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The Effect of Dioxane on the Dissociation and Activity of Glutamic Dehydrogenase*

JORGE E. CHURCHICH AND FINN WOLD†

From the Biochemistry Division, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana

Received December 7, 1962

Dioxane has been found to cause dissociation of the glutamic dehydrogenase molecule without interfering with the activity of the enzyme. For a 1% protein solution the apparent molecular weight of glutamic dehydrogenase is 990×10^3 ($s_{20,w} = 24$ S) in buffer, and decreases to 550×10^3 ($s_{20,w} = 16.5$ S) in 5% dioxane, and to 300×10^3 ($s_{20,w} = 13$ S) in 10% dioxane. There is no change in rotatory dispersion associated with the dissociation, and several properties of the subunits (absorption, fluorescence, DPNH binding, and enzymatic activity) appear to be unaffected by dioxane. Glutamic dehydrogenase is known to dissociate on dilution, and it has been found that regardless of the nature of any dissociating or associating reagents added (DPN, DPNH, diethylstilbestrol, dioxane), the extrapolated value of $s_{20,w}$ at infinite dilution is 13 S. It is therefore concluded that the active form of glutamic dehydrogenase *in vitro* must be the low (300×10^3) molecular weight unit.

The dissociation of beef liver glutamic dehydrogenase into subunits of different size can be affected by a number of reagents, and the current picture of the various forms of the enzyme is a rather complex one. At high concentration the apparent molecular weight of glutamic dehydrogenase (GDH)[1] is 10^6 g/mole, but, as was first shown by Olson and Anfinsen (1952), this high molecular weight molecule appears to dissociate on dilution. A number of workers have investigated the dissociation of the 10^6 mw GDH, and found that dissociation into 250×10^3 mw "subunits" can be caused by steroid hormones (Yielding and Tomkins, 1960; Tomkins *et al.*, 1961), by high concentration ($> 10^{-4}$ M) of DPNH (Frieden, 1959a, 1959b), by thyroxine (Wolff, 1961a, 1961b), by simple inorganic and organic anions (Frieden, 1962; Wolff, 1961a), and by extremes of *p*H (Frieden, 1962). ADP, DPN, and low levels of DPNH appear to stabilize the high molecular weight unit (Frieden, 1959a, 1959b). In all these cases the association - dissociation is reversible, and since the conditions favoring dissociation also seem to cause inhibition of the enzyme and since association is promoted by the coenzymes, several attempts have been made to correlate dissociation and loss of enzyme activity. Such correlations have recently been questioned (Mildvan and Greville, 1962). A number of reagents have also been found to cause irreversible dissociation of GDH beyond the 250×10^3 units. Thus urea and dodecylsulfate (Wolff, 1961a; Jirgensons, 1961) cause dissociation into units of 43×10^3 mw, and, at *p*H above 10, GDH dissociates into some 18 subunits (Fisher *et al.*, 1962).

In the present paper, we report the reversible dissociation of GDH into apparent 500×10^3 and 250×10^3 units in dioxane, without any loss in biological activity. The evidence presented favors the hypothesis that no unit larger than 250×10^3 mw can exist under the conditions of the *in vitro* enzyme assay, and any direct correlation between the dissociation process and enzyme activity thus appears very unlikely.

* Supported by USPH grants RG-8197, A-2905.

† National Institutes of Health Research Career Development Awardee.

[1] Abbreviations used in this paper: GDH, glutamic dehydrogenase.

EXPERIMENTAL

Materials.—Crystalline bovine liver GDH was obtained from Calbiochem (Lots 501180, 502398, and 502991 of Boehringer preparations) as ammonium sulfate suspensions. The enzyme was centrifuged, dissolved in 0.05 M sodium phosphate buffer *p*H 7.2, and dialyzed against the same buffer for at least 10 hours before use. Highest purity grade DPN, DPNH, and substrates were obtained from Sigma Chemical Company and from Calbiochem. Dioxane was reagent grade, was purified by reflux with KOH followed by distillation, and was stored in sealed, dark bottles.

