On the Chemistry of Rabbit Muscle α-Glycerophosphate Dehydrogenase*

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Crystalline rabbit muscle α-glycerophosphate dehydrogenase is rapidly inactivated in acid, in alkali, and on freezing. This inactivation seems to be caused by a dissociation of the protein, which is slowly reversible in the case of the freezing or the acid-induced processes. The composition of the protein and the fingerprint pattern are compatible with the theory that at least some subunits are identical or closely related. The protein has two to three acetyl groups and no free N-terminal amino groups. However, only 1 mole of adenosine diphosphate ribose is bound per mole of enzyme. Nonprotein moieties of the enzyme appear not to be involved in either the dissociation or the catalytic-activity process.

Crystalline rabbit muscle α-glycerophosphate dehydrogenase is a complex protein (Baranowski, 1949; Eys *et al.*, 1959; Eys, 1960; Ankel *et al.*, 1960; Celliers *et al.*, 1963). However, removal of the nonprotein moieties does not appear to result in loss of activity (Eys *et al.*, 1959; Ankel *et al.*, 1960). In the course of attempts to ascertain a dependence of catalytic activity on cofactors, a number of treatments designed to remove bound groupings resulted in inactive preparations. These often regained activity after addition of synthetic or natural cofactors. However, these reactivations could never be completely described as being caused by cofactors. This difficulty became explained finally when it was realized that reversible dissociation of the enzyme occurred. The dissociated enzyme appears inactive. Deal and co-workers (Deal and Holde, 1962; Deal *et al.*, 1963b) have presented evidence previously that conditions which dissociate aldolase also reversibly inactivate glycerophosphate dehydrogenase, though initially they did not find a dissociation of the enzyme (Deal *et al.*, 1963b). Recently, however, a dissociation was also reported by Deal, but this was attributed to a reduction of disulfide bonds (Deal and Holleman, 1964), in contrast to the data to be reported here. Preliminary accounts of part of this material have been presented (Eys, 1960, 1963).

Materials and Methods

Source and Analysis of the Enzyme.—The data in this paper were all obtained on commercial α-glycerophosphate dehydrogenase (C. F. Boehringer and Sons, Mannheim, West Germany). Several preparations were used, all of which gave similar results. The protein was maintained throughout in a solution 2×10^{-3} M in Versene and 10^{-3} M in mercaptoethanol. Desalting of the enzyme before analysis was accomplished by exhaustive dialysis or, later, by passage through a Sephadex G-25 column. Protein concentrations were determined by the biuret method (Weichselbaum, 1946) of the method of Lowry *et al.* (1951), using crystalline ovalbumin as a standard. No significant difference between the amount of protein estimated from dry weight and either the biuret or Lowry method was seen. The variable amount of nonprotein material makes optical density measurements for protein concentrations unreliable.

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Preparation of Protein Derivatives.—The protein was oxidized with performic acid as described by Bettelheim (1955) for chymotrypsinogen. Carboxymethyl-α-glycerophosphate dehydrogenase was prepared by treating the protein in urea and mercaptoethanol with iodoacetic acid following the method of Crestfield *et al.* (1963). Dinitrophenylation of the protein was accomplished using either trimethylamine or sodium carbonate as the base, essentially as described by Fraenkel-Conrat *et al.* (1955). For complete reaction, as judged by lysine or tyrosine disappearance, usually two treatments were necessary, each continued for 12 hours.

Amino Acid Determinations.—Amino acid analyses were made on the Beckman/Spinco amino acid analyzer Model 120 according to the procedure of Moore *et al.* (1958). All hydrolyses were performed in evacuated, sealed ampules using 6 N HCl. In addition, the following special methods were used: Tryptophan determinations were done microbiologically, using *Lactobacillus arabinosus* as the test organism. The medium used was that of Greene and Black (1944). For this analysis, the protein was hydrolyzed in 5 N NaOH (Krehl *et al.*, 1946) and complete racemization was assumed. Also, the tyrosine-tryptophan ratio was checked spectrophotometrically as described by Beaver and Holiday (1952). A single tryptophan analysis was made by the procedure described by Noltmann *et al.* (1962). Lysine was on occasion determined microbiologically by the method of Steele *et al.* (1949) using *Leuconostoc mesenteroides* as the assay organism.

N-Terminal acetyl groups were determined by the method of Ludowieg and Dorfman (1960). Sulfhydryl determinations were performed with *p*-mercuribenzoate in the spectrophotometric method of Boyer (1954). In addition, the method of Ellman (1959) as modified by Jocelyn (1962) was used. Previous observations using iodine titrations have been published (Eys *et al.*, 1959).

Chromatography.—Dinitrophenyl derivatives of amino acids were chromatographed in systems as described by Fraenkel-Conrat *et al.* (1955). To detect dinitrophenylcysteic acid the procedure of Bettelheim (1955) was followed. [^{14}C]dinitrofluorobenzene was used in several instances.

"Fingerprinting" of the protein was performed using a 50:1(w/w) dehydrogenase-trypsin ratio. The dehydrogenase was pretreated either by performic acid oxidation or by conversion to the carboxymethyl derivative. The digestion was continued for 12 hours at *p*H 8.5. The *p*H was maintained in a *p*H-stat using 5% aqueous trimethylamine as base. The digest was then lyophilized and approximately 2-mg aliquots (as origi-

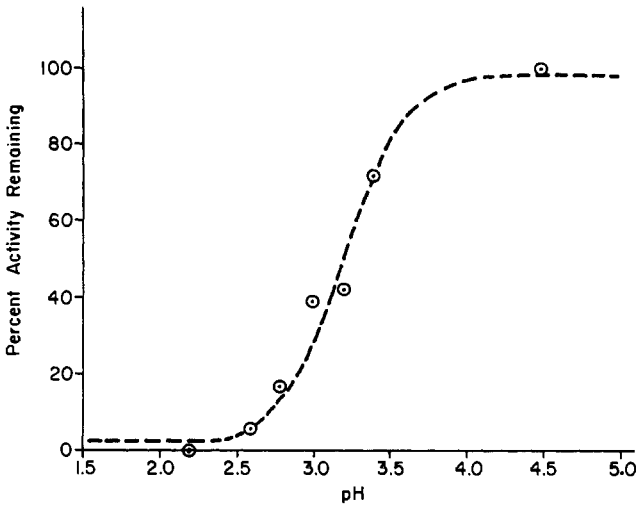

FIG. 1.—*p*H dependence of inactivation at acid *p*H values. Approximately 4 μg enzyme was diluted with glycine buffer of the *p*H indicated at 0.02 M final concentration. The enzyme was immediately assayed after dilution. The dotted line represents a theoretical curve which was drawn using the equation: $pH = 0.5 \log [f/(1 - f)] + 3.2$, where f = per cent activity remaining. This equation is derived from the equilibrium expression satisfying the relationship: active enzyme + 2 $H^+ \rightleftharpoons$ inactive enzyme. This equation implies no effect of a single proton addition.

