Sheep Liver 6-Phosphogluconate Dehydrogenase

INHIBITION BY NUCLEOSIDE PHOSPHATES AND BY OTHER METABOLIC INTERMEDIATES*

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SUMMARY

A variety of metabolic intermediates of the glycolytic pathway and tricarboxylic acid cycle have been found to be inhibitors of 6-phosphogluconate dehydrogenase isolated from sheep liver. All nucleoside 5'-triphosphates and 5'-diphosphates are inhibitors with $K_i$ values averaging 0.8 mM, nucleoside 5'-monophosphates are also inhibitory with $K_i$ values averaging 6 mM. The 2'- and 3'-monophosphates of adenosine and guanosine are also inhibitory, the 2'-isomers being especially potent inhibitors. The following compounds are also inhibitory with $K_i$ values averaging 6 mM: oxaloacetate, oxalate, fructose-1-P, fructose-6-P, glucose-6-P, P$_i$, PP$_i$, citrate, and sulfate. Fructose-1,6-P$_2$ is a potent inhibitor of the enzyme with a $K_i$ value of 70 $\mu$M (DYSON, J. E. D., and D'ORAZIO, R. E. (1971) Biochem. Biophys. Res. Commun. 43, 183). The presence of EDTA prevents inhibition by fructose-1,6-P$_2$, but not by the other inhibitory compounds.

All the nucleoside phosphates, with the exception of 2'-AMP, inhibit competitively with respect to both 6-phosphogluconate and NADP$^+$, as do citrate, PP$_i$, P$_i$, and sulfate. 2'-AMP inhibits competitively with respect to NADP$^+$ and noncompetitively with respect to 6-phosphogluconate. The hexose monophosphates inhibit competitively with respect to 6-phosphogluconate and noncompetitively with respect to NADP$^+$. Total inhibition caused by combinations of two or three nucleoside phosphates indicates that binding to the enzyme is mutually exclusive for all nucleoside phosphates except 2'-AMP, indicating that 2'-AMP binds at a different site from the other nucleoside phosphates. Binding of citrate and the hexose monophosphates is also mutually exclusive with the nucleoside phosphates, except 2'-AMP, indicating overlap of the binding site of citrate and the hexose monophosphates with that of the nucleoside phosphates.

Dietary effects (1, 2) on the levels of pentose phosphate pathway intermediates suggest control of this pathway at the two initial oxidative steps. The contribution of this pathway to carbohydrate metabolism is approximately 5% (3) (with the exception of adipose tissue), although the potential catalytic capacities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase considerably exceed this value.1 Thus, it appears that these two enzymes normally operate under a considerable degree of inhibition, or, alternatively, lack potential substrate. It has been shown that ATP (4) and 2'-AMP (5) inhibit 6-phosphogluconate dehydrogenase, and nucleoside phosphates (6-8) inhibit glucose 6-phosphate dehydrogenase, so that the activities of these two enzymes may possibly be inhibited under physiological conditions by the presence of nucleoside phosphates, thus reducing the rate at which glucose-6-P enters the pentose phosphate pathway. As part of our program to characterize sheep liver 6-phosphogluconate dehydrogenase we have, therefore, carried out a detailed kinetic study of the effect of nucleoside phosphates on this enzyme.

This study has also been extended to a variety of other compounds found to be inhibitors of 6-phosphogluconate dehydrogenase, with the object of delineating structural features of the active site, and also investigating the possible regulatory significance of inhibition by these compounds. A comparison of the inhibitory properties of these compounds with their ability to reduce the rate of catalytic function, due to cysteine modification, of the enzyme, has also been carried out, and a preliminary report on this investigation has been presented (9), and a complete report will be published elsewhere.

