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Steric Effects in the Glyceraldehyde 3-Phosphate Dehydrogenase Catalyzed Hydrolysis of Acyl Phosphates. An Example of Substrate-Induced Cooperativity*

David R. Phillips† and Thomas H. Fife

ABSTRACT: The acyl phosphatase activity of glyceraldehyde 3-phosphate dehydrogenase has been examined at 25°. This activity can conveniently be divided into three categories depending upon the steric bulk in the acyl group of the substrate, that of: (1) acetyl phosphate which follows apparent second-order kinetics, K_m being too large to measure; (2) propionyl, butyryl, isobutyryl, and isovaleryl phosphate which obey normal Michaelis–Menten kinetics; and (3) the highly branched compounds, trimethylacetyl and 3,3-dimethylbutyryl phosphate, which are not substrates. Arsenate increases the rate of hydrolysis of the compounds in group 2, showing the rate-determining step for those compounds to be deacylation of the acyl enzyme intermediate. Methyl phosphate, however, was found to be a poor catalyst for deacylation of the enzyme. This and other observations support a general base mechanism for deacylation of glyceraldehyde 3-phosphate dehydrogenase.

The highly branched compounds bind to the enzyme since they inhibit acetyl phosphatase activity. In addition, compounds in groups 2 and 3 inhibit the dehydrogenase activity. This inhibition was found to be sigmoidal and was related to a cooperativity effect on binding to the enzyme. A plot of log k_3 (deacylation) *vs.* E_s, the Taft steric effects constants, had a slope of 1.1.

Acyl phosphates are substrates for glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate: NAD oxidoreductase (phosphorylating), EC 1.2.1.12) (Malhotra and Bernhard, 1968; Harting and Velick, 1954). The C–O bond is broken (Park and Koshland, 1958), and a cysteine residue is acylated at the active site of the enzyme (Mathew *et al.*, 1967). This cysteine is also acylated by *p*-nitrophenyl acetate during reaction with the NAD^+-free enzyme (Mathew *et al.*, 1967; Harris *et al.*, 1963). Thus, an acyl enzyme intermediate is formed which is a thiol ester. Histidine has also been implicated in the catalytic process, and mechanisms have been proposed for acylation and deacylation involving cysteine and histidine (Olson and Park, 1964). However, conclusive mechanistic evidence is lacking. Behme and Cordes (1967) found from studies of the esterase activity of the NAD^+-free enzyme that deacylation proceeds much more slowly in D_2O than H_2O and suggested that a general base catalyzed proton transfer may be occurring. There is, of course, recognized ambiguity in the interpretation of D_2O solvent isotope effects in enzymatic reactions (Jencks, 1963).

The study of steric effects in α-chymotrypsin-catalyzed reactions has given results that could be directly related to the mechanism of deacylation of the enzyme (Fife and Milstien, 1967; Milstien and Fife, 1968). It was considered that a similar study of steric effects in glyceraldehyde 3-phosphate dehydrogenase reactions, combined with nonenzymatic studies of steric effects in acyl phosphate and thiol ester hydrolysis, might yield useful information. This paper reports work in which acyl phosphates with varying steric bulk in the acyl group have been examined as substrates for glyceraldehyde 3-phosphate dehydrogenase. With this series, profound differences among the compounds were observed in regard to the kinetics followed and to binding effects which are dependent upon the size of the acyl group.

Experimental Section

Materials. Acetyl phosphate was purchased from Sigma Chemical Co. The remaining acyl phosphates were prepared as previously reported (Phillips and Fife, 1968). The barium salt of DL-glyceraldehyde-3-P diethyl acetal was purchased from Calbiochem. It was converted into the free aldehyde and the concentration of the D isomer determined by the method of Kochman and Rutter (1968). NAD^+ was also purchased from Calbiochem. K & K Laboratories, Inc., supplied the sodium monomethyl phosphate, and its concentration was deter-