Methods.—All the sedimentation experiments were performed with the Spinco Model E ultracentrituge at constant temperatures in the range from 4° to 9°. The experimental sedimentation coefficients were normalized to water at 20° by correcting for viscosity and density in the normal manner. The temperature-viscosity relationships for buffer, 5% dioxane-buffer, and 10% dioxane-buffer were determined directly with Ostwald-Fenske viscometers and are given in Table I. For the density corrections, the contribution of dioxane to the density was disregarded and only the temperature corrections were considered. $\bar{V}$ was assumed to be constant and equal to 0.75 ml/g. The protein concentration was determined from the 280 mμ absorption using the extinction coefficient reported by Olson and Anfinsen (1952). Moving boundary electrophoresis was carried out in the Spinco Model H electrophoresis-diffusion apparatus. Absorption spectra were recorded with the Bausch and Lomb Spectronic 505 spectrophotometer, and measurements of fluorescence spectra and fluorescence polarization were carried out with the Aminco-Bowman fluorescence spectrophotometer sup-

TABLE I

THE VISCOSITY OF BUFFER AND BUFFER-DIOXANE MIXTURES

The data were obtained with an Ostwald-Fenske Viscometer with a flow time of 5 minutes at 25°; 0.05 M sodium phosphate buffer, *p*H 7.2, was used. The recorded temperature of the bath was accurate to ±0.1°.

t, °C.	Buffer	Viscosity in Centipoises Buffer-5% Dioxane	Buffer-10% Dioxane
4.4	1.55	1.64	—
5.4	1.50	—	1.83
6.4	1.45	—	1.77
7.1	1.42	—	1.72
8.2	1.38	1.46	1.68
9.0	1.346	—	1.62
9.7	1.32	—	—
10.4	1.29	1.36	—

plied with a high intensity xenon arc or with the Brice-Phoenix light scattering photometer modified according to Steiner and Edelhoch (1961) and also provided with a water jacket to insure good temperature control. The rotatory dispersion was determined with the Rudolph recording spectropolarimeter. Activity measurements were carried out manually with a Beckman DU spectrophotometer. Special care was taken to eliminate peroxides in the dioxane used for the kinetic experiments and the experiments involving measurements of the fluorescence of DPNH. In the ultracentrifuge there was no evidence of difference between peroxide free and fresh reagent grade dioxane, and the two were used interchangeably. The dioxane concentration is given as volume per cent.

Sodium phosphate buffer, 0.05 M at *p*H 7.2 was used for all the physical experiments, while the same buffer at a somewhat higher *p*H (7.4–7.6) was used for activity measurements. No attempt was made to compensate for the slight increase in the buffer *p*H in the presence of dioxane. The magnitude of this change is illustrated by the following values, representing the actual *p*H meter readings after dilution of the stock buffer solution: no dioxane, *p*H 7.6; 5% dioxane, *p*H 7.7; and 10% dioxane, *p*H 7.83. Only the *p*H of the dioxane free buffer will be given in the text.

RESULTS

The sedimentation patterns of GDH (10 mg/ml) in phosphate buffer containing 0%, 5%, and 10% dioxane are shown in Figure 1. Only a single peak is apparent.

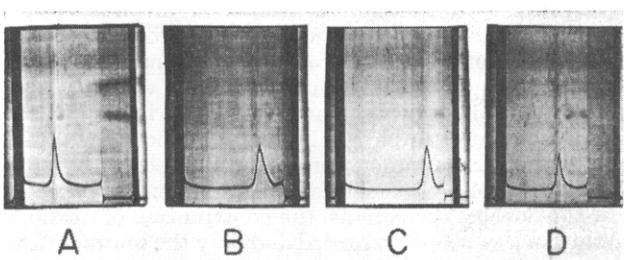

FIG. 1.—Sedimentation pattern of GDH (10 mg/ml) in buffer (0.05 M sodium phosphate, *p*H 7.2) and in buffer-dioxane mixtures. A, GDH in buffer; B, GDH in 5% dioxane; C, GDH in 10% dioxane; D, GDH exposed to 10% dioxane for several hours, and then dialyzed overnight against buffer (exposures made at A, B, C, 32 min. D, 16 min. after reaching constant speed). The experiments were conducted at 8° with a centrifuge speed of 42,040 RPM. The resulting sedimentation coefficients are given in Table II. It should be noted that after 90 minutes there was still no indication of separation of the asymmetrical boundaries into separate peaks.