MATERIALS AND METHODS

6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate: NADP oxidoreductase (decarboxylating), EC 1.1.1.44) was isolated from sheep liver employing the purification procedure previously described (10). The preparations used had specific activities of 16 to 18 units per mg of protein, at pH 7.7 and 30°C, and were homogeneous according to the criteria of the analytical ultracentrifuge and isoelectric focusing. Enzyme preparations were stored at 2°C in 50 mM phosphate buffer pH 7.0, plus 2.5 mM ammonium sulfate. Under these conditions the
enzyme is stable for several months. As both P$_1$ and sulfate are inhibitors of the enzyme, aliquots for the kinetic studies were prepared by dialysis overnight against 2 liters of the same buffer used for the rate measurements (see below). 6-Phosphogluconate, trisodium salt; NADP$^+$, sodium salt; NAD$^+$, sodium salt; the sodium salts of the nucleoside triphosphates, diphosphates, and monophosphates; fructose-1-P, sodium salt; fructose-6-P, sodium salt; glucose-6-P, sodium salt; fructose-1,6-P$_2$, trisodium salt; were purchased from Sigma Chemical Co., St. Louis. Tris(hydroxymethyl)aminomethane (Tris) was also purchased from Sigma Chemical Co., as Trizma base, primary standard. All other chemicals used were of reagent grade and met American Chemical Society standards. Solutions of substrates and inhibitors were prepared in the same buffer used for the rate measurements, with adjustment of the concentrations of KCl where necessary to maintain the same ionic strength. Substrate solutions and certain solutions of inhibitors were standardized by enzymatic assay assuming a molecular absorptivity of 6.22 x 10$^3$ for NADPH at 340 nm (11). Nucleoside phosphates$^3$ solutions were standardized by spectrophotometric measurement.$^4$ Reaction velocities were determined by following the rate of reduction of NADP$^+$ at 340 nm with a Beckman DB-G recording spectrophotometer equipped with thermostated cuvette chamber. Standard assay conditions of 50 mm Tris-acetate buffer adjusted to 0.10 ionic strength with KCl, pH 7.7, and 30°, were employed for all rate measurements. All buffers used were prepared with deionized, glass-distilled water. The pH values of reaction mixtures were determined immediately on termination of the reaction by means of a Radiometer model 26 expanded scale pH meter equipped with semimicro-electrodes. Within each set of reaction velocity determinations, the measured pH values agreed to better than ± 0.05 pH unit. One unit of enzyme is defined as that amount of enzyme which will convert 1 μmole of substrate to product in 1 min at 30°.

**CALCULATIONS**

Calculation of micromoles of substrate converted to product was carried out from optical density changes assuming a molecular absorptivity of 6.22 x 10$^3$ for NADPH at 340 nm (11). Michaelis constants (K$_m$), and apparent Michaelis constants (K$_{app}$) in the presence of inhibitor concentrations (I), were calculated from the intercept on the abscissa of extrapolations from enzyme rate measurements carried out in the absence and presence of inhibitor, the rate measurements being plotted in a double reciprocal manner (12). Dissociation constants (inhibitor constants) for the enzyme-inhibitor complexes (K$_i$ values) were calculated, when the inhibition was competitive, from the ratio $K_{app}/K_m$, and inhibitor concentration, as described by Dixon and Webb (13). When the inhibition was noncompetitive, K$_i$ values were calculated by means of Equation 6 of Nordlie and Lygre (14), the equation being used in the manner discussed in that reference.

For those experiments in which percentage inhibition due to the presence of two or more inhibitors was determined, theoretical values for the percentage inhibition that would occur if the inhibitors present all bind to the same site were calculated according to the equation described by Webb (15). Theoretical values for the percentage inhibition that would occur if the inhibitors present bind to separate sites, to give additive, or cumulative inhibition, were calculated according to the method described by Woolfolk and Stadtman (16).

A systematic investigation of the kinetic properties of sheep liver 6-phosphogluconate dehydrogenase has been carried out (10). This showed that under the assay conditions employed the $K_m$ value for one substrate was independent of the concentration of the other substrate. $K_m$ and $K_{app}$ values for one substrate were determined, therefore, by varying the concentration of that substrate in the presence of a single fixed concentration of the other substrate.

In all the plots presented in this paper the slopes of the lines are drawn as the best visual fit to the experimental points.

