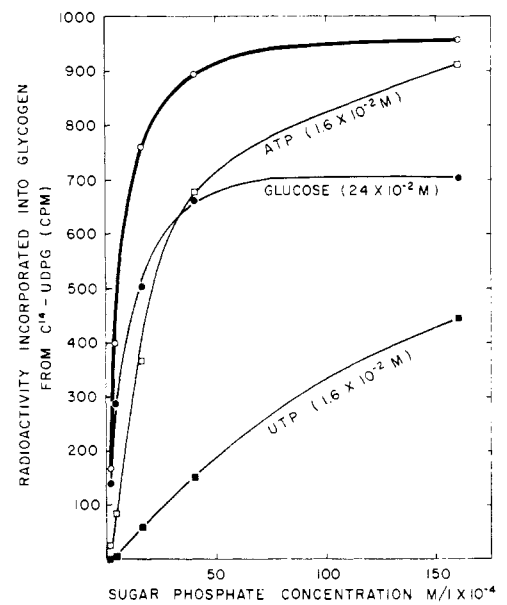

FIG. 7.—Activity concentration curves with a single concentration of ATP, UTP, and glucose. The inhibition by UTP and ATP is surmounted by increasing the concentration of glucose-6-phosphate; the inhibition produced by glucose is not competitive. The thick line between open circles represents the activity with glucose-6-phosphate alone.

in the absence of any activator. The dashes in Table I indicate activation in or below the range of the small activation due to inorganic phosphate.

Inhibition by Inorganic Phosphate and Sulfate.—The inhibition of the *D* form of transferase from dog muscle increased with increasing concentrations of inorganic sulfate and phosphate. Inorganic phosphate was the more-potent inhibitor under these experimental conditions.

In order to examine further the type of inhibition produced, experiments were done with one inhibitory concentration of either phosphate or sulfate in the presence of increasing concentrations of glucose-6-phosphate. It was found (Fig. 4) that phosphate inhibited competitively the activation by glucose-6-phosphate. The K_i for this inhibition was (calculated with the formula below) 1.9×10^{-3} M. The inhibition

$$\text{Slope} = \frac{K_m}{V}\left(1 + \frac{(I)}{K_i}\right)$$

produced by inorganic sulfate was also competitive (Fig. 4). The K_i was 1.4×10^{-2} M.

Inhibition by Fructose-6-phosphate and Other Sugar Phosphate Derivatives.—Transferase activity was measured with fructose-6-phosphate in the presence of increasing concentrations of glucose-6-phosphate to determine whether a competitive inhibition existed. A competitive inhibition was observed (Fig. 5). The K_i calculated was 1.3×10^{-3} M. However, at the lowest concentration of glucose-6-phosphate a significant activation was noted. It was not possible to decide whether in these experiments a small conversion of fructose-6-phosphate to glucose-6-phosphate had occurred (Leloir *et al.*, 1959; Algranati and Cabib, 1962). When the concentration of glucose-6-phosphate

TABLE I
ACTIVATION OF THE *D* FORM OF TRANSFERASE FROM DOG BY SEVERAL SUGAR PHOSPHATES AND PHOSPHATE DERIVATIVES[a]

Activator	K_m (M)	V (relative)	CA (× 100)
D-Glucose-6-phosphate	5×10^{-4}	1	100
D-1,5-Sorbitan-6-phosphate	7×10^{-4}	0.83	59.3
D-Galactose-6-phosphate	1.4×10^{-3}	0.93	33.3
D-Allose-6-phosphate	2.5×10^{-3}	0.91	18.2
D-Glucosamine-6-phosphate	1.6×10^{-3}	0.76	24.5
2-Deoxy-D-glucose-6-phosphate	2.6×10^{-3}	0.32	6.2
D-Mannose-6-phosphate	4×10^{-3}	0.42	5.0
D-Fructose-6-phosphate	1.2×10^{-3}	0.19	7.9
D-Gluconate-6-phosphate	6×10^{-3}	0.20	1.7
D-Glucose-1-phosphate	1×10^{-2}	0.15	0.75
D-Galactose-1-phosphate	—	—	—
D-Fructose-1-phosphate	0	0	0
D-Fructose-1,6-diphosphate	3.3×10^{-3}	0.10	1.5
Myoinositol-2-phosphate	4×10^{-2}	0.10	0.1
α-L-Sorbose-1-phosphate	3×10^{-3}	0.14	2.3
D-Glucoheptulose-7-phosphate	—	—	—
α-Methyl-D-glucoside-6-phosphate	—	—	—
D-Altroheptulose-7-phosphate (D-sedoheptulose-7-phosphate)	2.5×10^{-3}	0.94	18.8
D-Glucose	0	0	0
D-Gluconic acid	0	0	0
D-Glucuronic acid	0	0	0
D-Glucose + P_i	—	—	—
D-Ribose-5-phosphate	1.2×10^{-3}	0.40	18
D-Xylose-5-phosphate	3.7×10^{-3}	0.20	2.7
D-Erythrose-4-phosphate	—	—	—
α-Glycerophosphate	5.5×10^{-3}	0.12	1.1
β-Glycerophosphate	3.3×10^{-3}	0.05	0.8
Dihydroxyacetone phosphate	1×10^{-3}	0.06	3.3
DL-Glyceraldehyde-3-phosphate	4×10^{-3}	0.11	1.4
2-D-Phosphoglyceric acid	2×10^{-3}	0.15	3.8
3-D-Phosphoglyceric acid	3.3×10^{-3}	0.52	6.3
3-P-Hydroxypyruvic acid	—	—	—
Glycolaldehyde phosphate	4.5×10^{-3}	0.16	1.8
Methyl phosphate	1.4×10^{-3}	0.09	3.1
Inorganic pyrophosphate	1.6×10^{-3}	0.12	3.7
P_i	—	—	—