nal protein) were applied to Whatman No. 1 paper. They were chromatographed first, ascending, in butanol–acetic acid–water (200:75:30). After drying for 3 hours at room temperature the chromatograms were subjected to electrophoresis at *p*H 3.7 in 0.1 M pyridine acetate buffer at 2000 volts for 45 minutes. Peptide spots were located by spraying with ninhydrin.

Other Chemical Determinations.—Total and inorganic phosphate were determined by the method of King (1932) and Lowry and Lopez (1946), respectively. Reducing sugars were determined by the method of Park and Johnson (1949), while pentoses were estimated by the orcinol procedure as modified by Lampen (1953).

Enzyme Assays.—α-Glycerophosphate dehydrogenase activity was assayed routinely as described previously (Eys *et al.*, 1959). All activities refer to the initial change in optical density at 340 mμ/min. The enzyme was equilibrated with suitable buffers by passage through Sephadex G-25 with the buffer required. Again, all solutions, unless specifically stated otherwise, were 2×10^{-3} M in Versene and 10^{-3} M in mercaptoethanol.

Preparations.—Crystalline bovine serum albumin and crystalline ribonuclease were obtained from Armour and Co. Crystalline ovalbumin and chymotrypsinogen were gifts of Dr. L. W. Cunningham. [^{14}C]Dinitrofluorobenzene was a generous gift of Dr. S. Harshman. All other chemicals used were standard commercial preparations.

RESULTS

Effect of Freezing and Thawing.—In dilute solutions the enzyme loses activity on storage in the frozen state. However, a great deal of this activity will return on standing at room temperature. Thus, immediately after thawing less than half the original activity was often present, but activity returned to the original level in 0.5 hour. The enzyme is rather more stable at room temperature. Preparations still had an activity of 85% after 24 hours, and after 83 hours the activity often dropped only to 75% of the initial value. This brings to mind the original method of crystallizing the enzyme through the preparation of myogen A (Baranowski, 1949). In this procedure the enzyme is treated and maintained for long periods at room temperature.

The inactivation on freezing is dilution-dependent. It is difficult to obtain quantitative data since the recovery of activity is also dependent on protein concentration, but in general, dilution enhanced loss of activity and slowed down the recovery. This seemed to point to dissociation; therefore other means of studying this phenomenon were sought.

Effect of Acid and Salts.—The reversible loss of activity on treatment with acid had been reported by Deal and co-workers (Deal and Holde, 1962; Deal *et al.*, 1963b). When the *p*H of the enzyme solution is lowered an immediate loss of activity results, which is only slowly progressive if the ionic strength is low. The lowering of the *p*H can conveniently be accomplished with dilute acetic acid or through the use of glycine buffer of the desired *p*H. Addition of salt results in further loss of activity. Since NH_4Cl, $(NH_4)_2SO_4$, and Na_2SO_4 all have this effect, the salt appears to assert its influence solely through its ionic strength.

Figure 1 shows the per cent activity remaining immediately after adjusting the *p*H to the indicated level at low ionic strength. The data follow closely a theoretical curve calculated from the equilibrium expression which satisfied the relationship:

$$\text{active enzyme} + 2\ H^+ \rightleftharpoons \text{inactive enzyme}$$

The half-maximal effect is at *p*H 3.2, which might implicate a β-aspartyl or γ-glutamyl carboxyl group.

Effect of Dilution of Acid Inactivation.—If the enzyme solution is lowered to a fixed *p*H but the concentration of enzyme is varied, one obtains data which indicate that the acid inactivation is dilution sensitive. Figure 2 shows the dilution effect on the enzymic activity both at low and high ionic strength. It is clear that the protein is more sensitive to dilution at the higher ionic strength. It appears that at low ionic strength a dilution sensitivity is observed which is compatible with a dissociation into two subunits.

Recovery of Activity after Acid Treatment.—The theoretical treatment of the data used in Figure 2 implies that the reaction is reversible. This is indeed the case. Figure 3 illustrates this recovery of activity with time. The neutralization could be accomplished conveniently by adding a predetermined amount of phosphate buffer. As indicated in the legend of Figure 3, it is imperative to have both Versene and mercaptoethanol present during the whole process. Recoveries of up to 85% of the original activity have been realized, which appears to indicate that the process is totally reversible.

Alkali Treatment.—Alkali also inactivates the protein. In fact, the protein is far more sensitive to alkali treatment than it is to acid treatment. The transition from active to inactive protein is very sharp (Fig. 4). The abrupt change occurs in a concentration range of NaOH between 5.0×10^{-3} M and 6.5×10^{-3} M. This range corresponds to a *p*H difference of only 0.11 *p*H unit. The protein remains active in 5×10^{-3} M NaOH for over 15 minutes. The inactivation by ammonium hydroxide shows this same sharp transition. The alkali inactivation can be reversed somewhat by neutralization with dilute acetic acid, though the recoveries have not been more than a few per cent of the original activity.