**RESULTS**

**Inhibition by Nucleoside Phosphates at Constant Substrate Levels**—Preliminary measurements of the degree of inhibition as a function of nucleoside phosphate concentration, at constant substrate levels, are shown in Fig. 1A for nucleoside 5'-monophosphate.
Enzyme activity measurements plotted according to the method of Dixon (17). Concentrations of GTP present were as indicated in the abscissa to the figures. A, concentrations of 6-phosphogluconate employed as indicated in the figure, the concentration of NADP+ was 0.16 mM in all assays. B, concentrations of NADP+ employed as indicated in the figure, the concentration of 6-phosphogluconate was 0.001 mM in all assays. Reaction velocities in this and subsequent figures are expressed as enzyme units per ml of the diluted enzyme solution employed for the rate measurements.

Inhibitory compound | Ki x 10^4 M
--- | ---
ATP | 13.4 ± 6.0
ADP | 5.59 ± 1.84
AMP | 57.0 ± 20.0
2'-AMP | 3.55 ± 0.06
3'-AMP | 61.4 ± 15.0
GTP | 2.02 ± 0.31
GDP | 3.77 ± 2.55
GMP | 23.8 ± 11.0
2'-GMP | 5.70 ± 1.83
3'-GMP | 28.7 ± 8.9
CTP | 6.10 ± 1.18
CDP | 7.38 ± 0.87
CMP | 30.8 ± 7.2
ITP | 5.46 ± 3.07
IDP | 5.24 ± 2.07
IMP | 33.9 ± 15.4
UTP | 41.5 ± 25.0
UDP | 8.51 ± 2.35
UMP | 113 ± 20
TTP | 3.44 ± 0.83
TDP | 5.41 ± 0.97
TMP | 95.0 ± 37.1
Fructose-1,6-P_2 | 0.71 ± 0.31
Oxalacetate | 45.0 ± 17.9
Oxalate | 241 ± 12
Citrate | 56.6 ± 12.3
Fructose-1,6-P_2 | 91.0 ± 7.1
Glucose-6-P | 61.3 ± 35.0
Orthophosphate | 68.5 ± 44.9
Pyrophosphate | 60.0 ± 7.1
Sulfate | 89.1 ± 9.3
Nicotinamide | 1390 ± 250
Adenosine | 154 ± 31

TABLE I
Inhibitor constants (Ki values) for nucleoside phosphates, and various other compounds, for sheep liver 6-phosphogluconate dehydrogenase, determined at pH 7.7 and 30°C, and 0.1 ionic strength

Type of Inhibition and Ki Values of Nucleoside Phosphates—Enzyme activity measurements carried out as a function of GTP concentration are shown in Fig. 2 plotted according to the method of Dixon (17). In Fig. 2A, 6-phosphogluconate was the limiting substrate, and in Fig. 2B NADP+ was the limiting substrate, and in both cases it is evident that the criterion for competitive inhibition is clearly fulfilled (17). Enzyme activity measurements were also carried out as a function of substrate concentration, and the data plotted in double reciprocal manner. The plots obtained indicated, in confirmation of the results of Fig. 2, that GTP inhibits competitively, with respect to the binding of both 6-phosphogluconate and NADP+. Similar measurements were carried out in the presence of the nucleoside 5'-phosphates listed in Table I, and plots obtained from these measurements confirmed that all nucleoside 5'-phosphates inhibit competitively, with respect to the binding of both 6-phosphogluconate and NADP+. Average Ki values for the nucleoside 5'-phosphates, calculated from the various plots, are summarized in Table I.

Inhibition by Nucleoside 2'- and 3'-Monophosphates—In order to determine the extent to which the position of the phosphate group on the ribose moiety of the nucleoside phosphate affects the degree of affinity for the enzyme, and the type of inhibition, enzyme activity measurements were carried out in the presence and absence of the 2' and 3' isomers of the adenosine and guanosine monophosphates. The results obtained are presented in Fig. 3, and the Ki values for these compounds are summarized in Table I. As may be seen from Fig. 3, 2'-GMP, 3'-GMP, and 3'AMP display competitive inhibition with respect to both 6-phosphogluconate and NADP+ binding, and thus agree with the remainder of the nucleoside phosphates in this respect. In contrast it is apparent from Fig. 3 that 2'-AMP inhibits noncompetitively with respect to 6-phosphogluconate, although it does inhibit competitively with respect to NADP+. In view of the observation that 2'-AMP differs from the remainder of the nucleoside phosphates in this respect, it appeared appropriate to test adenosine, adenosine, and nicotinamide for possible inhibition. Enzyme activity measurements carried out in the presence and absence of adenosine and nicotinamide indicated that these two compounds inhibit in an identi-
Addition of Ilg of the results of these measurements are presented in Fig. 4.