[a] As described in the text.

was increased, the activation by fructose-6-phosphate was overcome and the competitive nature of the inhibition was clearly established. Another case of competitive inhibition was that of ribose-5-phosphate. At low concentrations of glucose-6-phosphate, an activation by ribose-5-phosphate was obtained. At higher concentrations of glucose-6-phosphate, the competitive inhibition was noted. By way of contrast two other compounds are shown. Inorganic phosphate inhibited competitively at all concentrations of glucose-6-phosphate tested. Glucose-1-phosphate was inhibitory, but the inhibition was not overcome by glucose-6-phosphate.

The K_i values of the different compounds tested in the presence of glucose-6-phosphate are shown in Table II. It can be seen that except for inorganic sulfate, the values varied between 9.4×10^{-4} M and 1.9×10^{-3} M.

TABLE II
K_i VALUES OF SEVERAL COMPOUNDS ON THE DEPENDENT FORM OF DOG MUSCLE TRANSFERASE[a]

Compound	Concentration Tested	K_i (M)	K_i (mean value)
Inorganic sulfate	8×10^{-3}	1.2×10^{-2}	1.4×10^{-2}
	1.6×10^{-2}	1.5×10^{-2}	
P_i	8×10^{-3}	1.7×10^{-3}	1.9×10^{-3}
	2.4×10^{-3}	2.0×10^{-3}	
Inorganic pyrophosphate	8×10^{-3}	1.5×10^{-3}	1.7×10^{-3}
	2.4×10^{-2}	1.9×10^{-3}	
Methyl phosphate	8×10^{-3}	7.8×10^{-4}	9.4×10^{-4}
	2.4×10^{-2}	1.1×10^{-3}	
D-Fructose-6-phosphate	8×10^{-3}	1.3×10^{-3}	1.3×10^{-3}
	2.4×10^{-3}	1.3×10^{-3}	
3-Phosphoglycerate	8×10^{-3}	8.2×10^{-4}	1.9×10^{-3}
	2.4×10^{-3}	3.1×10^{-3}	
2-Deoxy-D-glucose-6-phosphate	5×10^{-3}	1.2×10^{-3}	1.3×10^{-3}
	1.4×10^{-3}	1.4×10^{-3}	
D-Gluconate-6-phosphate	8×10^{-3}	0	0
	2.4×10^{-3}	0	
D-Ribose-5-phosphate	2.4×10^{-3}	1.8×10^{-3}	1.8×10^{-3}

[a] As described in the text.

Inhibition by Other Phosphate Compounds.—The inhibition produced by other phosphorylated compounds is shown in Figure 6. Of the compounds tested, UTP was the most effective inhibitor. Figure 7 shows that the inhibition produced by both ATP and UTP was surmounted by increasing the concentration of glucose-6-phosphate and was of a competitive type. Experiments with UDPG showed that the inhibition was not competitive with this substrate. Free glucose was also inhibitory (Fig. 7). The inhibition produced by glucose was not competitive with glucose-6-phosphate (Fig. 8). It was rather of the "anticompetitive" type (Dogson *et al.*, 1956; Jermyn, 1958). Pyrophosphate, by way of contrast, was shown to be a competitive inhibitor.