Behavior of the Enzyme on Sephadex Columns.—When the protein in 0.1 N acetic acid or in 1.5 M NH_4OH is applied to a column of Sephadex G-25 the protein appears at the salt boundary if the ionic strength is suf-

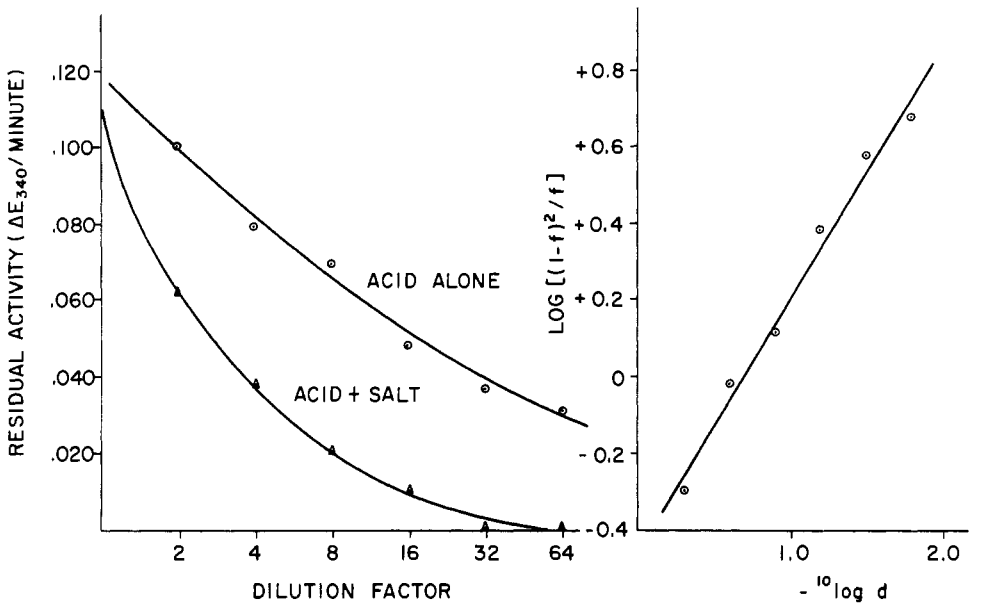

FIG. 2.—The effect of dilution on acid inactivation. (a, left) Several samples of enzyme were diluted with glycine buffer, final concentration 0.02 M, at a 2-fold decrease in concentration between each tube. The activity was assayed immediately. Then enough sodium sulfate was added to give a molarity of 0.15 M and the tubes were immediately reassayed. (b, right) The data for acid alone in Fig. 2a treated using the equation: $-(n-1)\log D = \log[(1-f)^n/f] + \text{constant}$; where D = dilution factor, f = fraction of activity remaining, and n = the number of subunits. This equation can be derived from a dilution effect on a reversible dissociation of a polymer, satisfying the relationship: active enzyme + 2 $H^+ \rightleftharpoons$ inactive enzyme $\rightleftharpoons n$ subunits. The data are given for $n = 2$ and the constant = 0.46. This constant is derived empirically, and is a composite of the two equilibrium constants in the equation and the *p*H of the experiment. Substituting higher numbers of subunits gives serious deviations from linearity.

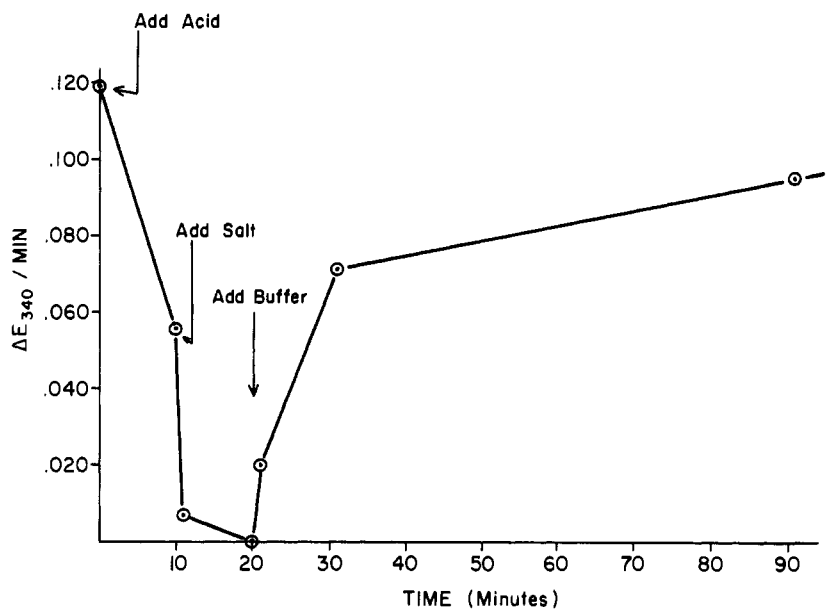

FIG. 3.—Reversibility of acid inactivation. Enzyme (10 μg) was brought to *p*H 3.2 glycine buffer, final strength 0.01 M (final volume 3.0 ml). Ten minutes later the tube was assayed and enough Na_2SO_4 was added to bring the molarity to 0.2 M. Ten minutes later, when no activity remained, the solutions were neutralized with phosphate buffer to *p*H 8.0 and the activity was estimated at the times indicated.

ficiently high (Fig. 5). This does not occur at low ionic strength; in that case, the protein is still excluded by the column. A number of model proteins were tested for similar behavior, and under the identical conditions described in Figure 5 bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease all were excluded by the column (though ovalbumin liberated what appears to be carbohydrate). Thus the change in ionic strength severely modified the behavior of glycerophosphate dehydrogenase.

Ultracentrifugation.—Both in acid and alkali, evidence for a dissociation of the enzyme can be observed on ultracentrifugation. The undissociated protein has a sedimentation constant $s_{20,w}$ = 4.9 S. At *p*H 2.6, in 0.01 M glycine buffer, a sedimenting peak was observed for which $s_{20,w}$ = 2.6 S. On prolonged centrifugation it was observed that two components were present with approximate sedimentation constants of 2.4 S and 5.2 S. Similar results are obtained in alkali. At low ionic strength in 1.5 M NH_4OH one