Inhibitory properties of nucleoside phosphates with respect to sheep liver 6-phosphogluconate dehydrogenase, enzyme of ATP to Mg2+ were present in the assay mixtures. Examples 7). In order to establish, therefore, the effect of Mg2+ on the dehydrogenase by ATP is reduced by the presence of Mg2+ (6, 7). It has been shown, moreover, that inhibition of glucose 6-phosphate dehydrogenase by ATP is reduced by the presence of Mg2+ (6, 7). In order to establish, therefore, the effect of Mg2+ on the dehydrogenase by ATP is reduced by the presence of Mg2+ (6, 7).

Effect of Mg2+ on Inhibition by Nucleoside Phosphates—In many cases the kinetic and inhibitory properties of the magnesium complexes of the nucleoside phosphates differ appreciably from the properties of the free nucleoside phosphates. It has been shown, moreover, that inhibition of glucose 6-phosphate dehydrogenase by ATP is reduced by the presence of Mg2+ (6, 7). In order to establish, therefore, the effect of Mg2+ on the inhibitory properties of nucleoside phosphates with respect to sheep liver 6-phosphogluconate dehydrogenase, enzyme activity measurements were carried out in which variable ratios of ATP to Mg2+ were present in the assay mixtures. Examples of the results of these measurements are presented in Fig. 4. Addition of Mg2+ to the assay medium did result in a decrease in the level of inhibition caused by the presence of ATP, and at a Mg2+/ATP ratio of 1:1 the inhibitory effect of ATP was almost completely alleviated. Further increase in the Mg2+ to ATP ratio to a value of 3:1, however, led to an increase in the level of inhibition as compared to that observed at a ratio of 1:1. It is probable, however, that this increase was not caused by the ATP present, but was due to the increased levels of free Mg2+ in the assay medium, since concentrations of Mg2+ above 10 mM have been found to inhibit the enzyme (10). The effect of Mg2+ on the inhibition caused by other nucleoside phosphates was identical with that observed for ATP, the optimum effect having been observed at a nucleoside phosphate:Mg2+ ratio of 1:1.

Inhibition by Citrate and by Hexose Monophosphates—It has been shown in this laboratory that fructose-1,6-P2 is a potent inhibitor of sheep liver 6-phosphogluconate dehydrogenase (18). As both fructose-1,6-P2 and 6-phosphogluconate have negative charges at each end of the molecule, this suggested that molecules with this type of structure could be potential inhibitors of the enzyme. Accordingly a variety of molecules with structural similarities to 6-phosphogluconate and fructose-1,6-P2 were tested for inhibitory properties.

Of the compounds tested adipic acid (butane 1,4-dicarboxylic acid), succinate, fumarate, malate, and α-ketoglutarate, were found to cause no detectable inhibition under the standard assay conditions employed. Citrate, oxalacetate, and oxalate were found to inhibit competitively with respect to both 6-phosphogluconate and NADP+, as illustrated by the results shown in Fig. 5. Fructose-6-P, fructose-1-P, and glucose-6-P were found to inhibit competitively with respect to 6-phosphogluconate, and noncompetitively with respect to NADP+, as shown in Fig. 6. K values for these various compounds are summarized in Table I.

Inhibition by P, PP, and Sulfate—It has been shown that P is an inhibitor of 6-phosphogluconate dehydrogenase from Can- dida utilis (19), and since both 6-phosphogluconate and NADP+ contain phosphate moieties, this suggested that P, PP, and sulfate should be tested for inhibitory properties. All three anions were found to inhibit competitively with respect to the binding of both 6-phosphogluconate and NADP+ to the enzyme. K values for P, PP, and sulfate are presented in Table I.

