Studies on the Mechanism of Action of Glyceraldehyde 3-Phosphate Dehydrogenase. Absorbance and Fluorescence Properties of Reduced Nicotinamide–Adenine Dinucleotide Complexes with Glyceraldehyde 3-Phosphate Dehydrogenase*

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ABSTRACT: When glyceraldehyde 3-phosphate is added to muscle glyceraldehyde phosphate dehydrogenase (GPD)–oxidized nicotinamide–adenine dinucleotide (NAD^+) complex in the absence of acceptor molecules, an acyl–GPD–NADH ternary complex is formed. The wavelength of maximum absorption of reduced nicotinamide–adenine dinucleotide (NADH) of this complex is about 333 mμ. There is no significant change in the wavelength of maximum fluorescence emission of NADH of this complex, but the fluorescence intensity is about 80% quenched. The addition of arsenate to this complex reverses the shift in the absorption spectrum and enhances the fluorescence intensity of the NADH to that observed previously for the binary GPD–NADH complex. NAD in concentrations from 22,500 to 45,000 times that of the directly determined dissociation constant (0.02 μM) and from 5 to 10 times that of the kinetically determined dissociation constant (90 μM) will not release any of the bound NADH from the ternary complex as judged by an absence of either a shift in the absorption spectrum or an enhancement in the fluorescence intensity. These data are consistent with a mechanism in which the acyl group is transferred from the complex while the NAD^+ is still in the reduced form.

The formation of this complex, however, does not appear to be dependent upon the order of addition of the substrates, *i.e.*, the same absorbance and fluorescence properties are observed when glyceraldehyde 3-phosphate is added to apo-GPD followed by the addition of NAD^+ as are observed when the glyceraldehyde 3-phosphate is added to the GPD–NAD^+ complex.

D-Glyceraldehyde 3-phosphate:NAD^+[1] oxidoreductase (phosphorylating), EC 1.2.1.12 (referred to here as GPD)[1] crystallized from rabbit muscle (Cori *et al.*, 1948), contains 3–4 equiv of NAD^+/mole of protein. The first detectable step in the reaction of this GPD–NAD^+ complex is the formation of an acyl–GPD–pyridine nucleotide complex with reduction of an amount of NAD presumably equivalent to the amount of acyl–enzyme formed (Velick and Hayes, 1953). Even in the presence of excess NAD but in the absence of acceptor phosphate or arsenate, NADH formation is limited by the number of acyl acceptor sites available on the enzyme. It has not been established unequivocally whether the acyl–enzyme–pyridine nucleotide complex contains NADH or NAD^+. It is known, however, that an acyl–GPD prepared in the absence of pyridine nucleotide undergoes no reaction unless either NAD^+ or NADH is added (Krimsky and Racker, 1955, 1963). Arsenolysis or phosphorolysis can occur if NAD^+ is added or reduction can occur if NADH is added. The data presented below give spectral evidence indicating that an acyl–GPD–NADH complex is formed when glyceraldehyde 3-phosphate is added to the GPD–NAD^+ complex and that NAD^+ will not displace NADH from this complex.

Materials and Methods

Enzyme Preparation. GPD was prepared from rabbit muscle by the method of Cori *et al.* (1948) except that the extraction fluid and all other solutions contained 0.004 M EDTA (Velick, 1955). The enzyme was recrystallized five times. The crystals were washed after the second crystallization with 0.66 saturated (at 3°) $(NH_4)_2SO_4$ at pH 8.2. Before use, aliquots of 10–15 mg of the crystals were collected by centrifugation, dissolved in 1 ml of 0.05 M Tris–acetate buffer at pH 7.5 containing 0.002 M EDTA, and put through a 1 × 15 cm column of Sephadex G-25 which had been equilibrated with the same buffer. The enzyme, free of $(NH_4)_2SO_4$, was recovered quantitatively from

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[1] Abbreviations used: GPD, glyceraldehyde 3-phosphate dehydrogenase; PCMS, *p*-chlorophenylmercurisulfonate; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotide; ADP, adenosine diphosphate.