TABLE I
RATIO COMPOSITION OF α-GLYCEROPHOSPHATE DEHYDROGENASE

Amino Acid[a]		Molar Ratio to Aspartic Acid in 24-Hour Hydrolysates[b]	Ratio Extrapolated[c]	Probable Composition (aspartic acid = 28)	Residue Weight	Calculated Weight Per Cent	Observed Weight Per Cent[d]
Lysine[e]	(6)	1.052 ± 0.048	1.06	30	3,845.70	9.12	10.31
Histidine	(2)	0.325 ± 0.049	0.33	9	1,234.44	3.61	3.90
Ammonia	(6)			(41)			
Arginine	(6)	0.275 ± 0.022	0.28	8	1,249.68	3.29	3.65
Aspartic acid	(11)	1.000	1.00	28	3,223.08	8.49	9.08
Threonine	(11)	0.444 ± 0.012	0.44	13	1,314.56	3.46	3.68
Serine[f]	(6)	0.351 ± 0.009	0.41	12	1,045.20	2.75	2.22
Glutamic acid	(11)	1.441 ± 0.025	1.44	41	5,294.33	13.61	14.35
Proline	(11)	0.547 ± 0.018	0.55	16	1,554.08	4.35	4.25
Glycine	(11)	1.364 ± 0.021	1.41	40	2,282.80	5.86	6.46
Alanine	(11)	1.144 ± 0.016	1.20	34	2,417.40	5.99	6.48
Cysteine[g]	(6)	0.363 ± 0.026		10	1,031.50	2.72	2.10
Valine	(10)	1.007 ± 0.380	1.15	32	3,172.80	7.31	8.97
Methionine[h]	(11)	0.270 ± 0.017	0.27	8	1,049.68	2.77	2.99
Isoleucine	(11)	0.958 ± 0.030	0.96	27	3,055.59	8.05	8.34
Leucine	(11)	1.058 ± 0.017	1.08	30	3,395.10	8.95	9.24
Tyrosine	(5)	0.138 ± 0.053	0.14	4	652.76	1.72	1.70
Phenylalanine	(11)	0.536 ± 0.012	0.54	15	2,207.85	5.82	6.13
Tryptophan[i]				2	372.44	0.98	1.10
Total				359	38,398.99[j]	98.85	104.97

[a] Figures in parentheses give the number of 24-hour hydrolysis values which were averaged. [b] Figures are given with their standard deviations. [c] Extrapolated graphically from 24-, 48-, and 72-hour hydrolysates. [d] Average of four determinations except for tryptophan. [e] Includes ε-dinitrophenyllysine figures. [f] Serine was extensively destroyed under certain conditions; therefore several values were excluded. [g] As either cysteic acid or carboxymethylcysteine. No cystine is present (see Table III). [h] Includes methionine sulfone obtained from performic acid–oxidized samples. Tryptophan was determined separately (see Table II). The observed weight per cent is the figure calculated from microbiological determinations. [i] To this figure one needs to add approximately 430 as the contribution of the nonprotein material. The cofactors therefore comprise 1.13% of the total weight.

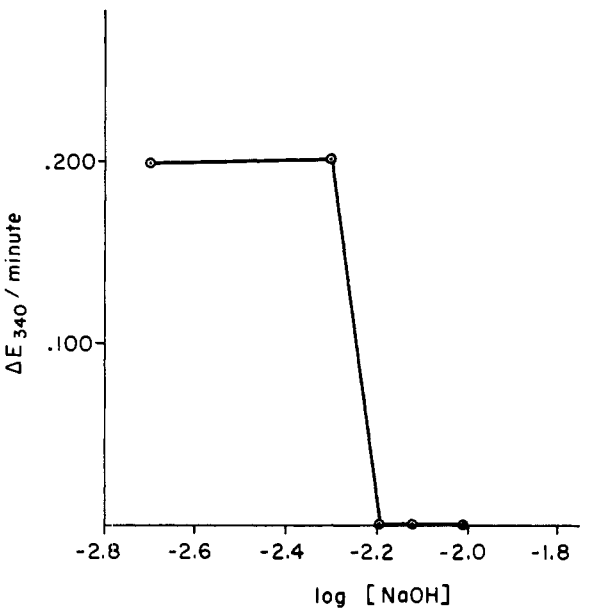

FIG. 4.—Inactivation by sodium hydroxide. Enzyme (5 μg) was added to 0.1 ml of solutions of sodium hydroxide to give the final molarity indicated. The solutions were assayed 2 minutes later.

boundary was seen for which $s_{20,w}$ = 2.7 S. Both in acid and in alkali at high ionic strength no specific boundary was observed. These results are illustrated in Figure 6.

Effect of Other Agents on Dissociation.—Sodium dodecylsulfate, an agent which frequently is found to give dissociation in other proteins, is a strong inhibitor of the enzyme. Fifty % inhibition of activity is observed at 7×10^{-6} M. However, at levels which give complete inhibition, no dissociation is seen as judged from sedimentation patterns in the ultracentrifuge at neutral *p*H values. Equally without effect on molecular size are the inhibitors *p*-mercuribenzoate, urea, or zinc ions.

Composition of the Protein.—Table I summarizes the results of analyses obtained on the protein. These figures include results on performic acid-oxidized, carboxymethylated, and dinitrophenylated protein preparations. Therefore, fewer values are obtained for histidine, cysteine, and tyrosine. Lysine figures include ε-dinitrophenyllysine, while methionine figures include those obtained as methionine sulfone. Corrections for loss during hydrolysis have been made from observations on 48- and 72-hour hydrolysates. The most serious loss was found in serine and proline, and to some degree in glycine. Little further liberation of leucine and isoleucine occurred on further hydrolysis, though some was found for valine. Serine gave special variations above and beyond losses resulting from hydrolysis, for reasons not quite clear at present. Valine also had a large variation in the 24-hour hydrolysates. However, valine was the one amino acid which was incompletely liberated at 24 hours. Tryptophan and cysteine values are discussed separately.

Adjustment of the ratios to the nearest integer gives a minimal chemical molecular weight of 38,400 on the assumption of 28 aspartic acids. Aspartic acid was chosen as standard since the values obtained were constant at all times of hydrolysis. Leucine and isoleucine could have served equally well. As discussed later, tryptophan serves to set a limit to the minimal size of the protein at approximately half the figure given, while tyrosine, the next lowest amino acid, would restrict the minimal molecular weight to one-fourth or one-fifth this figure given. However, if one sets aspartic acid at 14, greater deviations from integral values are obtained, so the figure of 28 was preferred.