Prevention of Fructose-1,6-P2 Inhibition by Presence of EDTA—
It has been shown by Bridges and Wittenberger (20) that the presence of EDTA can prevent the inhibition by fructose-1,6-P₂ of 6-phosphogluconate dehydrogenase from *Streptococcus faecalis*. This confirmed an earlier observation with respect to the enzyme from rat liver by Carter and Parr.⁵ As shown in Fig. 7 essentially all inhibition of sheep liver 6-phosphogluconate dehydrogenase by 2 mM fructose-1,6-P₂ could be alleviated by 6 μM EDTA, thus confirming that in the case of the sheep liver enzyme inhibition by fructose-1,6-P₂ can be prevented by EDTA. As shown in Fig. 7, similar measurements carried out with respect to 2'-AMP, ATP, fructose-6-P, and citrate indicated that EDTA had no effect whatever on the inhibition of 6-phosphogluconate dehydrogenase by these compounds. It will be noted from Fig. 7 that EDTA alone has no detectable effect on the catalytic activity of the enzyme. In addition, further experiments in which the enzyme was incubated with EDTA, and other chelating agents, for 24 hours at 30° showed that the catalytic activity of the enzyme was unaffected by such treatment.⁶ The fructose-1,6-P₂ solution employed in these experiments had been treated with cation exchanger (Bio-Rad AG 50W X8), the inhibition observed was, therefore, not due to the presence of heavy metals. Moreover, as shown in Fig. 1A, when a comparison was made between the extent of inhibition caused by untreated fructose-1,6-P₂ and that caused by fructose-1,6-P₂ treated with cation exchanger, no difference could be observed.

⁵ J. E. D. Dyson and M. H. Hatlelid, unpublished results.
⁶ J. E. D. Dyson and M. H. Hatlelid, unpublished results.
Inhibitory effect observed on sheep liver 6-phosphogluconate dehydrogenase due to simultaneous presence of two or three inhibitory compounds

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<th>Inhibition (%)</th>
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a Determined, as described in the text, at pH 7.7, 30° and 0.1 ionic strength. Concentration of NADP+ used, except in the case of AMP + adenosine, 0.17 mM. Concentration of 6-phosphogluconate used as noted below in Footnotes d to g. With the exception of the experiment employing AMP + adenosine the limiting substrate in all cases was 6-phosphogluconate.

b Calculated according to Reference 15, using the K, values of Table I, and the concentrations of inhibitors employed as noted below in Footnotes k to ee. The first value given in these footnotes is, in all cases, the concentration of inhibitor employed. K, values of 16.0 µM for 6-phosphogluconate and 7.0 µM for NADP+ were used (10).

c Calculated according to Woolfolk and Stadtman (16), using the concentration for percentage inhibition observed in the presence of a single added inhibitor as noted below in Footnotes k to ee. In all cases the second value given in these footnotes is the percentage inhibition.

d Concentration of 6-phosphogluconate used 0.11 mM.

e Concentration of 6-phosphogluconate used 36.1 µM.

Inhibitory effect observed on sheep liver 6-phosphogluconate dehydrogenase due to simultaneous presence of 2'-AMP and other inhibitory compound

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<th>Inhibitors present</th>
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<th>Calculated for binding at common site</th>
<th>Calculated for cumulative inhibition</th>
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<td>51.2</td>
<td>51.7</td>
<td>62.6</td>
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a Determined, as described in the text, at pH 7.7, 30° and 0.1 ionic strength. Concentration of 6-phosphogluconate 18.0 mM. Concentration of NADP+ 33.5 µM. NADP+ was, therefore, the limiting substrate.

b Calculated according to Reference 15, using the K, values of Table I, and the concentrations of inhibitors employed as follows: 2'-AMP 1.80 mM; 2'-GMP 2.50 mM; 3'-AMP 0.00 mM; CTP 0.67 mM; 3'-AMP 8.81 mM; AMP 16.7 mM; ATP 5.01 mM; PP 13.3 mM; citrate 16.6 mM; nicotinamide 0.17 M; adenosine 11.1 mM. A K, value for NADP+ of 7.0 µM was used (10).