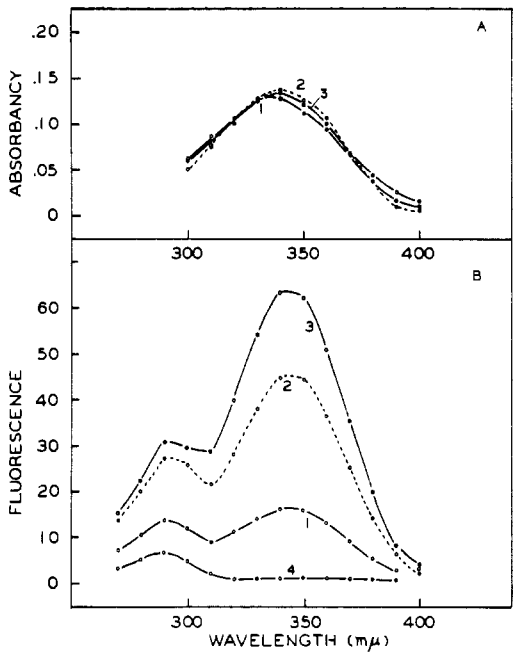

FIGURE 1: Absorbance and fluorescence activation spectra of the ternary 3-phosphoglyceryl–GPD–NADH complex. The reaction mixture contained 268 μM glyceraldehyde 3-phosphate, 8.47 μM apo-GPD, 25.7 μM NAD^+, and 30 mM Tris–acetate buffer at pH 6 in a final volume of 1 ml. The number of each curve in part "a" (absorbance spectra) has a counterpart in part "b" (fluorescence activation spectra). Emission was measured at 460 mμ. This notation is the same for Figures 1–4. Curves 1 are the absorbance and fluorescence spectra observed upon addition of the glyceraldehyde 3-phosphate to the mixture described above. Curves 2 are the spectra obtained after the addition of 2 μmoles of arsenate. Curves 3 are the spectra obtained after making the reaction mixture 100 μM with respect to PCMS. Reference cuvetts for the absorbance spectra contained the same mixtures except glyceraldehyde 3-phosphate was omitted. The fluorescence curve 4 is the activation spectrum of GPD–NAD^+ alone.

the column. The 280/260 mμ absorbancy ratio was about 1.08, corresponding to a ratio of NAD^+/protein of approximately 3.25 based on a molecular weight of 136,000 (Fox and Dandliker, 1956) and a molar extinction coefficient of 138,200 at 280 mμ.

In some cases the bound NAD^+ was removed from the GPD by treatment with neutral (acid-washed) Norit. Norit (100 mg) was added to an enzyme solution containing 10–15 mg of protein at 0°. The mixture was filtered with the aid of Celite through Whatman No. 50 paper after about 10 min. The enzyme solution had a 280/260 mμ absorbancy ratio of 1.9 or greater. This material was then filtered through a 1 × 15 cm column of Sephadex G-25 under the same conditions as those used for the native enzyme. Enzyme solutions desalted in this manner, both holo- and apoenzyme, were used within a few hours of chromatography.

Substrate. Glyceraldehyde 3-phosphate was purchased from the Sigma Chemical Co. as the diethyl acetal barium salt. The free aldehyde was obtained by hydrolysis in a 100° water bath for 3 min and absorption of the barium on Dowex 50-x4.

Optical Measurement. All fluorescence spectra were obtained in an Aminco-Bowman spectrophotofluorometer. No corrections have been applied to the activation or emission spectra. In some cases, fluorescence was measured in a Farrand Model A photoelectric fluorometer using a Corning No. 7-37 as primary filter and No. 3-73 and 4-70 as secondary filters. A Leeds and Northrop Speedomax H AZAR recorder was used as a readout.

All absorption spectra were obtained on a Bausch and Lomb Spectronic 505. Where indicated, reaction rates were measured in a Beckman DU spectrophotometer.

Results

Effects of pH on the Hydrolysis of the Acyl Group from GPD. When glyceraldehyde 3-phosphate and excess NAD^+ are added to GPD in the absence of an acceptor (phosphate, arsenate, or SH compounds) NAD^+ is reduced very rapidly in an amount equivalent to the three available acyl acceptor sites[2] (Velick and Hayes, 1953). Following the fast reaction is a slow reduction of NAD^+. (See the review by Velick and Furfine, 1963.) This slow reduction is due to hydrolysis of the acyl–enzyme complex, thus making more acceptor sites available for the aldehyde substrate. The speed of the second reaction increases with pH. However, the acyl complex appears relatively stable at pH values lower than 6.5.

Spectral Properties of the Ternary Complex. Because the acyl–GPD–pyridine nucleotide complex appeared stable under more acidic conditions, an attempt was made to determine whether this complex has any characteristic spectral properties. It was observed that at pH 6 the addition of glyceraldehyde 3-phosphate to the GPD–NAD^+ complex results in the reduction of NAD^+ but the characteristic NADH absorption no longer peaks at 340 mμ but is shifted to 333 mμ (Figure 1a, curve 1). The addition of arsenate to this complex reverses the hypsochromic shift and the maximum absorption of the NADH is again at 340 mμ (curve 2 of Figure 1a). The addition of *p*-chlorophenylmercurisulfonate (PCMS) after arsenate has no additional effect on the absorption spectrum (curve 3 of Figure 1a). This hypsochromic shift in the absorption spectrum of NADH appears to be due to the formation of a 3-P-glyceryl–GPD–NADH ternary complex. The reverse shift in the presence of arsenate is appar-