TABLE II

THE EFFECT OF DIOXANE ON THE SEDIMENTATION COEFFICIENT AND THE MOLECULAR WEIGHT OF GLUTAMIC DEHYDROGENASE

The data were obtained with 10 mg/ml GDH, at 8°, and in 0.05 M sodium phosphate buffer, *p*H 7.2. V was assumed constant and equal to 0.75 ml/g, (Olson and Anfinsen, 1952). Centrifuge speed was 42,040 rpm for the sedimentation velocity runs and 8766 rpm for the Archibald runs.

Dioxane[a]	$s_{20,w}$	mw (Archibald)
0	23.7	990,000
5%	16.4	550,000
10%	13.1	300,000
10% (dialyzed back to 0)	23.7	—

[a] Dialyzed against phosphate buffer overnight after exposure to 10% dioxane for several hours at 6°.

As should be expected for a molecule dissociating on dilution the peaks show some trailing, and this trailing appears to become less pronounced with increasing dioxane concentration. Sedimentation coefficients for GDH in the three different solvents, together with molecular weights, are given in Table II. The latter were obtained by the Archibald (1947) approach to equilibrium method. The molecular weights were calculated from the meniscus only, either according to the conventional Archibald (1947) method, determining C_0 from a synthetic boundary run (for GDH in buffer), or according to the method of Trautman (1956). The Trautman plot for GDH in 5% dioxane, given in Figure 2, shows no indication of the heterogeneity one might expect to observe if the apparent 500 × 10^3 mw species were a mixture of 10^6 and 250 × 10^3 mw species.

The electrophoretic behavior of GDH in 5% dioxane is given in Figure 3, and no heterogeneity is apparent. Under the conditions of this experiment, the molecule should be essentially completely dissociated (see Figure

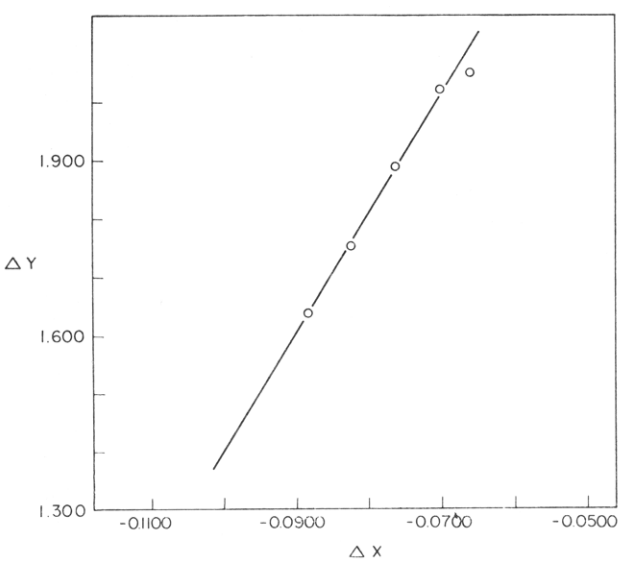

FIG. 2.—A Trautman plot of 10 mg/ml GDH in buffer–5% dioxane. The approach to equilibrium patterns were obtained at 8766 rpm under the conditions given in Table II.

$$\Delta y = K\left(\frac{\delta c}{\delta r}\right)_{r = r_m}$$

$$\text{and } \Delta x = \frac{-K}{r^2_m} \int_{r_m}^{r_p} r^2 \left(\frac{\delta c}{\delta r}\right)_t dr$$

in the Trautman (1956) equation.