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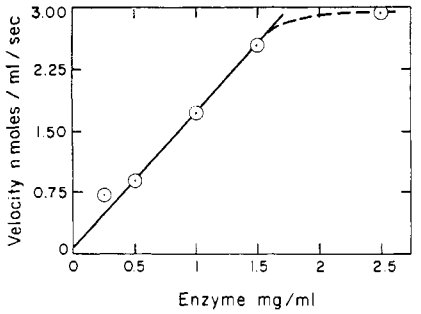


FIGURE 1: A plot of the acetyl phosphatase activity of glyceraldehyde 3-phosphate dehydrogenase *vs.* enzyme concentration at 25°. Activity is expressed in *n* moles of acetyl phosphate hydrolyzed per ml per sec. The procedure followed was as outlined in the Experimental Section (with 6.06 mM acetyl phosphate).

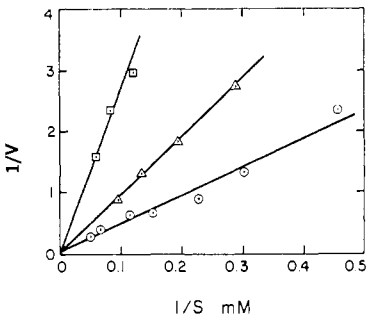


FIGURE 2: Lineweaver and Burk plot of the acetyl phosphatase activity of glyceraldehyde 3-phosphate dehydrogenase in the absence of inhibitor (⊙) and in the presence of 0.00329 M trimethylacetyl phosphate (⊡) and 0.00424 M 3,3-dimethylbutyryl phosphate (△) at 25°. Velocity is expressed in *n* moles of acetyl phosphate hydrolyzed per mg of protein per sec.

mined by means of the King phosphate test (King, 1932). Glass-distilled water was employed for all runs. The remainder of the chemicals were reagent grade.

Enzyme Preparations. Glyceraldehyde-3-P dehydrogenase, rabbit muscle enzyme, was from Worthington Biochemical Corp., Code 8 GB. Enzyme preparations were made by spinning the crystalline suspension at 3° for 20 min at 14,500*g*. The crystals were then dissolved in 0.025 M sodium barbital buffer (pH 7.20) which was also 5×10^{-3} M in EDTA. The solution was respun to remove any solid material, and was stored at 0°. Enzyme concentrations were determined by measuring the absorbance at 280 mμ of a 1:30 dilution of this solution. An extinction coefficient of 1.002 $cm^2\ mg^{-1}$ and the molecular weight of 140,000 employed by Fox and Dandliker (1956a,b) were used for all calculations. The ratio of absorbance at 280 mμ to that at 260 mμ for all enzyme preparations was 1.20 ± 0.05. This enzyme preparation was employed as the stock solution for determining acyl phosphatase activity and was utilized within 2 hr after its preparation.

The stock enzyme solution for determination of glyceraldehyde-3-P dehydrogenase activity consisted of a 1:500 dilution of the previous solution at pH 7.40 and contained 10^{-3} M mercaptoethanol and 10^{-3} M EDTA. This enzyme preparation was stable for several hours when stored at 0°.

Kinetic Measurements. In determining acyl phosphatase activity the hydroxamic acid assay of Lipmann and Tuttle (1945) was employed to measure the amount of remaining acyl phosphate. In each run, the enzyme was diluted in 0.025 M sodium barbital buffer at pH 7.00 and equilibrated at 25.0° for 5 min in a circulating water bath. This stabilized enzyme activity (Ferdinand, 1964). The acyl phosphate, also dissolved in 0.025 M sodium barbital buffer at pH 7.00, was then added to the enzyme solution to initiate the reaction. In the inhibition studies, the inhibitor was added together with the substrate. Aliquots were withdrawn at appropriate time intervals and introduced into the hydroxylamine reagent. After complete formation of the corresponding hydroxamic acid, the aliquots were developed through addition of $FeCl_3$, and the absorbance was measured at 540 mμ. Each reaction was followed to approximately 15% completion, and the velocity was determined from ten points. A plot of absorbance *vs.* time was used to calculate the velocity.