c Calculated according to Woolfolk and Stadtman (16), using the values for percentage inhibition observed in the presence of a single added inhibitor as follows: 2'-AMP 50.0%; 2'-GMP 52.0%; 3'-AMP 48.0%; AMP 49.8%; ATP 49.5%; PP 41.9%; citrate 40.0%; nicotinamide 21.9%; adenosine 17.7%.
inhibitory properties from the remainder of the nucleoside phosphates, the results obtained when 2'-AMP was combined with other nucleoside phosphates, PPi, citrate, or nicotinamide (shown in Table III) indicate that 2'-AMP binds at a site distinct from all these compounds. The results would indicate, however, that adenosine binds to a common site with 2'-AMP (Table III).

The results obtained when fructose-1, 6-P2 was combined with another inhibitor can be divided into two groups according to the type of the inhibitor. Firstly, when fructose-1, 6-P2 was combined with a nucleoside 5'-monophosphate, or with fructose-6-P, the experimental values obtained for percentage inhibition were in good agreement with theoretical values calculated for cumulative inhibition, indicating a separate binding site for fructose-1, 6-P2. When fructose-1, 6-P2 was combined with an inhibitor from the second group, however, which included the nucleoside 5'-triphosphates and 5'-diphosphates, PPi, and citrate, very anomalous results were obtained, the percentage inhibition resulting from the combination of inhibitors being consistently less than that which resulted from each inhibitor separately. Possible reasons for these results are considered under "Discussion."

**DISCUSSION**

There appear to be three possible interpretations of the observation that all nucleoside phosphates, with the exception of 2'-AMP, display competitive inhibition with respect to the binding of both 6-phosphogluconate and NADP+. (a) The binding of 6-phosphogluconate and NADP+ to the enzyme takes place in an ordered manner. The possibility of the ordered addition of substrates in the case of this enzyme has been demonstrated (21). (b) Nucleoside phosphates do not bind at the active site, but elsewhere on the enzyme, and affect the affinity of both substrates by altering the conformation of the active site. (c) Nucleoside phosphates bind at the active site in such a manner as to interfere with the binding of both 6-phosphogluconate and NADP+ to the enzyme.

Kinetic evidence alone cannot unequivocally demonstrate that an inhibitor is binding at the active site, in the case of the nucleoside phosphates, however, structural similarity to NADP+, the competitive nature of the inhibition, and their effect on cysteine modification of the enzyme, all suggest that they do bind at the active site. The same considerations, together with data for 6-phosphogluconate dehydrogenase from Candida utilis (5) suggest that 2'-AMP also binds at the active site. Combinations of 2'-AMP with other nucleoside phosphates, however, show additive inhibition (Table III), indicating a separate binding site for 2'-AMP. A possible explanation for these conflicting observations is that the nucleoside phosphates, other than 2'-AMP, bind at the nicotinamide ribose binding area, whereas 2'-AMP binds at the adenosine binding area of the NADP+ binding site. Binding at the nicotinamide ribose area could result in intrusion of a ring substituent, or part of the ring structure, of the nucleoside phosphate into the 6-phosphogluconate binding site, thus preventing binding of both 6-phosphogluconate and NADP+. The different binding position of 2'-AMP would prevent intrusion so that it would not interfere with 6-phosphogluconate binding. Similar reasoning would explain the observation that the nucleoside phosphates, with the exception of 2'-AMP, are able to reduce the rate of loss of catalytic function on cysteine modification of the enzyme. Since an ordered addition of substrates to the enzyme, or the binding of nucleoside phosphates at some point other than the active site, would not explain the behavior of 2'-AMP the final interpretation c appears more in accord with the present observations than either a or b. The binding of inhibitors to different areas of the NADP+ binding site has also been suggested for yeast alcohol dehydrogenase (22).

The binding of nucleoside phosphates (except 2'-AMP), citrate, and hexose monophosphates, to the enzyme, is mutually exclusive (Table II), indicating overlap in their binding sites, and suggesting binding of the latter compounds to the 6-phosphogluconate binding site. The hexose monophosphates inhibit noncompetitively with respect to NADP+ binding, in contrast to citrate which inhibits competitively, possibly because the carboxyl group on C-3 of citrate overlaps into the NADP+ binding site. PPi, P3, and sulfate, would appear to be too small for their presence at a single point at the active site to prevent binding of both substrates, these three anions may bind, therefore, at both the 6-phosphogluconate and NADP+ binding sites.