DISCUSSION

The 100,000 × *g* particulate fraction of transferase from dog muscle was a particularly suitable source of the *D* form of the enzyme for activation and inhibition studies. With the present series of compounds tested glucose-6-phosphate was the most effective. The K_m for glucose-6-phosphate was found to be 5×10^{-4} M. This may be compared with the value of 6×10^{-4} M reported by Leloir *et al.* (1959) and Leloir and Goldemberg (1960) for the enzyme from rat liver and muscle. In the present study, it was shown that Mg^{2+} (0.008 M) significantly decreased the K_m.

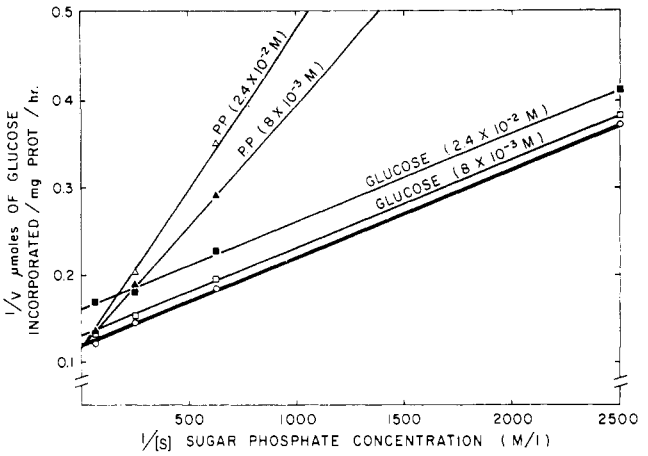

FIG. 8.—The inhibition by glucose and inorganic pyrophosphate. The thick line between open circles represents the activity with glucose-6-phosphate alone.

Among the compounds tested, 1,5-sorbitan-6-phosphate was the most effective after glucose-6-phosphate. This is of interest because this compound is presumably not utilized by glycolytic enzymes and, therefore, can be used as an activator that does not undergo further modification. It would appear from this experiment that the hydroxyl at carbon-1 of glucose-6-phosphate is not crucial. However, since α-methyl-D-glucoside-6-phosphate was inactive, an α-methyl substituent on the hydroxyl of carbon-1 blocks action.

The decrease in effectiveness with mannose-6-phosphate and with fructose-6-phosphate points out the importance of the hydroxyl at carbon-2. With fructose-6-phosphate the greater proportion of the furanose ring form may also play a role in its decreased effectiveness. Glucosamine-6-phosphate was also a less effective activator than glucose-6-phosphate, in keeping with the hydroxyl at carbon-2 as a binding point. The elimination of the carbon-2 hydroxyl in 2-deoxy-glucose-6-phosphate produced a decrease in the power of activation.

Since galactose-6-phosphate was less effective than 1,5-sorbitan-6-phosphate, the hydroxyl at carbon-4 may represent a site of attachment. The hydroxyl at carbon-3 appears to exert some influence in determining the effectiveness of the activator. Thus allose-6-phosphate was almost as effective as glucosamine-6-phosphate.

Two pentose phosphates were tested and proved to be weak activators. The change from pyranose to furanose ring structure plays a role, as well as the position of the hydroxyls at carbon-3 of the pentose. 6-Phosphogluconate was essentially inactive as an activator (Table I) or as an inhibitor (Table II). The slight activation noted may be due to contamination by glucose-6-phosphate. Thus the activation by glucose-6-phosphate showed no significant variation in the presence of added 6-phosphogluconate (Fig. 5). This indicates that the pyranose ring is of importance in the activator molecule. The importance of the pyranose ring would be in keeping with the activation by sedoheptulose-7-phosphate. The ineffectiveness of glucoheptulose-7-phosphate remains unexplained.

The attached phosphate appears to be a rather strict

requirement. Inorganic phosphate or glucose alone, or inorganic phosphate plus glucose together did not activate. Phosphate bound to the hydroxyl of carbon-1 of glucose, fructose, or galactose markedly decreased activation. Glucose-1-phosphate, one of the poorest activators, inhibited at all concentrations of glucose-6-phosphate tested. The inhibition was not competitive

We cannot explain the anomalous lack of activation by erythrose-4-phosphate when other molecules of shorter chain length, such as methyl phosphate and pyrophosphate, did activate. A similar case was observed with 3-phosphohydroxypyruvic acid. No activation was detected with this compound, although 3-phosphoglyceric acid gave a considerable stimulation. The activation by triosephosphate derivatives (with the exception of 3-phosphoglyceric acid) was significant.