From these data a weight per cent can be calculated, based on the ratios. The data so far given were some-

TABLE II
TRYPTOPHAN CONTENT OF α-GLYCEROPHOSPHATE DEHYDROGENASE

Method	Experiment	Residue Weight (%)	Ratio Tyrosine/ Tryptophan	Ratio Lysine + Ornithine/ Tryptophan	Moles/Mole Enzyme
Microbiological	1	1.023			4.22
	2	1.180			4.86
	3	1.074			4.43
Spectrophotometric	4		3.1		2.60
	5		2.59		3.80
	6		2.42		3.32
Column chromatographic	7			18.4	4.13
Average					3.81

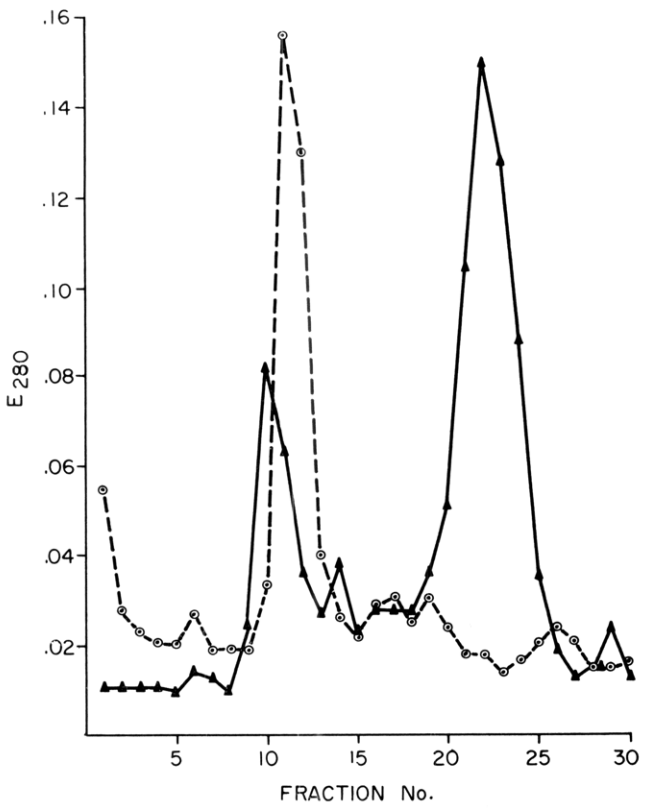

FIG. 5.—Sephadex chromatography of alkali-treated enzyme. α-Glycerophosphate dehydrogenase (800 μg) in 1 ml of 0.35 M $(NH_4)_2SO_4$ was applied to a 25 × 1–cm column of Sephadex G-25, and the column was developed with distilled water. The broken line indicates the elution pattern so obtained. The solid line shows the elution pattern when the original enzyme was dissolved in 1 ml of 0.35 M $(NH_4)_2SO_4$, 1.5 M in NH_4OH.

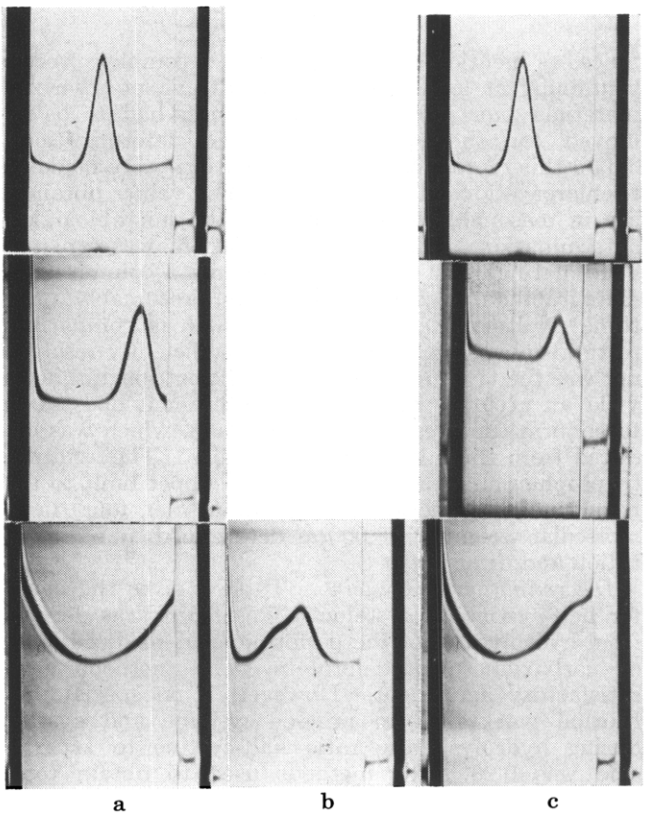

FIG. 6.—Ultracentrifuge patterns of α-glycerophosphate dehydrogenase. (a) The effect of acid. The first frame represents a control in phosphate buffer *p*H 6.5; $s_{20,w}$ = 4.9 S. The second frame is from a run in 0.01 M glycine, *p*H 2.6; $s_{20,w}$ = 2.6 S. The third frame results from the enzyme in 0.01 M glycine, *p*H 2.6, 0.2 M in Na_2SO_4. All photographs were 50 minutes after reaching speed (59,780 rpm). Sedimentation is from left to right. (b) Prolonged centrifugation in acid. 0.01 M glycine buffer, *p*H 2.6, 124 minutes after reaching speed (59,780 rpm). Sedimentation is from left to right. (c) The effect of alkali. The top picture is a control run, as in (a). The second frame comes from an experiment in which the enzyme was centrifuged in 1.5 M NH_4OH, 0.01 M in Na_2SO_4; $s_{20,w}$ = 2.6 S. The third frame represents enzyme dissolved in a solution 0.25 M in Na_2SO_4, and 0.025 M in NaOH. All figures are 50 minutes after attaining speed (59,780 rpm). Sedimentation is from left to right. The third frame is distinctly different from a control run of buffer alone and indicates material at the top boundary.

times obtained on preparations for which the absolute amounts were not known, owing to various pretreatments. However a number of determinations were made where the weight of protein was known. Table I also compares the composition derived from ratio data with directly determined figures. Since the only significant discrepancies which occur are for valine and serine, the ratio data were considered satisfactory; and, since ratio data were based on more observations, they were in fact preferred. The minimal molecular weight figure from ratios of 38,400 can also be compared to an average minimal molecular weight calculated from weight per cent data of 18,710 ± 642. Twice the latter, i.e., 37,420, constitutes good agreement, especially since the latter is not corrected for losses during hydrolysis. One should add approximately 860 for the contribution of prosthetic groups (*vide infra*). Since the molecular weight of the protein appears to be 70–78,000 as determined by physical methods (Eys *et al.*, 1959; Ankel *et al.*, 1960), a molecular weight from composition of 76,800 seems indicated.