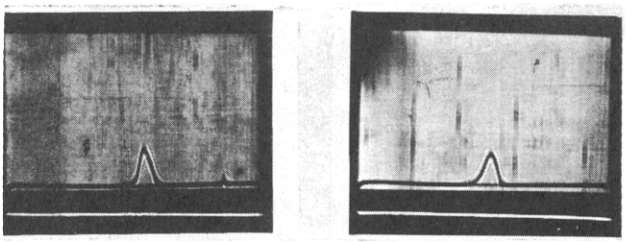

FIG. 3.—Electrophoretic pattern of GDH (2 mg/ml) in 5% dioxane–buffer (*p*H 7.2) after 6 hours at 50 v, 8 ma, and 5°. The data were obtained with the Spinco Model H electrophoresis-diffusion apparatus.

9). A number of starch and agar gel electrophoresis experiments in high and low *p*H buffers in the absence and presence of dioxane did not give any indication of more than one electrophoretic component.

The specific rotation of GDH in buffer and in 10% dioxane is given in Table III, and is in good agreement with the values reported by Jirgensons (1961). It is interesting to note that there is no rotational change associated with the dissociation process. Jirgensons (1961) pointed out the difficulty of turbidity formation in the course of the experiment, and the same difficulty was encountered in this work, since no temperature control was available with the spectropolarimeter. It was partially circumvented by cooling all samples at 4°, filling the cell very rapidly, and performing the experiment as fast as possible, while blowing dry air over the cell to prevent moisture from condensing on the cell windows. The temperature of the sample increased from 10° to 13° in the course of the experiment, but turbidity formation was essentially completely prevented.

A comparison of absorption and fluorescence spectra and of DPNH binding in 0%, 5%, and 10% dioxane is shown in Figures 4 and 5. As will be shown (see Figure 9), the enzyme is essentially completely dissociated in all three solvent systems at the concentration used in these experiments, and the data in Figures 4 and 5 thus have no direct relation to the dissociation process. The fact that there is no significant effect of dioxane in these experiments must mean that these properties of the GDH subunits are not affected by 5% and 10% dioxane. The number of DPNH binding sites (15) obtained from Figure 5 is in agreement with the value determined for GDH in the presence of diethylstilbestrol (Tomkins *et al.*, 1962), but is higher than that determined by Frieden (1961) for GDH alone. It should be noted that there was a slow change in the fluorescence of DPNH in dioxane solutions, and that all the values in Figure 5 were obtained immediately after preparing the solutions.

TABLE III

SPECIFIC ROTATION OF GDH IN THE ABSENCE AND PRESENCE OF DIOXANE

10 mg/ml GDH in 0.05 M phosphate buffer (*p*H 7.2) with and without dioxane. Temperature: 10–13°. Rotations obtained with a Rudolph Recording Polarimeter.

	$[\alpha]_\lambda$		
λ (mμ)	In Buffer	in 10% Dioxane	Jirgensons' (1961) Data (25°, 8 mg/ml GDH)
546	+14	+14	+13
435	+22	+22	+21
404	+21	+21	+20
366	+10	+10	+9

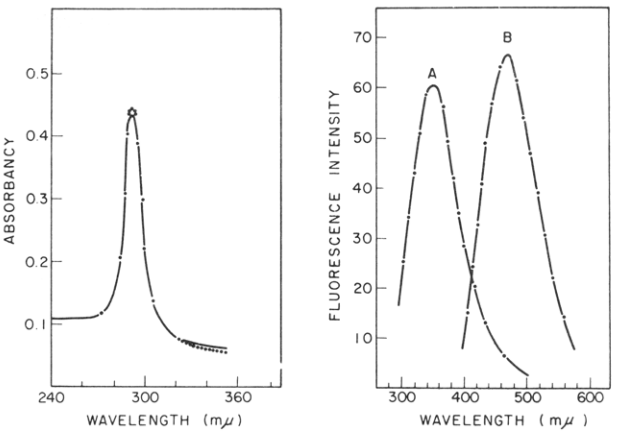

FIG. 4.—Absorption and fluorescence properties of 0.5 mg/ml GDH in buffer and 5% dioxane (———), and 10% dioxane (· · · ·), at 7°. Curve A is the emission spectrum of GDH alone, excited at 280 mμ. Curve B is the emission spectrum of GDH alone, excited at 280 mμ. Curve B is the emission spectrum of GDH in the presence of 0.8×10^{-5} M DPNH (16 moles of DPNH per 10^6 g GDH) excited at 340 mμ. The fluorescence spectra were obtained with the Aminco-Bowman fluorescence spectrophotometer. It should be noted that the fluorescence properties of free DPNH are not affected by 10% dioxane in short-time experiments.