Perusal of Table I shows that the presence of certain structural features results in increased affinity for the enzyme. (a) The presence of a PPi (or triphosphate) moiety. The binding of Mg2+ to the PPi group results in loss of affinity of the nucleoside phosphate for the enzyme. (b) The presence of a 2'-phosphate on the ribose moiety of the nucleoside (NAD+ has no detectable affinity for the enzyme). (c) The guanine ring structure results in increased affinity, possibly because the C-6 oxygen and adjacent N1 form a group analogous to the amide group of nicotinamide. (d) Citrate has a K1 value comparable to those of the hexose monophosphates, whereas adipic acid causes no detectable inhibition, thus neither terminal negative charges nor the presence of terminal phosphate groups appear to be necessary or sufficient criteria for binding at the 6-phosphogluconate binding site. Citrate and the hexose monophosphates, however, have a hydroxyl group on C-3, whereas adipic acid does not, so the presence of this group possibly contributes to binding affinity.

Fructose-1, 6-P2 inhibits competitively with respect to 6-phosphogluconate, to which it has certain structural similarities, thus it was suggested in an earlier paper (18) that fructose-1, 6-P2 binds at the 6-phosphogluconate binding site, the data of Table II, however, indicate binding at a point other than the active site. The prevention of fructose-1, 6-P2 inhibition by EDTA (Fig. 7) suggests that a metal is necessary for it to bind to the enzyme (as pointed out by Bridges and Wittenberger (20)). However, fructose-1, 6-P2 can still reduce the rate of loss of catalytic activity, due to cysteine modification, of EDTA-treated enzyme, with about 35% of its effect on modification of the untreated enzyme. This suggests that a metal is not an absolute requirement for fructose-1, 6-P2 to bind, but necessary for transfer of the effect of binding to the active site where it causes an increase in K1 for 6-phosphogluconate. As mentioned under "Results" when fructose-1, 6-P2 was combined with certain other inhibitory compounds the total inhibition determined was found to be less than that caused by the individual inhibitors. The compounds concerned were all weak chelating agents and thus capable of removing a metal required for the inhibitory properties of fructose-1, 6-P2 to be manifest, although not of preventing it binding. A combination of any of these compounds, therefore, with fructose-1, 6-P2 would result in the total inhibition observed being less than either fructose-1, 6-P2 or the compound would be capable of causing separately.

**Physiological Aspects**—The K1 values of ATP and ADP, and GTP and GDP are quite similar (Table I) so that relative levels of these compounds do not appear to constitute a mechanism for regulation of 6-phosphogluconate dehydrogenase. It appears probable, however, that cellular concentrations of these com-
pounds are sufficiently high (23, 24) that the enzyme operates under a certain degree of inhibition due to their presence, although the extent of the inhibition will be modified by the concentration of Mg++. The $K_i$ for ATP is rather high for it to be of significance in regulating 6-phosphogluconate dehydrogenase, although under certain conditions (26) the $P_i$ concentration might reach a level where it could influence the activity of the enzyme.

The $K_m$ for 6-phosphogluconate (16 $\mu$m (10)), the $K_i$ for fructose-1,6-P$_2$ (71 $\mu$m (18)), and their cellular concentrations in liver (23, 27), are such that inhibition of 6-phosphogluconate dehydrogenase by fructose-1,6-P$_2$ may be of regulatory significance under physiological conditions. Stimulation of glycolysis, due to activation of phosphofructokinase, would result in a marked increase in fructose-1,6-P$_2$ levels, and this increase would tend to be reduced. A balance in the passage of glucose-6-P into the pentose phosphate pathway, thus favoring passage of glucose-6-P into glyceraldehyde, the increased levels of fructose-1,6-P$_2$ would also activate pyruvate kinase (36), thus not be of significance in regulating G6P. The increased concentrations of Mg$^{2+}$ (25).

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