Determination of Tryptophan.—Table II summarizes the results obtained for tryptophan determinations.

TABLE III
CYST(E)INE BALANCE IN GLYCEROL PHOSPHATE DEHYDROGENASE

Treatment of Protein	Derivative Measured	Moles Found/Mole Enzyme[a] Before Dinitrofluorobenzene	After Dinitrofluorobenzene
p-Mercuribenzoate	—SH groups	11.8[b]–16.6[c]	
Di-(3-carboxy-4-nitrophenyl)-disulfide	—SH groups	11.9	
Untreated	Cystine/2	14.6	0
Performic acid oxidized (after dinitrofluorobenzene)	Cysteic acid	19.5	1.68
Performic acid oxidized (before dinitrofluorobenzene)	Cysteic acid	19.5	25.5
Carboxymethylated	Carboxymethyl-cysteine	20.7	17.8

[a] Assuming a molecular weight of 76,800 Table I. [b] Data from this study. [c] Data from Ankel *et al.* (1960).

Since, as mentioned, the enzyme is a complex protein containing at least two different ultraviolet-absorbing materials (*vide infra*), these substances had to be removed for the spectrophotometric determinations. To do this reproducibly the observations were made on trichloroacetic acid precipitates. The values obtained are in reasonable agreement with the microbiological determinations, though the variation was greater. The final figure in Table II represents a column-chromatographic run. Since lysine plus, presumably, ornithine (Noltmann *et al.*, 1962) are high in comparison to tryptophan, the peaks were not sufficiently resolved nor was the tryptophan peak of sufficient magnitude to yield an accurate figure. The experiment does serve to confirm the presence of tryptophan, which was deduced from the microbiological results. The results of tryptophan analyses serve to set an upper limit to the number of possible identical subunits (i.e., four) for a molecular weight of 78,000 as determined by sedimentation and diffusion.

Determination of Cysteine.—Table I gives the figure for half-cystine as cysteine. This figure was derived from hydrolysates of the performic acid–oxidized and/or carboxymethylated protein. The methods gave satisfactory agreement. However, if no special precaution was taken to protect cysteine and cystine during hydrolysis the ratio half-cystine to aspartic acid was 0.26. The methods used to obtain total cysteine values do not distinguish between cysteine and cystine, since the carboxymethylation was done after reduction of the protein. Therefore an attempt was made to ascertain the contribution of both –S–S– and –SH forms. Previously it was reported that about 6 moles –SH/78,000 g was found on iodine titration (Eys *et al.*, 1959).[1] Ankel *et al.* (1960) found an average of about 15.1 moles/70,000 of protein with *p*-mercuribenzoate. In our hands protein in 8 M urea gave results similar to Ankel's figure: with *p*-mercuribenzoate 11.8 moles/78,000 g, and with (di-3-carboxy-4-nitrophenyl)disulfide 11.9 moles/78,000 g are found. Without urea values as low as 7.8 moles/78,000 g of protein have been obtained. However, even the highest figure obtained by –SH analyses allows only about sixteen –SH groups, while there are about twenty total half-cystine residues per 78,000 g.

It was observed that the protein, when dinitrophenylated but otherwise untreated, contained no cystine. Similarly, a dinitrophenylated protein failed to yield cysteic acid after performic acid oxidation. However, if the order was reversed, first performic acid oxidation, and then dinitrophenylation, all the cystine was accounted for as cysteic acid. Also, dinitrophenylation of a carboxymethylated protein did not remove any carboxymethylcysteine. These results are summarized in Table III. Thus the conclusion must be reached that all the half-cystine is as cysteine and that some –SH groups are unavailable to –SH reagents, even in 8 M urea. This view is strengthened by the following experiment. The protein was treated with [^{14}C]dinitrofluorobenzene, followed by performic acid oxidation and acid hydrolysis. Carrier dinitrophenylcysteic acid was added. On reisolation of the dinitrophenylcysteic acid no significant radioactivity remained after several repeated purifications; therefore the loss on dinitrophenylation is not owing to an N-terminal half-cystine.

N-Terminal Amino Acids.—On exhaustive dinitrophenylation of the protein, followed by acid hydrolysis no definite assignment of α-dinitrophenylamino acids could be made. Even with [^{14}C]dinitrofluorobenzene no stoichiometrically significant quantity of N-terminal amino acids was found. The amino acid analysis of a dinitrophenylated protein showed, as expected, complete disappearance of cysteine, histidine, and tyrosine, and almost complete disappearance of lysine. Two new ninhydrin-positive compounds appeared during analysis, both on the short column. One has been definitely identified as ϵ-dinitrophenyllysine. This emerges from the column at approximately 58–60 ml after arginine as a somewhat trailing peak. It gives characteristically a higher extinction at 440 mμ than the attenuated 570 mμ recording. The second unknown chromatographed in the position of tryptophan. This peak was quantitatively too high for tryptophan and is unidentified at present. All other amino acids remained unchanged.

Acetyl determinations on the protein suggested an explanation for the unavailability of the terminal amino groups. On multiple analyses, between 2 and 3 moles of acetyl groups per 78,000 g of protein were found (Table IV). Adenosine diphosphate ribose, which is a normal prosthetic group on the enzyme, yields some color in the determination, so that the figure of 2 moles of "acetyl" per mole of enzyme is probably closer to the correct one (Table IV).

Fingerprinting.—The molecular weight of 76,800 represents 76 lysine plus arginine residues. On digestion with trypsin, if there were no subunits, or if the subunits were different, one should see about 77–78 peptides. However, in repeated experiments chromatography of trypsin digests gave no more than 16

[1] This figure may in reality represent 12 moles —SH/78,000 g. Both β-lactoglobulin and ovalbumin, used as standards, yield sulfenyl iodides (Cunningham and Nuenke, 1959). However, this is not the case for all proteins, and disulfide formation would require but half the iodine consumption per —SH group (L. W. Cunningham, personal communication).