Glyceraldehyde 3-phosphate dehydrogenase activity was determined as reported by Velick (1955). Each rate was initiated by adding glyceraldehyde-3-P to the enzyme solution which had been equilibrated for 5 min in the buffer. In all inhibition studies the inhibitor was included in the equilibration process. The reaction was followed by observation of the increase in absorbance at 340 mμ, due to the conversion of NAD^+ to NADH, with a Beckman DU spectrophotometer equipped with a Gilford Model 2000 recording attachment. An extrapolation of the rate to zero time was used to obtain the initial rate of change of absorbance. The concentration of NADH was determined using an extinction coefficient of $6.22 \times 10^6\ cm^2\ mole^{-1}$ (Horecker and Kornberg, 1948). Sodium barbital buffer (0.025 M) at pH 8.43 was employed for all dehydrogenase experiments.

Results

Acetyl Phosphate. Figure 1 shows a plot of the velocity of the acetyl phosphatase activity of glyceraldehyde-3-P de-

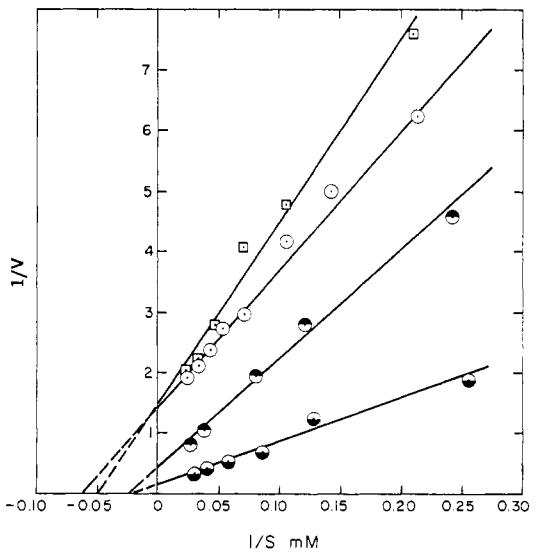


FIGURE 3: Lineweaver and Burk plots of the propionyl (◒), butyryl (◓), isobutyryl (⊙), and isovaleryl (⊡) phosphatase activities of glyceraldehyde 3-phosphate dehydrogenase at 25°. Velocity is expressed in *n* moles of acyl phosphate hydrolyzed per mg of protein per sec.

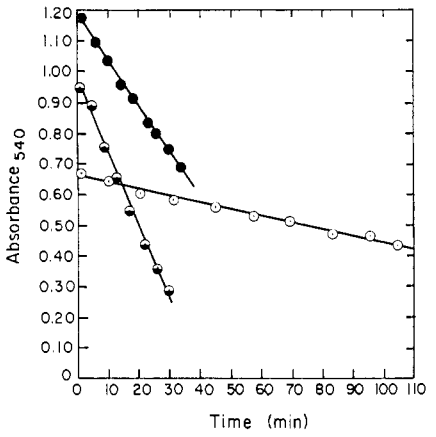


FIGURE 4: The rate of disappearance of propionyl (◒), butyryl (●), and isovaleryl (⊙) phosphate measured by the hydroxamic acid assay at 540 mμ, in the presence of glyceraldehyde 3-phosphate dehydrogenase and 0.01 M sodium arsenate at 25°. Initial substrate concentrations were as reported in Table II.

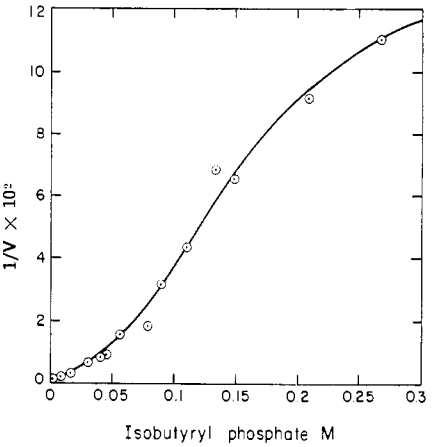


FIGURE 5: The dehydrogenase activity of glyceraldehyde 3-phosphate dehydrogenase, measured as n moles of NADH formed/mg of protein per sec in the presence of varying amounts of isobutyryl phosphate as inhibitor at 25°. Initial concentrations are as follows: NAD^+, 5.64×10^{-4} M; glyceraldehyde 3-phosphate, 3.82×10^{-4} M; glyceraldehyde 3-phosphate dehydrogenase, 2.0×10^{-4} mg/ml.