[2] More recent studies on the rabbit and pig muscle enzymes (Harris and Perham, 1965; Harrington and Karr, 1965) have shown that GPD is composed of four identical subunits each containing an acyl acceptor site.

ently due to arsenolysis of the 3-P-glyceryl group from the complex. Additional support for this interpretation is seen in the fluorescence activation spectra shown in Figure 1b. The fluorescence activation spectra represented by curves 1–3 were made in microcuvets on the same mixtures used to obtain the absorption spectra shown in curves 1–3, respectively, of Figure 1a. It can be seen that the NADH fluorescence is about 80% quenched in the ternary complex (curve 1 of Figure 1b). The addition of arsenate resulted in arsenolysis of the 3-P-glyceryl moiety of the ternary complex, yielding the binary GPD–NADH complex (curve 2) and 3-phosphoglycerate. Titration binding studies have shown that the fluorescence intensity of NADH in the binary GPD–NADH complex is approximately 70% of the fluorescence intensity of free NADH and that sulfhydryl agents such as PCMS will disrupt the complex and thus release free NADH in solution (Velick, 1958). Curve 3 of Figure 1b shows that the addition of PCMS to the binary complex obtained in these studies enhanced NADH fluorescence but resulted in no appreciable change in the absorption spectrum (curve 3 of Figure 1a). These results with GPD, like those of Velick (1958), show that the binding of NADH by GPD results in a reduced fluorescence yield from NADH with no change in its absorption spectrum. But in addition, the present data indicate that the formation of a ternary 3-P-glyceryl–GPD–NADH complex causes a hypsochromic shift in the absorption spectrum of the bound NADH and a very marked decrease in its fluorescence intensity. Neither complex resulted in any measurable change in the wavelength of maximum fluorescence emission for NADH. Furthermore, the order of addition of reactants was not important for the development of the spectral characteristics demonstrated. The addition of NAD^+ to the mixture containing apo-GPD and glyceraldehyde 3-phosphate yielded a complex having spectral and fluorescence properties indistinguishable from that produced when glyceraldehyde 3-phosphate was added to the GPD–NAD complex.

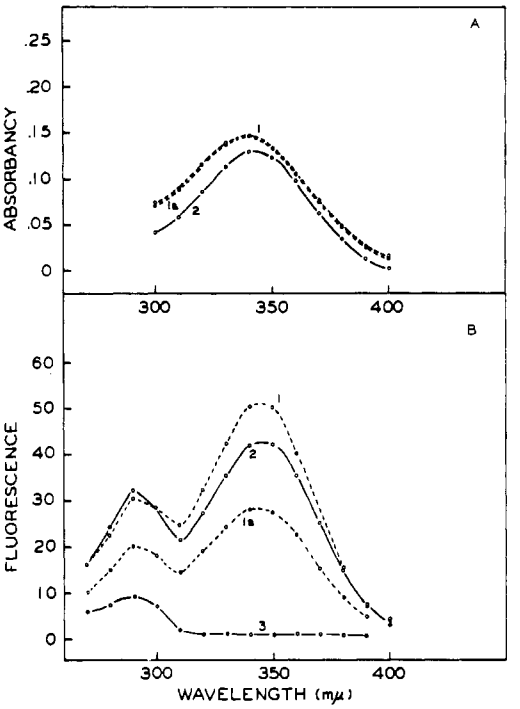

FIGURE 2: Absorbance and fluorescence activation spectra of the GPD–NADH binary complex in the presence of glyceraldehyde 3-phosphate. Curves 1 are the absorbance and fluorescence activation spectra of 8.47 μM charcoal-treated NAD^+-free GPD reconstituted with 3 equiv of NADH, immediately after the addition of 268 mμmoles/ml of glyceraldehyde 3-phosphate. Curves 1a are the same mixture 5 min later. Curves 2 are the absorbance and fluorescence activation spectra of the GPD preparation before addition of the glyceraldehyde 3-phosphate. The fluorescence spectrum in curve 3 is apo-GPD.