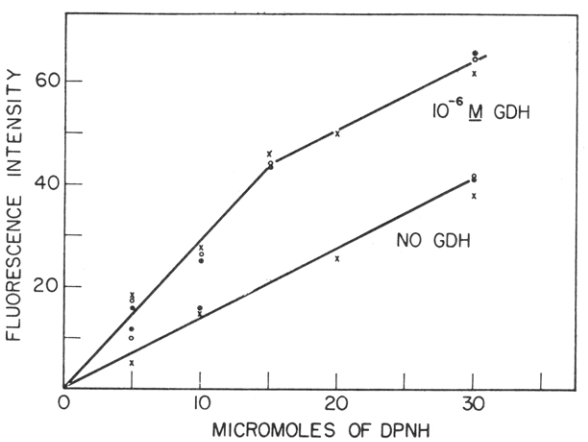

FIG. 5.—Titration of GDH with DPNH in buffer (×), in 5% dioxane (●) and in 10% dioxane (○) at 7°. The protein concentration was 1 mg/ml (10^{-6} M). The number of binding sites obtained in this experiment is about 15 per 10^6 g of GDH, which is somewhat higher than the reported values. The data were obtained with the Aminco-Bowman fluorescence spectrophotometer (excitation at 340 mμ, emission at 460 mμ), using 1-ml samples.

Depolarization of fluorescence is a sensitive way of detecting structural alterations which lead to changes in the rotational relaxation of either the whole molecule or parts of the molecule. Although GDH is too large for optimal sensitivity, it was felt that the method would detect any small change in the structure which could lead to greater degree of freedom of the protein bound fluorescent dye. Thus Steiner and Edelhoch (1961) were able to detect large changes in the rotational relaxation time of thyroglobulin (mw 700×10^3) when the molecule was denatured by a number of reagents. In the case of GDH, labeled with dimethylaminonaphthalene sulfonyl chloride, no significant effect of 10% dioxane on the polarization could be detected (Figure 6). Under the conditions of this experiment, too, the molecule was in the dissociated form in both solvents, and the data again illustrates the absence of any effects of dioxane on the GDH subunits. The

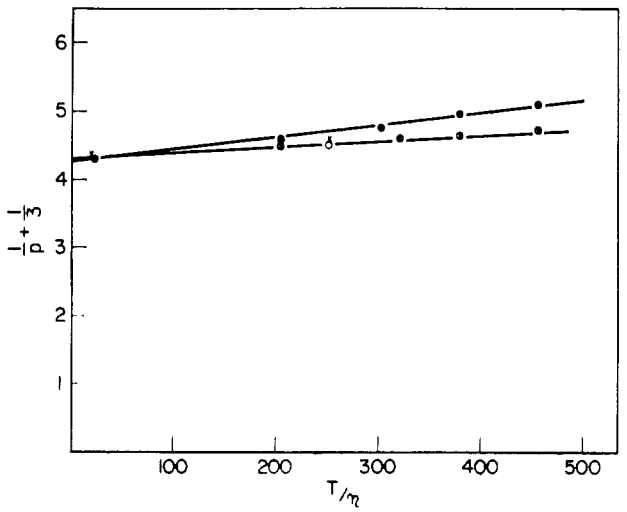

FIG. 6.—The polarization of fluorescence of 1-dimethylamine-naphthalene-5-sulfonyl chloride-labeled GDH in buffer (×), in 10% dioxane (○), and in 10^{-4} M sodium dodecyl sulfate (●). The experimental points were obtained in the range from 5° to 38° with a protein concentration of 1 mg/ml. Excited at 365 mμ (filter # CS 7-59) with natural light and emission read above 400 mμ (filter CS # 3-73). The dye-protein conjugate was prepared according to Weber's (1952) method and after dialysis the protein contained 2 moles of dye per 10^6 g of protein. The absence of free dye was established by paper chromatography. The data were obtained with the modified Phoenix light scattering photometer.