hydrogenase *vs.* enzyme concentration. The plot is linear to an enzyme concentration of 1.5 mg ml^{-1}, a concentration that was routinely used for all acyl phosphatase studies. This high enzyme concentration facilitates the hydrolysis of the more branched compounds and stabilizes the enzyme in the reaction vessel (Ferdinand, 1964). A Lineweaver and Burk (1934) plot of the glyceraldehyde-3-P dehydrogenase catalyzed hydrolysis of acetyl phosphate is shown in Figure 2. This plot shows that either classical Michaelis–Menten kinetics are not followed or that the K_m is too large to measure. Treating the reaction as a second-order reaction according to eq 1, the

$$\text{velocity} = k_b(\text{enzyme})(\text{substrate}) \qquad (1)$$

second-order rate constant was calculated by varying substrate, holding the enzyme concentration constant (Figure 2) and by varying enzyme, holding the substrate concentration constant (Figure 1). The value of k_b from the plot of Figure 2 was found to be 31 M^{-1} sec^{-1} while k_b calculated from Figure 1 was 35 M^{-1} sec^{-1}, in reasonably good agreement. It is, of course, likely that binding is occurring but that K_m has a value much greater than the highest substrate concentration. A concentration of 0.01 M arsenate had little effect on the rate, a very slight decrease being observed.

Intermediate Compounds. The glyceraldehyde-3-P dehydrogenase catalyzed hydrolysis of propionyl, butyryl, isobutyryl, and isovaleryl phosphate appears to be of the classical Michaelis–Menten type. Double-reciprocal plots according to Lineweaver and Burk (1934) are linear as shown in Figure 3 and yield reasonable values for K_m and V_{max} which are reported in Table I. The effect of arsenate on the hydrolysis of propionyl, butyryl, and isovaleryl phosphate is presented in Table II. Figure 4 shows that the data used to calculate these rate constants are linear, even to 70% of the reaction. Previous studies of other acyl phosphates (Malhotra and Bernhard, 1968; Mathew *et al.*, 1967) indicate that the hydrolysis of the present series of compounds should follow the reaction

TABLE I: Kinetic Constants for the Hydrolysis of Various Acyl Phosphates by Glyceraldehyde 3-Phosphate Dehydrogenase at 25°.[a]

Acyl Group	V_{max} (nmoles/mg of Protein per sec)	K_m (M)	k_3 (sec^{-1})
Propionyl	6.3	0.046	0.89
Butyryl	2.2	0.039	0.30
Isobutyryl	0.70	0.016	0.097
Isovaleryl	0.67	0.020	0.093

[a] Constants were determined from five or six points on a computer plot of $1/V$ *vs.* $1/S$ designed to give intercept and slope values by regression analysis of the least-squares line. Substrate concentration was varied from approximately 4 to 40 mM for each substrate. Each reaction vessel contained 1.5 mg of protein/ml and 0.025 M sodium barbital (pH 7.00).

TABLE II: The Glyceraldehyde 3-Phosphate Dehydrogenase Catalyzed Hydrolysis of the Acyl Phosphates in the Presence of Arsenate and Phosphate at 25.0°.[a]

Acyl Group	Substrate (mM)	Velocity (nmoles/mg of Protein per sec) V_0	$V_{arsenate}$[b]	$V_{phosphate}$[c]
Propionyl	17.6	1.9	4.3	
Butyryl	18.7	0.72	2.7	
Isovaleryl	10.2	0.20	0.39	0.17

[a] Each reaction vessel contained 1.5 mg of protein/ml and was 0.025 M in sodium barbital (pH 7.0). [b] In the presence o 0.01 M sodium arsenate. [c] In the presence of 0.001 M sodium phosphate.