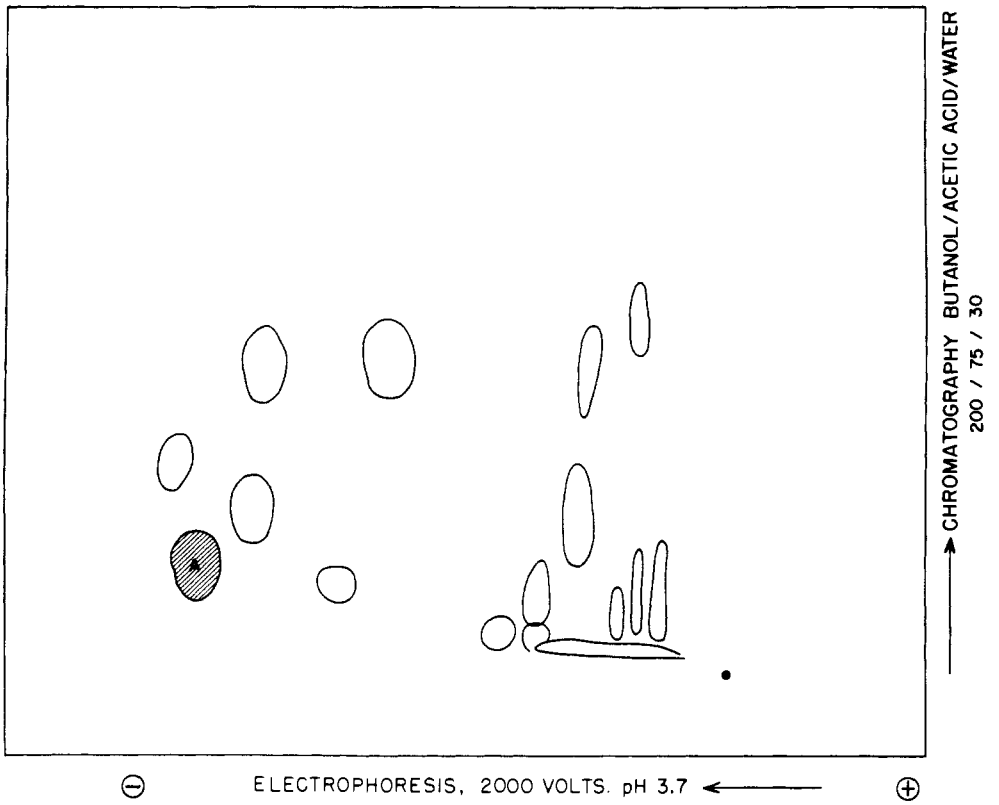

FIG. 7.—A representative tracing of a "fingerprint" of α-glycerophosphate dehydrogenase. The spot labeled A was always dark on ninhydrin development and easily discernible.

TABLE IV
ACETYL DETERMINATIONS ON α-GLYCEROPHOSPHATE DEHYDROGENASE

Preparation	Sample Size (mg)	Acetyl/ Sample (μmoles)	Moles Acetyl/ 78,000 g
α-Glycerophosphate dehydrogenase	13.2	0.35	2.1
	8.6	0.32	3.0
	8.6 (μmoles)	0.32	3.0
Adenosine diphosphate ribose	0.25	0.20	0.81
	0.24	0.18	0.65
Average acetyl groups/mole of protein corrected for ADPR			1.92

TABLE V
ANALYSIS OF α-GLYCEROPHOSPHATE DEHYDROGENASE[a]

Components	Enzyme (moles/ 78,000 g protein)	Supernatant (moles/ mole adenine)[b]	Adenosine Diphosphate Ribose (moles/mole adenine)
Ribose	2.10	1.73	2.12
Phosphate[c]	2.19	1.75	1.91
Reducing power[d]	0.75	0.62	0.31
Adenine	1.25	1.00	1.00

[a] The crystals were collected by centrifugation and were redissolved in distilled water. On occasion this was dialyzed for 4 hours against 2000 volumes of distilled water. Then an equal volume of trichloroacetic acid was added and the supernatant was analyzed. The precipitate was redissolved and analyzed by the biuret test. Adenine was calculated from the extinction at 262.5 mμ. [b] Assuming all ultraviolet-absorbing material is adenine. [c] Corrected for inorganic phosphate. [d] Xylose was used as standard.

peptides (Fig. 7). Even though some of the elongated ninhydrin-positive areas could represent more than one peptide, it is unlikely that 78 peptides are present. If the protein were a tetramer, one would expect 20 peptides, while for a dimer one would expect 39. To help distinguish between the two possibilities the peptide area designated A in Figure 7 was eluted and hydrolyzed. Qualitative analysis showed that it contained lysine. Quantitative analysis gave less than 2 moles of lysine per mole of enzyme. If the assumption is made that the subunits are identical, then a dimer structure seems indicated.

Stoichiometry of the Prosthetic Groups.—Part of the nonprotein components was identified by Ankel *et al.* (1960) as adenosine diphosphate ribose. Table V gives a typical analysis of the supernatant of the protein. It is clear that the analysis is not satisfactory for adenosine diphosphate ribose, nor does the spectrum of the supernatant agree with the spectrum of an adenine derivative. However, hydrolysis of the supernatant does yield a base which on cochromatography with [8-^{14}C]adenine coincides in three solvents. Also the adenosine diphosphate–ribose can be isolated from the supernatant of a $HClO_4$-precipitated protein. The difference in analysis between pure adenosine diphosphate–ribose and enzyme supernatant lies in the presence of additional material (Eys, 1960; Celliers *et al.*, 1963.)[2] It is certain, however, that not more than *one* adenosine diphosphate ribose is present per mole of enzyme. While there is too much absorbing material and far too much reducing power, the phos-

[2] The other prosthetic group was tentatively identified initially as 4-methyl-5-(β-hydroxyethyl)thiazolyl succinic acid (Eys, 1960, 1961). Experiments of Celliers *et al.* (1963) have suggested that this structure may be in error. Our own experiments with [^{14}C]thiamin also leave the structure less certain that it appeared previously.