rotational relaxation time for GDH in buffer and in 10% dioxane at 25° was calculated (lifetime of excited state for dimethylaminonaphthalene sulfonate = 1.2×10^{-8} sec (Steiner and Edelhoch, 1961) to be of the order of 6×10^{-7} sec, which corresponds to an ellipsoid molecule of about 300×10^3 mw. When 10^{-4} M sodium dodecylsulfate was added, the relaxation time decreased (2.3×10^{-7} sec) indicating that smaller units (about 100×10^3 mw) were formed, in agreement with literature reports (Jirgensons, 1961). For a molecule of this size these values are clearly very approximate.

The next obvious question to ask was to what extent dioxane interferes with the activity of the enzyme. Testing the activity of different enzyme samples at a single constant level of substrates and coenzymes gave a very confused picture with poor reproducibility. Some of the rate curves are given in Figure 7. At constant enzyme concentration these curves are representative of the relative rates in 0%, 5%, and 10% dioxane for the forward and the reverse reactions. The findings that the velocity of forward reaction starts to decrease long before either substrate or coenzyme has become limiting (indicating that the concentration of active enzyme decreases as the reaction proceeds) and that the rate of the reverse reaction accelerates with time (Fig. 7) both indicate a very strong DPNH inhibition of the enzyme. This has been demonstrated earlier by Frieden (1959a), and is also reminiscent of a number of dehydrogenases, notably lipoic dehydrogenase (Massey and Veeger, 1960). There is also a strong *apparent* inhibition of the reverse reaction by dioxane (Fig. 7). However, when the activity was tested in this system over a range of coenzyme concentrations at constant substrate concentration a different picture was obtained, and Figure 8 shows that at low coenzyme concentration the activity of GDH is independent of the added dioxane. At higher coenzyme concentrations, dioxane appears to enhance strongly the DPNH inhibition of the enzyme. There is some evidence for a slow stimulation of GDH activity by dioxane. Thus Table IV shows that after storage in the cold the enzyme stored in 10% dioxane shows greater activity than an identical sample from the same stock solution stored at the same concentration in buffer alone. This phenomenon has not been studied further. It is also of interest to note that if stored at room temperature the enzyme in dioxane precipitates, and irreversible loss of activity is very rapid as compared to the parallel sample in buffer.

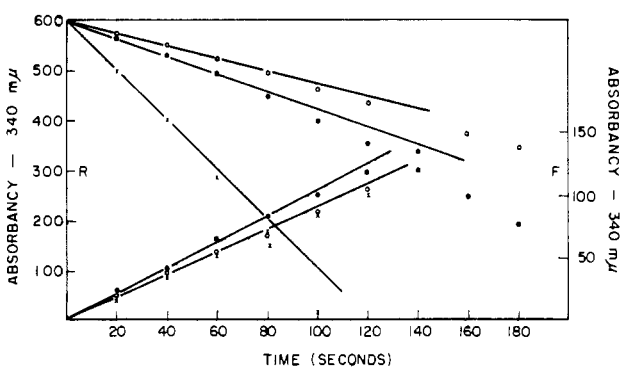

FIG. 7.—The activity of GDH in buffer (×), 5% dioxane (●), and 10% dioxane (○), in the forward (F) reaction, measured with 3×10^{-2} M L-glutamate and 3×10^{-4} M DPN; and in the reverse (R) reaction measured with 3×10^{-2} M α-ketoglutarate, 3×10^{-2} M NH_4Cl, and 3×10^{-4} M DPNH, all in 3-ml reaction volumes. The enzyme concentration was approximately 10^{-3} mg/ml (10^{-9} M assuming a molecular weight of 10^6). The buffer for the activity measurements was 0.05 M sodium phosphate, *p*H 7.5, containing 10^{-4} M Versene. The rates were determined at 25°.