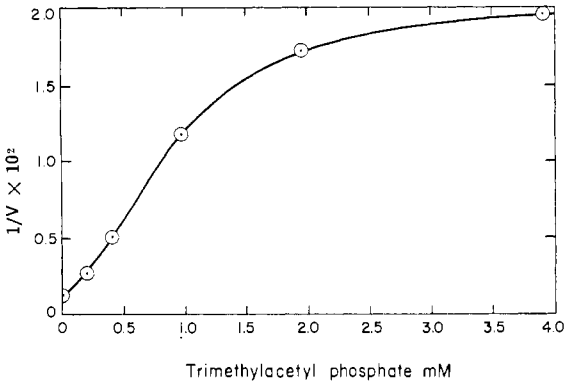


FIGURE 6: The dehydrogenase activity of glyceraldehyde 3-phosphate dehydrogenase, measured as n moles of NADH formed per mg of protein per sec in the presence of varying amounts of trimethylacetyl phosphate as inhibitor at 25°. Initial concentrations are as follows: NAD^+, 2.56×10^{-4} M; glyceraldehyde 3-phosphate, 6.86×10^{-5} M; glyceraldehyde 3-phosphate dehydrogenase, 3.16×10^{-4} mg/ml.

scheme in eq 2, where P_1 represents inorganic phosphate, P_2

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES' + P_1$$
$$ES' \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P_2 \qquad (2)$$

the organic acid, and ES′ the acyl enzyme intermediate. Arsenate must be exerting its effect by catalyzing deacylation of the acyl enzyme (Mathew *et al.*, 1967). The linearity ob-

$$ES' + \text{arsenate} \underset{k_{-3}'}{\overset{k_3'}{\rightleftharpoons}} E + P_2 + \text{arsenate} \qquad (3)$$

served in Figure 4 indicates that the rate of disappearance of acyl phosphate is concentration independent and that eq 3 is therefore the rate-determining step. Since k_3' must be greater than k_3, rate-determining deacylation must also occur in the absence of arsenate. The kinetic expression for V_{max} then reduces to eq 4, which was used to calculate the values for k_3 reported in Table I.

$$V_{max} = k_3 E_0 \qquad (4)$$

An attempt was made to show that these compounds are reacting at the active site by demonstrating inhibition of the dehydrogenase activity of glyceraldehyde 3-phosphate dehydrogenase. The inhibition by isobutyryl phosphate is shown in Figure 5. The plot is sigmoidal, with maximal inhibition at approximately 0.5 M isobutyryl phosphate.

Highly Branched Compounds. Trimethylacetyl and 3,3-dimethylbutyryl phosphate are not substrates. Even with an enzyme concentration of 7 mg/ml, no catalysis could be observed with either compound. It is possible that the enzyme could be acylating and not deacylating. This appears unlikely, however, since catalysis could not be observed even in the presence of 0.015 M sodium arsenate, a reagent which favors deacylation. Both compounds are inhibitors of the acetyl phosphatase activity of glyceraldehyde 3-phosphate dehydrogenase as illustrated