Studies with two activators of medium potency such as 2-deoxyglucose-6-phosphate and ribose-5-phosphate were of interest. When these two activators were tested in the presence of glucose-6-phosphate, a competitive inhibition was shown only at the highest concentrations of glucose-6-phosphate. At lower concentrations of glucose-6-phosphate, the activation which they produced increased the activation produced by glucose-6-phosphate. It is possible that this interpretation may also be applied to the activation that was detected with fructose-6-phosphate at low concentrations of glucose-6-phosphate.

The data presented in Table II show a close agreement of the K_i values for all the inhibitors tested (with the exception of inorganic sulfate). Despite the fact that each of the compounds tested had a different half-maximal activation value, the K_i values were very similar. This finding is in keeping with the idea that the K_i reflects the attachment of the phosphate portion of the compound to the enzyme.

The K_i for sulfate was higher than that of phosphate or of the other phosphate compounds tested. It appears that inorganic phosphate may fit best at that site of activation on the enzyme where the phosphate of the sugar phosphate is attached.

ACKNOWLEDGMENT

The authors wish to express their thanks for the skillful help of Miss Helene Sasko in these studies.

REFERENCES

Algranati, I. D., and Cabib, E. (1962), *J. Biol. Chem. 237*, 1007.
Dogson, K. S., Spencer, B., and Williams, K. (1956), *Nature 117*, 432.
Jermyn, M. A. (1958), *Australian J. Biol. Sci. 11*, 114.
Leloir, L. F., and Goldemberg, S. H. (1960), *J. Biol. Chem. 235*, 919.
Leloir, L. F., Olavarría, J. M., Goldemberg, S. H., and Carminatti, H. (1959), *Arch. Biochem. Biophys. 81*, 508.
Rosell-Perez, M., and Larner, J. (1964a), *Biochemistry 3*, 75.
Rosell-Perez, M., and Larner, J. (1964b), *Biochemistry 3*, 81.
Rosell-Perez, M., Villar-Palasi, C., and Larner, J. (1962), *Biochemistry 1*, 763.
Sols, A., and Crane, R. K. (1954), *J. Biol. Chem. 210*, 581.
Steiner, D. F., Rauda, V., and Williams, R. H. (1961), *J. Biol. Chem. 236*, 299
Traut, R. R. (1962), Ph.D. dissertation (unpublished), The Rockefeller Institute.
Villar-Palasi, C., and Larner, J. (1961), *Arch. Biochem. Biophys. 98*, 436.

The Amino Acid Sequence of a Hexapeptide Containing an Essential Sulfhydryl Group of Rabbit Muscle Glyceraldehyde-3-Phosphate Dehydrogenase*

ALVIN H. GOLD† AND HAROLD L. SEGAL‡

From the Department of Pharmacology, St. Louis University School of Medicine, St. Louis, Missouri

Received January 23, 1964

The preferential reactivity of the essential sulfhydryl groups of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase with the yellow sulfhydryl reagent, *N*-(4-dimethylamino-3,5-dinitrophenyl)-maleimide (DDPM) has been utilized for the isolation of peptides containing these groups. After pepsin digestion of the DDPM-treated enzyme, the label was contained predominantly in a single hexapeptide of sequence ala-ser-(DDPM-cys)-thr-thr-aspNH_2. It is thus concluded that the structure of the several active sites of the enzyme are identical, at least in part, and that the reactive sulfhydryl groups are not components of glutathione moieties.

The elucidation of the chemical mechanism of action of an enzyme requires the characterization of transition states, which in turn depends upon a knowledge of the participating atoms in the enzyme molecules and their geometry. The determination of amino acid residues in the active sites of enzymes can provide some information in this regard.

Serine-specific reagents, such as DFP,[1] have been successfully employed for the labeling and isolation of peptide fragments containing part of the catalytically active centers of several proteases and esterases (reviewed in Koshland, 1960). Experiments with a variety of sulfhydryl-specific reagents have demonstrated that the activities of a large number of enzymes are dependent upon the presence of free sulfhydryl groups. Recently the use of DDPM has been de-

* Supported by a grant (AM-03642) from the United States Public Health Service.

† Present address: Biology Department, State University of New York, Buffalo 4, N. Y.

‡ Career Development Awardee (GM-5111) of the United States Public Health Service.

[1] Abbreviations are: DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)-maleimide; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl; PTC, phenylisothiocyanate; PTH, phenylthiohydantoin; DFP, diisopropylphosphorofluoridate; EDTA, ethylenediaminetetraacetic acid.