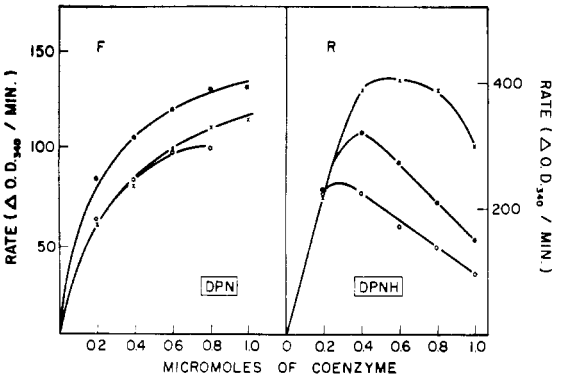

FIG. 8.—The effect of coenzyme concentration on the initial rate of the forward and reverse reaction catalyzed by GDH. The conditions and symbols are the same as those given in Figure 7.

TABLE IV

THE EFFECT OF DIOXANE ON THE ACTIVITY OF GDH AFTER STORAGE

Two mg/ml GDH stored at 4° in 0.05 M phosphate buffer with and without dioxane; assayed with L-glutamic acid (3×10^{-2} M) and DPN (3×10^{-4} M) in 0.05 M phosphate buffer (*p*H 7.6) after proper dilution in buffer. The rates were determined at 25°.

Experiment	Hours	Relative Activities OD/Min Stored in Buffer	Stored in Dioxane
1	0	80	80
	17	80	140
2	0	53	—
	9	55	180

In connection with the activity measurements, it must be noted that Versene has a stimulatory effect on the rate of the reaction, and that all the experiments in Figures 7 and 8 and in Table IV were carried out in the presence of 10^{-4} M Versene. It has also been found that higher concentrations (up to 10^{-2} M) of Versene do not cause inhibition of GDH activity. Further studies are required to understand the dioxane enhancement of the DPNH inhibition, and apparent stimulation of the forward reaction by 5% dioxane (Figs. 7 and 8) and the effect of dioxane on the stored enzyme, and also to explain the role of Versene. The main purpose here is to present evidence for the contention that the enzyme is fully active in dioxane.

The data in Figures 7 and 8 reemphasize the hazards involved in comparing relative enzyme activities in a complex system like the one at hand. It is obvious that the measured rate is extremely sensitive to the ratio of enzyme to coenzyme, especially in the reverse reaction, and due to the strong DPNH inhibition the true first-order rates are difficult to obtain. Only by comparing *initial* velocities over a range of coenzyme concentrations can one obtain a significant picture of the effect of the reagent under study. It should be emphasized that the data in Figures 7 and 8 have all been obtained at a fixed concentration of substrates and one cannot exclude the possibility that this modification may itself lead to erroneous conclusion.

The effect of dioxane in causing dissociation of GDH without a simultaneous loss of activity appeared to be contrary to most of the other cases of GDH dissociation, and the question whether the dioxane effect represents the normal or the abnormal behavior needed to be clarified. In view of Olson and Anfinsen's (1952) observations on the concentration dependence of the sedimentation coefficient of GDH, it appeared desirable to carry out complete sedimentation-concentration curves for GDH in the presence of a number of reagents affecting the dissociation of the enzyme. The results of such a study are given in Figure 9, and show that at infinite dilution there is no form of GDH larger than the 13 S unit. While the different reagents have the ability to shift the equilibrium between the 13 S units and the higher molecular weight units in the concentration range used for hydrodynamic studies, their effect disappears on dilution, and at the concentration used for enzyme activity assay it seems reasonable to conclude that the dissociation is shifted completely to the 13 S units regardless of the presence of "dissociating" or "associating" reagents.