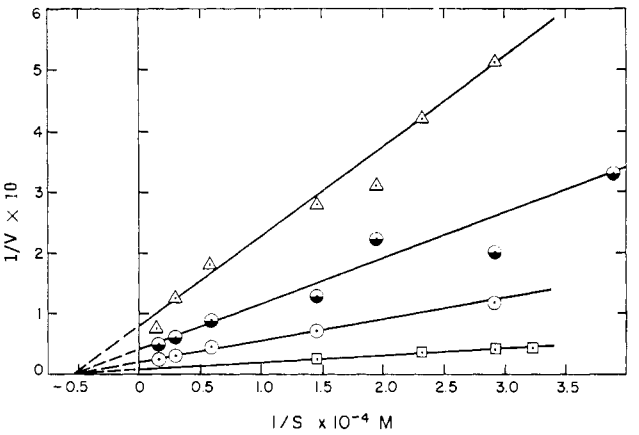


FIGURE 7: The effect of acyl phosphates on the dehydrogenase activity of glyceraldehyde 3-phosphate dehydrogenase varying the initial glyceraldehyde 3-phosphate concentration. The following inhibitor concentrations were employed: no inhibitor added (⊡), 0.0243 M isobutyryl phosphate (◒), 3.27×10^{-4} M trimethylacetyl phosphate (⊙), and 1.64×10^{-3} M trimethylacetyl phosphate (△). All reaction mixtures contained 3.43×10^{-4} mg of protein/ml and 4.22×10^{-4} M NAD^+, 0.025 M sodium barbital buffer (pH 8.43), 4.9×10^{-3} M sodium arsenate, and 8.2×10^{-4} M EDTA. Rates are expressed in n moles of NADH formed per mg of protein per sec.

in Figure 2. Further evidence that the highly branched compounds are bound to the enzyme can be seen in Figure 6 where it is shown that trimethylacetyl phosphate inhibits the dehydrogenase activity. It is readily apparent that this curve is sigmoidal, resembling the inhibition by isobutyryl phosphate. Maximal inhibition is at a much lower concentration (0.003 M) than that for isobutyryl phosphate. In Figure 7 is shown a plot of $1/V$ *vs.* $1/S$ for the dehydrogenase activity at varying concentrations of glyceraldehyde-3-P in the presence of constant concentrations of either trimethylacetyl phosphate or isobutyryl phosphate. It would appear from this plot that acyl phosphate inhibition is noncompetitive. A similar plot of $1/V$ *vs.* $1/S$ (not shown) was obtained when NAD^+ concentration was increased in the presence of constant concentrations of glyceraldehyde-3-P and trimethylacetyl phosphate,

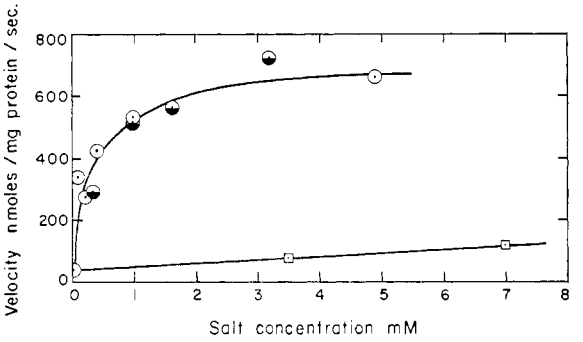


FIGURE 8: The effect of arsenate (⊙), phosphate (◒), and methyl phosphate (⊡) on the dehydrogenase activity of glyceraldehyde 3-phosphate dehydrogenase at 25°. Velocity is measured in n moles of NADH formed/mg of protein per sec. Initial concentrations are as follows: NAD^+, 2.48×10^{-4} M; glyceraldehyde 3-phosphate, 2.85×10^{-4} M; and glyceraldehyde 3-phosphate dehydrogenase, 3.38×10^{-4} mg/ml.

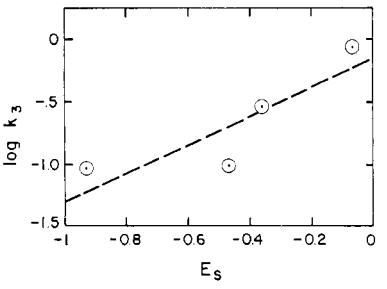


FIGURE 9: Plot of log k_3 from Table I *vs.* Taft's steric constants, E_s.

again indicating noncompetitive inhibition by the acyl phosphate.

The concentration dependence of the dehydrogenase activity of glyceraldehyde 3-phosphate dehydrogenase on arsenate and phosphate is shown in Figure 8. The two reagents appear to be equally effective with maximal activity at 0.004 M. Methyl phosphate is also included on this graph for comparative purposes.