The Interaction between Glyceraldehyde 3-Phosphate and the GPD–NADH Complex. The formation of aldehyde from acyl phosphate requires a GPD–NADH enzyme (Krimsky and Racker, 1955). Moreover, glyceraldehyde 3-phosphate will competitively inhibit the reverse reaction catalyzed by GPD (Furfine and Velick, 1965). It is expected, therefore, that some interaction will occur between glyceraldehyde 3-phosphate and the GPD–NADH enzyme. Figure 2 shows the spectral changes observed at pH 6 with a GPD preparation in which NAD^+ had been removed by charcoal treatment as described and the reduced complex had been reconstituted by the addition of 3 equiv of NADH. The absorbance spectra in Figure 2a show that the addition of glyceraldehyde 3-phosphate to this reconstituted GPD–NADH complex (curve 1) does not cause a hypsochromic shift in the absorbance spectrum of NADH, and that the absorbance spectrum is unchanged over a 5-min interval (curve 1a). A slight increase in absorbance of bound NADH was observed after addition of glyceraldehyde 3-phosphate. Consistent with the small increase in absorbance, there is also an increase in fluorescence as shown by the difference between curve 2 of Figure 2b (GPD–NADH complex alone) and curve 1 of Figure 2b (GPD–NADH complex immediately after addition of glyceraldehyde 3-phosphate). Fluorescence polarization measurements (Velick, 1958) indicated that large amounts of the NADH probably were not being released by the aldehyde substrate. The possibility that some was released causing an increased fluorescence cannot be excluded. However, reduction of a small amount of NAD^+ that was either not removed from the enzyme by the Norit treatment or that contaminated the NADH used to reconstitute the binary complex may be an equally good explanation for the increased absorbance and fluorescence observed. By contrast with the absorbance spectra, however, is the observation that a considerable decrease in fluorescence intensity occurs after a 5-min incubation of the complex (curve 1a of Figure 2b).

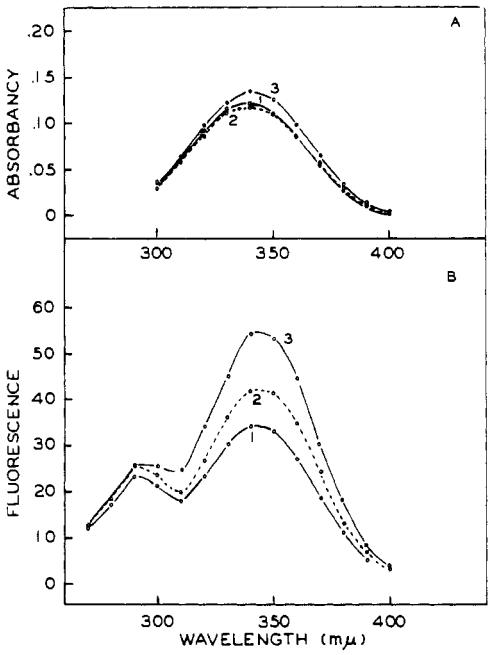

FIGURE 3: Effect of arsenate and PCMS on the glyceraldehyde 3-phosphate–GPD–NADH complex. Curves 1 are the same as curves 1a of Figure 2. Curves 2 were obtained after the addition of 1 μmole of arsenate to the reaction mixture of curve 1. Curves 3 were obtained after the addition of 100 mμmoles of PCMS to the reaction mixture of 1.

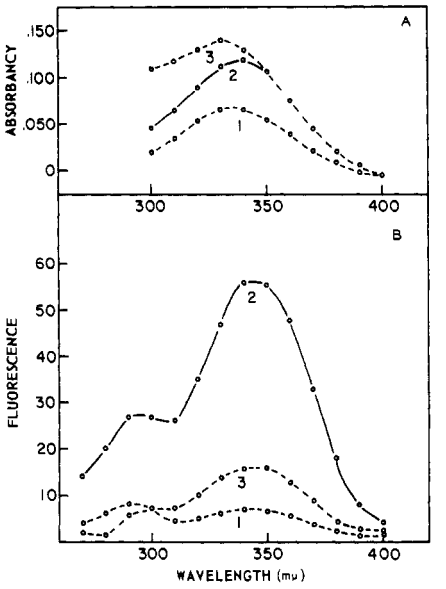

FIGURE 4: Effect of NAD^+ on the absorbance and fluorescence activation spectra of the ternary 3-phosphoglyceryl–GPD–NADH complex. Curves 1 are the spectra obtained after addition of 338 mμmoles of glyceraldehyde 3-phosphate to 8.97 mμmoles of GPD–NAD^+ at pH 6 as described in Figure 1. Curves 2 were obtained when 2 μmoles of arsenate was added to the same mixture. Curves 3 was obtained when 444 mμmoles of NAD^+ was added to the same mixture *instead* of arsenate.

What has happened to the NADH of this complex during the 5-min incubation? In an attempt to answer this question the absorbance and fluorescence spectra (curves 1a) of Figure 2 are replotted in Figure 3a and b and labeled with the numeral 1. The effects of arsenate and PCMS on this complex are shown. There is little to no effect of arsenate on the absorbance spectrum (curve 2), but PCMS addition did cause a slight increase in absorbance. However, the fact that the additions of arsenate (curve 2 of Figure 3b) and of PCMS (curve 3 of Figure 3b) restored most of the "lost" NADH fluorescence of this complex indicates that NADH had not undergone changes to a nonfluorescent species. Thus, NADH-x formation is not a likely explanation for these changes since NADH-x shows comparatively little absorbance at 340 mμ (Chaykin *et al.*, 1956). Kinetic studies have shown that glyceraldehyde 3-phosphate rapidly inhibits acyl phosphate reduction by GPD (Furfine and Velick, 1965). However, the change in the fluorescence properties of the aldehyde–GPD–NADH complex took up to 5 min or more to develop. This indicates that the fluorescence changes observed do not emanate from the initially inhibited complex of 3-phosphoglyceraldehyde–GPD–NADH but result from secondary changes in the structure of this complex in the vicinity of the fluorescence groups.