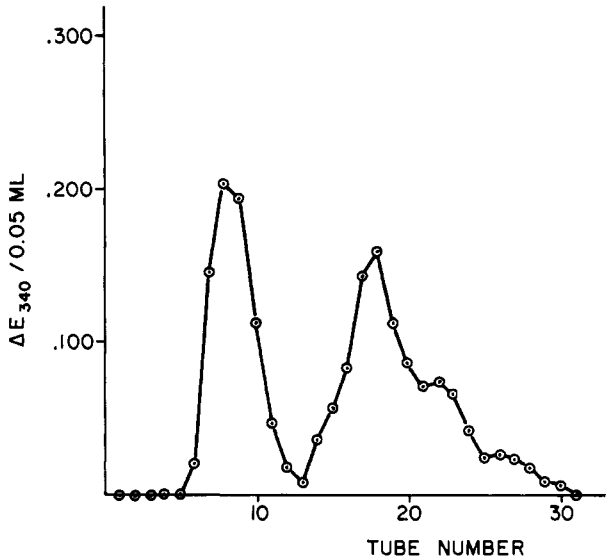

FIG. 8.—Amberlite chromatography. A 15 × 1-cm column of Amberlite IR-120 was washed with 100 ml of 0.01 M NaOH in 2 × 10^{-3} M Versene, 10^{-3} M in mercaptoethanol. This was followed with Versene-mercaptoethanol until the *p*H returned to about 5–6. The sample shown in this figure was 2 ml of enzyme desalted with Sephadex G-25, containing 5 mg of protein. Elution was performed with a gradient of 100 cc of 0.02 M phosphate, *p*H 8.0 in Versene-mercaptoethanol, into 100 cc of Versene-mercaptoethanol. Fractions (1 ml) were collected and aliquots were assayed in the standard manner. On rechromatography each fraction reemerged at its own position. The ratio between the fractions is variable, partially because the column is easily overloaded.

phate and orcinol analyses exclude any other interpretation.

Isozymes and the Role of the Nonprotein Moieties.—If, on acidification, one of the nonprotein moieties dissociated and such dissociation resulted in loss of activity, the results shown in Figure 2b could be explained. This possibility can be ruled out however. The enzyme can be fractionated on Amberlite IR 120 (Fig. 8). Two main peaks are obtained which are both fully active. The second peak is devoid of any nonprotein material. This second peak is inactivated by freezing, acid, and alkali in a manner completely analogous to the nonchromatographed protein. Thus the protein itself and not the nonprotein material is responsible for the observed effects. The nonprotein components emerge in the position of the first peaks, so that this procedure does not allow a decision as to whether a total dissociation of cofactors is effected.[3]

DISCUSSION

The data presented in this paper are all compatible with the theory that α-glycerophosphate dehydrogenase as isolated is composed of multiple polypeptide chains. The kinetic analysis of inactivation, the acetyl determinations and the amino acid analysis are compatible with a dimer structure. However, a trimer or a tetramer is not ruled out by the data. It seems clear from the "fingerprint" analysis that at least some of the subunits must be identical. The finding of subunits suggests a comparison with other dehydrogenases in this class. Beef heart lactic dehydrogenase with a molecular weight of 72,000 (Millar, 1962) or double that figure (Neilands, 1952) will yield subunits of about 35,000 mw each (Apella and Markert, 1961). Yeast-alcohol dehydrogenase is composed of four subunits of about 36,000 mw each (Kägi and Vallee, 1960; Hersh, 1962). Glutamic dehydrogenase can be split into subunits of that size (Fisher *et al.*, 1961; Jirginsons, 1961; Frieden, 1962; Fisher *et al.*, 1962a,b). Malic dehydrogenase is another example (Harrison, 1963). There are many additional examples of proteins other than dehydrogenases which dissociate into subunits, the molecular weight of which often converges around a limit of 35–50,000.

The nature of agents and conditions which dissociate the protein are fairly standard, since acid and alkali are now well-recognized means for the demonstration of subunits. Freezing as a cause of dissociation has also been observed for glucose-6-phosphate dehydrogenase (Kirkman and Hendrickson, 1962). But the subunits must be held together with extreme tenacity in α-glycerophosphate dehydrogenase, since reactivation occurs at extraordinarily high dilution. Furthermore, standard protein-denaturing agents such as urea and sodium dodecylsulfate have little effect.

It is interesting that most dehydrogenases have a stoichiometry of DPN-binding which is close to 1 mole per 35–40,000 mw (see Eys *et al.*, 1958; Pfleiderer and Auricchio, 1964). α-Glycerophosphate may be an exception to this. Ankel *et al.* (1960) reported a stoichiometry of 1 mole per 78,000 g and our data are not in disagreement with this (Eys *et al.*, 1959), though Pfleiderer and Auricchio (1964) suggest 2 moles per 78,000 g. In addition, it is reasonable to assume that adenosine diphosphate ribose is bound at the DPN-binding site (Ankel *et al.*, 1960) and again only 1 mole per 78,000 g is bound. Dissociation of α-glycerophosphate dehydrogenase could then represent a dissociation beyond the minimally unexpected size. There are examples in the literature where an enzyme dissociates into more subunits than there are active sites on the molecule. Aldolase is a well-described example. In that enzyme, two active sites exist (Horecker *et al.*, 1963) but three subunits are demonstrable (Stellwagen and Schachman, 1962; Deal *et al.*, 1963a). Yeast hexokinase is another example of an enzyme dissociating into more subunits than active sites (Schachman, 1960; Ramel *et al.*, 1961; Kenkare and Colowick, 1963).

The nature of the effect of high salt on glycerophosphate dehydrogenase is not clear. It is obvious from the ultracentrifugal analysis and Sephadex chromatography that an extreme deformation occurs. Whether this represents further dissociation is problematical. Only two to three N-terminal acetyl groups were found per mole and no free amino groups. Therefore it is unlikely that this salt effect represents further dissociation unless an actual breaking of covalent peptide linkages occurs. This seems unlikely because of the free reversibility of the inactivation when the experiment is carried out on the acid side. We must conclude therefore that the observed phenomenon is due to a total unfolding of the molecule.

[3] It is of interest that chromatography of the enzyme on Sephadex G-50 at high ionic strength gives two peaks with enzyme activity. This phenomenon is not observed on G-25 or on G-75. The two peaks are separated from the salt fraction. The two fractions have a different 260/280 ratio in their ultraviolet-absorption spectra and contain different quantities of adenosine diphosphate ribose.

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