Discussion

The acyl phosphates employed would be expected to acylate or bind to glyceraldehyde 3-phosphate dehydrogenase. Such widely varying compounds as β-(2-furyl)acryloyl phosphate (Malhotra and Bernhard, 1968) and acetyl phosphate (Park and Koshland, 1958) have shown active site specificity. Trimethylacetyl phosphate and 3,3-dimethylbutyryl phosphate inhibit the acetyl phosphatase activity. In addition, trimethylacetyl phosphate and isobutyryl phosphate were found to inhibit glyceraldehyde 3-phosphate dehydrogenase activity. Therefore, these compounds must bind strongly to the enzyme, although the compounds with highly branched acyl groups are not substrates.

The sigmoidal nature of the plots in Figures 5 and 6 indicate that the acyl phosphate inhibition of the dehydrogenase activity of glyceraldehyde 3-phosphate dehydrogenase may be cooperative. The enzyme is a tetramer consisting of identical subunits (Harris and Perham, 1965). Cooperativity in binding of NAD^+ to yeast glyceraldehyde 3-phosphate dehydrogenase has been demonstrated by the kinetic experiments of Kirschner *et al.* (1966), and recently substantiated by Chance and Park (1967). Similar experiments are not possible on the muscle enzyme since the NAD^+ is more firmly bound to that species (Velick, 1958). By the use of other techniques, however, subunit interactions have been noted. Velick (1958) has observed that 1 mole-equiv of *p*-mercuribenzoate/mole of protein weakens the affinity of the protein for NAD^+ at all the remaining binding sites. Listowsky *et al.* (1965), employing optical rotatory dispersion, reported that the major changes in conformation of the protein upon NAD^+ binding occurred when 1 equiv of the cofactor was bound to the enzyme. Using β-(2-furyl)acryloyl phosphate as a substrate, Malhotra and Bernhard (1968) have demonstrated some cooperative subunit interactions. The present experiments illustrate that a substrate which appears to possess active site specificity for glyceraldehyde 3-phosphate dehydrogenase, *i.e.*, isobutyryl phosphate, also exhibits cooperativity in binding to the enzyme. Furthermore, this cooperativity is initially positive as indicated by the sigmoidal curves in Figures 5 and 6, the initial binding of acyl phosphate making subsequent binding easier, in contrast to the negative cooperativity observed by Conway and Koshland (1968) for the binding of NAD^+ to enzyme. The noncompetitive nature of the inhibition of the dehydrogenase reaction may simply indicate that aldehydes and acyl phosphates bind at different sites which are probably adjoining since a common acyl enzyme intermediate is formed with the two types of compounds.

At high inhibitor concentration there is a decreasing effect of further increases in concentration. The residual dehydrogenase activity remaining at high acyl phosphate concentration is not unexpected. Values ranging from 0.8 to 3.8 have been obtained for the number of sites acylated by acyl phosphates (Malhotra and Bernhard, 1968). Experiments by Chance and Park (1967) indicate that while all monomers of the tetrameric yeast glyceraldehyde 3-phosphate dehydrogenase have identical primary structures, they are not all equivalent in their ability to be acylated by acetyl phosphate. Sites which are unfavorable to acetyl phosphatase activity also have a decreased dehydrogenase activity. The dehydrogenase activity remaining at maximal isobutyryl and trimethylacetyl phosphate concentration may therefore be due to monomers which are not readily subject to inhibition. Chance and Park (1967) found one highly active monomer in the yeast enzyme while Malhotra and Bernhard (1968) present evidence that half of the monomers of the muscle enzyme are highly active while half are less active. Since cooperativity is observed in the present inhibition experiments with the muscle enzyme, it appears that more than one of the monomers is highly active, and at least one is less active.