Order of Dissociation of the Ternary Complex. The data above give absorbance and fluorescence evidence for a 3-phosphoglyceryl-GPD–NADH complex. In such a complex the wavelength of maximum absorption of NADH has been shown to be shifted to 333 mμ and the NADH fluorescence to be about 80% quenched. The optical properties of this complex observed at pH 6 make it possible, therefore, to determine whether free NAD^+ can displace NADH in the ternary complex with the same efficiency as it does in the binary complex. If NAD^+ can displace NADH of the ternary complex, it should enhance the fluorescence of the NADH to the same level as that of an equivalent amount of the free reduced pyridine nucleotide. The addition of NAD^+ to the ternary complex prepared under the same conditions as in Figure 1 (shown here as curves 1 of Figure 4a and b) increases the absorbance of NADH (curve 3, Figure 4a), and this is accompanied by an exactly proportional increase in the fluorescence intensity (curve 3, Figure 4b), so that the fluorescence per molecule of NADH remains the same. This increase results from a shift in the equilibrium of the reaction in favor of the acyl–GPD–NADH complex. When arsenate alone is added to the ternary complex, *i.e.*, to the reaction mixture used to obtain the absorption and fluorescence spectra shown in curves 1 (Figure 4a and b) but in the absence of excess NAD^+, the absorption and fluorescence spectra shown in curves 2 (Figure 4a and b) are obtained. Thus NAD^+ at concentrations

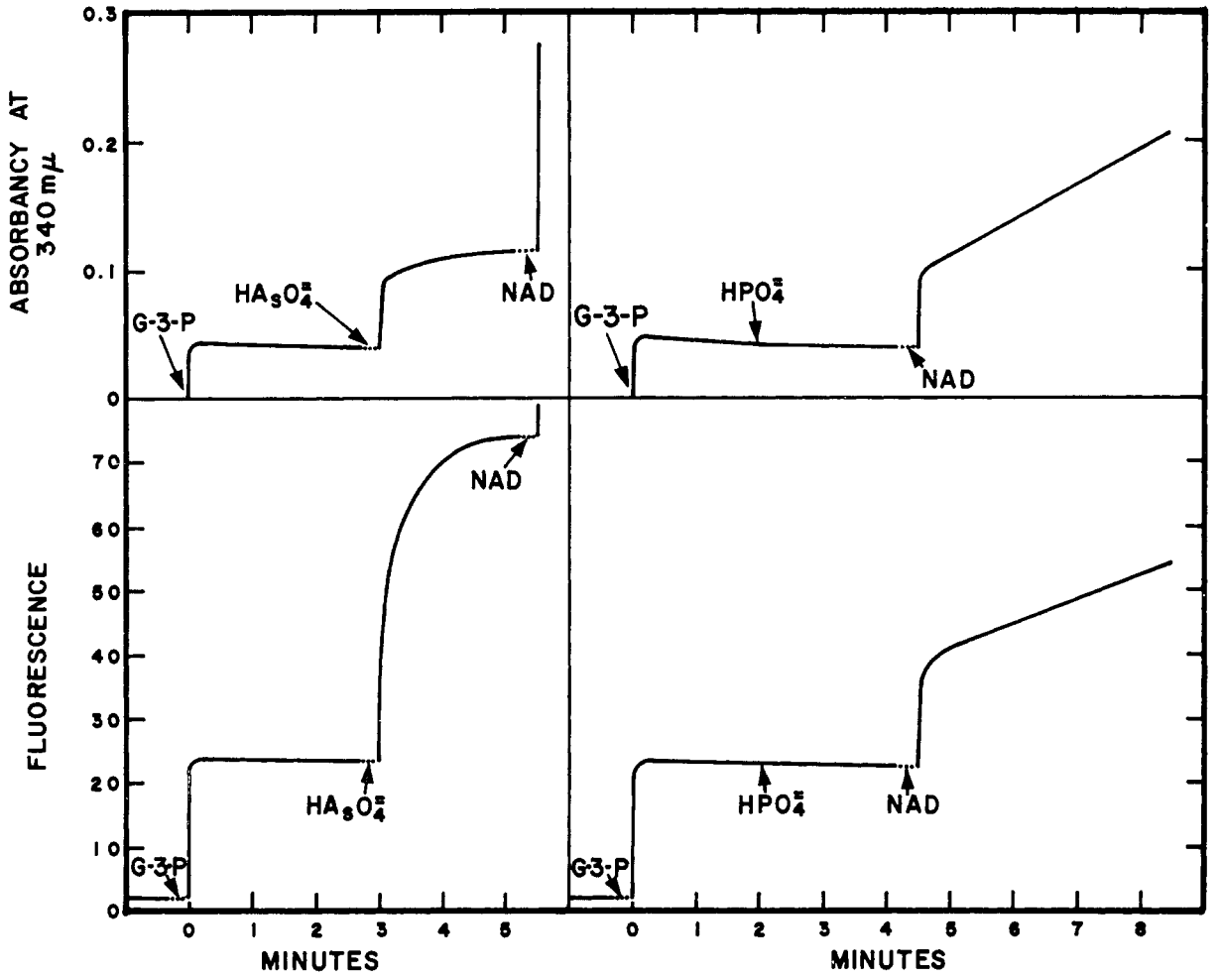

FIGURE 5: Reduction of bound NAD^+ at pH 7.5 after addition of substrates and acceptors. Each cuvet contained 7.81 μM native GPD containing about 3 equiv of bound NAD^+ and 0.16 M Tris–acetate buffer at pH 7.5. Where indicated, the solution was made to the indicated concentrations with the compounds listed: 118 μM glyceraldehyde 3-phosphate, 1 mM arsenate, 5 mM phosphate, and 944 μM NAD^+.

five times greater than the dissociation constant of NAD^+ from acyl–GPD–NAD^+ (Furfine and Velick, 1965) and about 10^4 times the dissociation constant as determined by titration procedures (Velick, 1958) does not disrupt the ternary complex. Arsenate, however, results in arsenolysis of 3-P-glycerate from the complex with formation of the binary GPD–NADH complex.

Since all the spectra described were observed at pH 6, it appeared important to determine whether the ternary complex can exist at pH 7.5 and if so, whether it is dissociated in an ordered sequence. Therefore, under identical conditions, except for pH, the NADH absorbance and fluorescence changes were observed as a function of time at constant wavelengths. The addition of glyceraldehyde 3-phosphate to GPD in the absence of excess NAD^+ results in the reduction of approximately one-third of the bound NAD^+ as judged by the increase in absorbance at 340 mμ (Figure 5a). This was accompanied by a concomitant increase in fluorescence intensity. By analogy with the results obtained at pH 6, this should represent the absorbance and fluorescence changes due to formation of a ternary 3-P-glyceryl–GPD–NADH complex. The addition of arsenate to this reaction vessel should result in arsenolysis of the ternary complex to yield the binary GPD–NADH complex. It is obvious from this figure that the addition of arsenate did just that. The ratio of the increase in fluorescence units to the increase in absorbance units increased from approximately 440 when only glyceraldehyde 3-phosphate was added to approximately 730 when arsenate was added. Thus, it is demonstrated again that the fluorescence *per NADH molecule* in the ternary complex was quenched significantly as compared to that in the binary GPD–NADH complex.

Phosphate at 5 mM concentration did not act like arsenate in this system (Figure 5b), presumably because the system was too near the phosphorylation equilibrium. The addition of excess free NAD^+ to a final concentration of 944 μM resulted in the formation

TABLE I: Fluorescence Properties of NADH in the Ternary and Binary Complexes of GPD.

Addn to Std Mixture[a]	Theoretical Complex	Fluorescence (intensity)	NADH[b] (μM)	Fluorescence/ NADH (μM)	Fluorescence of Complex/ Fluorescence of Free NADH
1. None	Ternary	16	7.8	2.05	0.43
2. NAD^+	Ternary	54	25.1	2.15	0.45
3. Arsenate	Binary	71	18.8	3.77	0.78
4. Phosphate + kinase	Binary	50	15.1	3.31	0.69
Standard reference NADH + buffer alone	None	82	17.0	4.82	...

[a] The standard incubation mixture contained 7.11 μM GPD–NAD^+ and 118 μM glyceraldehyde 3-phosphate in 0.16 M Tris–acetate at pH 7.5. Where indicated additions of the following compounds to the stated concentrations were made: (2) 944 μM NAD^+, (3) 2 mM arsenate, (4) 2.5 mM phosphate, 17.5 units of yeast phosphoglycerate kinase, 2 mM ADP, and 2 mM $MgSO_4$. [b] Apparent NADH concentrations were calculated from the change in absorbance at 340 mμ.

of additional 3-P-glyceryl–GPD–NADH complex. Under these conditions also, the ratio of the increase in fluorescence units to the increase in absorbance units was the same (445) as it was before or after phosphate was added as long as the total NADH concentration did not exceed the binding capacity of the enzyme. This ratio is consistent with that obtained for the ternary complex that presumably existed in Figure 5a before the addition of arsenate to the reaction mixture. Thus, NAD^+ at this concentration level does not displace NADH from the 3-P-glyceryl–GPD–NADH complex.