The acetyl phosphatase activity of glyceraldehyde 3-phosphate dehydrogenase appears to be a second-order reaction in view of the similarity in rate constants obtained by varying the substrate or the enzyme concentration. The apparent second-order nature of the reaction implies that acetyl phosphate is only weakly bound to glyceraldehyde 3-phosphate dehydrogenase. This is further substantiated by the decrease in K_m obtained with increased branching, as seen in Table I, and by observation that trimethylacetyl phosphate gives maximal inhibition at a much lower concentration than isobutyryl phosphate. Acetyl phosphate, which does not have a branched acyl group, would fit into this trend as a compound which is very weakly bound to the enzyme. These results indicate that the mode of attachment of acyl phosphates is not strictly an ionic attraction between a positive charge on the enzyme and the phosphate moiety but may be aided by a hydrophobic region in juxtaposition to the active site.

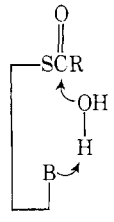
Increased steric bulk in the acyl group of the acyl phosphates has a similar influence on the enzymatic reaction and on nonenzymatic reactions involving nucleophilic attack at the carbonyl carbon. The rate constant for hydroxide ion catalyzed hydrolysis of the acyl phosphates is greatly reduced by branching in the acyl group, and imidazole catalysis can not be detected in the nonenzymatic hydrolysis of trimethylacetyl phosphate or 3,3-dimethylbutyryl phosphate (Phillips and Fife, 1969), although imidazole readily attacks the carbonyl group of acetyl phosphate (Di Sabato and Jencks, 1961). The hydrolysis of trimethylacetyl and 3,3-dimethylbutyryl phosphate is also not catalyzed by glyceraldehyde 3-phosphate dehydrogenase even though these compounds bind strongly to the enzyme.

Steric influences on the deacylation reaction are also similar to those observed in certain nonenzymatic hydrolysis reactions of thiol esters. The inductive effects constants σ^* in eq 5 (Taft, 1956) are nearly the same over the series studied.

$$\log \frac{k_3}{k_0} = \sigma^*\rho^* + \delta E_s \qquad (5)$$

Thus the differences in reactivity are mainly due to the differences in the steric parameter, E_s. In Figure 9 is shown a plot of log k_3 *vs.* E_s for the compounds obeying Michaelis–Menten kinetics. The slope of the line, δ, reflecting the susceptibility of deacylation to steric effects is 1.1, quite similar to the δ value obtained in deacylation of acyl-α-chymotrypsins (Fife and Milstien, 1967). In comparison, the value of δ for hydroxide ion catalyzed hydrolysis of esters of thiophenol is 1.15 (T. H. Fife and D. M. McMahon, unpublished data).

The mechanism for deacylation suggested by Olson and Park (1964) in the dehydrogenase reaction involved nucleophilic attack by the imidazole ring of histidine on the thiol ester intermediate, followed by transfer of the acyl group to a suitable acceptor. Some evidence does exist for involvement of histidine in reactions of the enzyme. Loss of dehydrogenase activity upon photooxidation has been correlated with loss of histidine (Friedrich *et al.*, 1964). Also, it has been reported (Halcomb *et al.*, 1968) that photooxidation of the enzyme, which destroys four to five histidine residues per tetramer, will not affect acylation by *p*-nitrophenyl acetate, but will inhibit deacylation of the enzyme. Destruction of the histidine residues also affects the acyl phosphatase activity. It is more probable that a general base mechanism is occurring similar to the general base catalyzed attack of water suggested by Behme and Cordes (1967) to explain the D_2O solvent isotope effect for deacylation of the NAD^+ free enzyme. This



mechanism is supported by the almost total lack of effect of monomethyl phosphate on the dehydrogenase activity, a reaction which has rate-limiting deacylation. In contrast, a marked enhancement is observed in the presence of arsenate or phosphate. Both arsenate and phosphate possess an ionizable proton at pH 8.4. Partial removal of this proton by general base would thereby facilitate catalysis by allowing attack of a more basic species. Monomethyl phosphate, which is completely ionized at that pH, cannot participate in such a reaction. If an uncatalyzed nucleophilic attack was taking place all three reagents should be effective. The greater size of monomethyl phosphate than P_i could, of course, also be an important factor in explaining its lack of effect.

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