The difference in intensity of NADH fluorescence between the ternary complex, the binary complex, and free NADH is not as dramatic at pH 7.5 as at pH 6, and phosphate alone was not sufficient for extensive removal of the acyl group as was arsenate. By analogy with the arsenate system, it appeared possible to relieve the apparent phosphate inhibition of the 3-P-glyceryl–GPD–NADH complex and to mimic the arsenate effect by the addition of ADP, Mg^{2+}, and phosphoglycerate kinase together with phosphate. In the absence of excess NAD^+, addition of the aforementioned reagents would be expected to favor phosphorolysis of the ternary complex in essentially the same manner as arsenate favors arsenolysis. The data of Table I show that, in fact, this is the case. The ratio of the fluorescence of free NADH to that of NADH in the binary complex is reasonably constant whether the binary GPD–NADH complex is formed by decomposition of the ternary complex by arsenolysis or phosphorolysis in the presence of phosphoglycerate kinase, Mg^{2+}, and ADP. At pH 7.5, as at pH 6, NAD^+ does not displace NADH of the acyl–GPD–NADH complex even in concentrations 10 times the kinetically determined dissociation constant and 4.5×10^4 times the directly determined dissociation constant for the GPD–NAD^+ complex. However, excess NAD^+ will allow essentially complete reduction of the bound NAD^+. Either in the presence or absence of excess NAD^+, the ratio of NADH fluorescence in the ternary complex to that of free NADH is about 0.44 as compared to about 0.74 for the binary complex. These data are consistent with the idea of an ordered dissociation of the acyl–GPD–NADH complex.

Discussion

These studies show that a ternary 3-phosphoglyceryl–GPD–NADH complex is an obligatory intermediate in the oxidation of glyceraldehyde 3-phosphate by GPD, and that the exchange of NADH of this complex does not occur unless an acyl acceptor molecule is present. Since acyl transfer is a rate-limiting step in this reaction as shown here and by kinetics (Furfine and Velick, 1965), it follows, therefore, that the energy-rich 1,3-diphosphoglycerate is formed from a reduced rather than an oxidized system. Although it has been shown that the 3-phosphoglyceryl–GPD–NAD^+ complex can form 1,3-diphosphoglycerate and can undergo arsenolysis (Krimsky and Racker, 1955) and that NAD^+ can act as coenzyme in the arsenolysis of the acetyl–GPD complex (Harting and Velick, 1954) it should be emphasized that those experiments were done under conditions that do not mimic the natural sequence of the forward reaction for this enzyme and that addition of the reduced form of the coenzyme would have favored reduction of the acyl group. It may be of significance also that if NADH cannot dissociate from GPD while the acyl group is bound, inherent in the reaction sequence would be a mechanism that would prevent the cysteine to lysine acyl-transfer reaction which causes inactivation (Mathew *et al.*, 1965; Park *et al.*, 1966). Therefore, the reaction sequence demonstrated

here is the one expected.

The exact chemical nature of the complex which produces the absorbance and fluorescence anomalies in the bound NADH is open to speculation. Two possibilities can be considered in light of these findings and some of those of other investigators. Firstly, let us consider the native muscle GPD–NAD^+ complex. This complex has a broad absorption band showing a maximum absorbance around 360 mμ (Racker and Krimsky, 1952). This 360-mμ absorption band has been attributed to a charge-transfer complex which probably involves a tryptophan residue and the pyridinium ring of pyridine nucleotide (Shifrin, 1964a).[3] Should such a complex exist in the native GPD–NAD^+, the pyridinium ring would serve as acceptor and the tryptophan as donor of electrons. Secondly, iodoacetate and model substrates for GPD bind a cysteine residue of the native protein (Harris *et al.*, 1963; Perham and Harris, 1963). Acetylation of the enzyme by model substrates such as *p*-nitrophenyl acetate or by iodoacetate is inhibited by glyceraldehyde 3-phosphate (Taylor *et al.*, 1963) also indicating that this thiol group is the acyl acceptor of the native enzyme. Furthermore, when iodoacetate is bound to this cysteine residue, the 360-mμ band is bleached (Racker and Krimsky, 1952). It is probable, therefore, that an acetyl–GPD–NAD complex is formed and that it is analogous to the 3-phosphoglyceraldehyde–GPD–NADH complex formed in these studies as shown by the data of Figures 2 and 3, the difference being that in the former case both the NAD and iodoacetate are electrophilic and in the latter case the thiolhemiacetal resulting from glyceraldehyde 3-phosphate addition and the NADH are nucleophilic. Thirdly, Kosower (1956) has suggested that NADH might serve as a donor of electrons in a charge-transfer complex involving NADH and carbonyl groups, in this case the 3-phosphoglyceryl–GPD–NADH complex. Thus, the assignment of a charge-transfer complex to the intermediate whose absorbance and fluorescence properties were presented here is consistent with existing knowledge. It is proposed, therefore, that the 3-phosphoglyceryl–GPD–NADH complex is of the charge-transfer type in which the NADH moiety acts as donor of electrons and the newly formed carbonyl group acts as acceptor of electrons. No charge-transfer complex forms when glyceraldehyde 3-phosphate interacts with the GPD–NADH species because both the NADH and the thiolhemiacetal formed are electronegative. By analogy, no charge-transfer complex of this type is formed when iodoacetate interacts with the GPD–NAD^+ species. In the latter case, both groups are electropositive.

[3] Although nicotinamide complexed with other amino acid side chains shows intramolecular charge transfer complexes (Shifrin, 1964b) the absorption spectrum observed for indolethylnicotinamide was more similar to that observed for muscle GPD. Moreover, since most of the protein fluorescence occurs as a result of the excitation of tryptophan, quenching of GPD fluorescence by addition of NAD^+ (Velick, 1958) probably can be explained by the formation of a tryptophan–NAD charge-transfer complex (Shifrin, 1964a).

The 3-phosphoglyceryl–GPD–NADH complex described here is not analogous to the binary complexes of NADH and lactic dehydrogenase (Neilands, 1952) nor NADH and liver alcohol dehydrogenase (Theorell and Bonnichsen, 1951) even though the shift in the absorption maximum for NADH is in the same direction. Kosower (1962) has suggested that the shift in NADH absorbance spectrum upon its binding to enzymes such as alcohol dehydrogenase may be due to a repulsion of the potentially positively polarized nitrogen atom in the 1,4-dihydropyridinium group by the approach of an ε-amino group (quaternary nitrogen) of lysine. This repulsion decreases the electron mobility and raises the excitation energy. It is worth noting that in the binary NADH–liver alcohol dehydrogenase complex, the fluorescence of NADH is shifted to a shorter wavelength and its intensity is much greater than that of free NADH (Sund and Theorell, 1963). However, the NADH of the 3-phosphoglyceryl–GPD–NADH complex exhibits much less fluorescence with no apparent shift in the wavelength of maximum emission, thus indicating some differences in its properties relative to the alcohol dehydrogenase–NADH system. Since fluorescence quenching is a general property of charge-transfer complexes involving molecules capable of fluorescence (Orgel and Phil, 1954), it is more reasonable to assume that the ternary 3-phosphoglyceryl–GPD–NADH complex observed is of the charge-transfer type rather than of the electrostatic repulsion type.

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The Effect of Metal Ions on Labile Phosphates. I. The Hydrolysis of Acetyl Phosphate Dianion*

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ABSTRACT: The hydrolysis of acetyl phosphate dianion is catalyzed by the divalent ions of Zn, Co, Mn, Ni, Ca, and Mg in aqueous solutions of pH 5.8–8.8. The rate of hydrolysis is dependent upon the specific metal catalyst employed, catalyst concentration, pH, temperature, and the particular buffer if one is employed. The rate of reaction has been determined in three aqueous media by an indirect spectrophotometric procedure and by titration of the acid generated in the absence of buffer. The observed rate constants for the magnesium- and calcium-catalyzed systems are interpreted in terms of three simultaneous reactions: an uncatalyzed reaction, attack on an intermediate metal complex by water, and attack on the intermediate metal complex by hydroxide ion. Formation constants for the metal complexes of acetyl phosphate dianion with calcium and magnesium ions have been estimated to be 7.7 and 5.7, respectively.

The catalytic (nonenzymatic) reaction of acetyl phosphate dianion,[1] AcP^{2-}, with water has been examined by several investigators (Lipmann and Tuttle, 1944; Koshland, 1952; Kurz and Gutsche, 1960; Di Sabato and Jencks, 1961a). The objective of the present work was a more detailed and extensive investigation of the role of metal ions in this reaction. The pH and the temperature dependence of the catalytic and the noncatalytic reactions have been established in part for a variety of catalysts in several different aqueous media. A comparison of the catalytic activity of six metal ions in the same system has been achieved. The principal chemical reaction under investigation is

$$CH_3COOPO_3^{2-} + H_2O = CH_3COO^- + HPO_4^{2-} + H^+$$

A secondary reaction takes place under certain conditions

$$2HPO_4^{2-} + 2OH^- + 3M^{2+} = M_3(PO_4)_2 \downarrow + 2\ H_2O$$

Experimental Procedures

Reagent grade metal perchlorates were employed and lithium acetyl phosphate (Worthington Biochemical Corp.) was used without further purification. The infrared spectrum of the latter is essentially the same as reported in the literature (Jencks *et al.*, 1960).

Reaction Media. Three aqueous media were em-

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[1] The following abbreviations are used: acetyl phosphate dianion, AcP^{2-}; divalent metal ions, M^{2+}; reaction products, RP.