It is interesting to note that the sedimentation behavior of GDH in 10% dioxane is independent of protein concentration and that the 13 S unit thus is the stable form of the enzyme in the concentration range studied. The molar concentration of dioxane in this system is approximately 1 M but its effect at high GDH concentration is completely reversed by 10^{-2} M DPN. Similarly, in the absence of dioxane one must go to very dilute GDH solutions to detect the dilution-induced dissociation in the presence of 10^{-2} M DPN. Thus DPN appears to stabilize the associated form of the enzyme quite efficiently and with high specificity. These experiments were carried out at the very high DPN concentration in an attempt to study the hydrodynamic properties of the enzyme at an enzyme-coenzyme ratio similar to that used in the activity assay.

Discussion

The many variables encountered in the GDH system makes it difficult to make direct comparisons between the results obtained by different groups of workers.

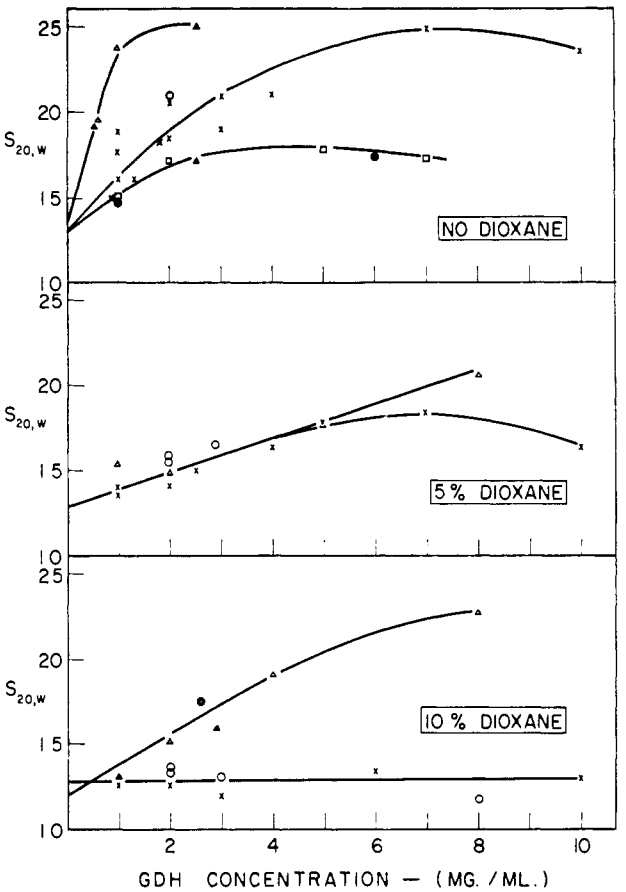

FIG. 9.—The variation of the sedimentation coefficient of GDH with GDH concentration in the presence of a number of reagents. GDH alone (×), in the presence of 5×10^{-5} M DPNH (○), 10^{-3} M DPNH (●), 10^{-2} M DPNH (▲), 5×10^{-5} M DPNH and 7.5×10^{-5} M diethylstilbestrol (□), 10^{-2} M DPN (△), 10^{-2} M DPN and 10^{-2} M Versene (⊘).

It was found early in this work that variables such as temperature and the age of the enzyme preparation were quite critical, and these variables were consequently controlled without any attempt to reproduce conditions used in other laboratories. Thus, all the physical experiments were carried out below 10°, and the enzyme samples used were removed from the stock ammonium sulfate suspensions in the evening and dialyzed overnight for use next morning. The complications encountered at room temperature have already been mentioned, and may be characteristic of the dioxane-containing system. The effect of age, however, appears to be general, and can be illustrated by the fact that a number of dilute buffer solutions of GDH were found to give a consistent decrease of 15–20% in the sedimentation coefficient upon storage at 4° for 12 hours. Furthermore, at the only time in this work when a double peak was observed in the ultracentrifuge, an old sample of GDH, which had also been exposed to room temperature for several hours, was used. In spite of the discrepancies in conditions, however, there appears to be good qualitative agreement between the observations reported here and those in the literature.

There are two separate phenomena to be considered in this work; the effect of dioxane on the properties of the protein, and the relation of the dissociation of the protein to its biological activity.

The first of these phenomena is of rather descriptive nature, and its interpretation must necessarily be speculative. Dioxane has been found to have a pro-

nounced effect on a number of proteins, but the mechanism of its effect is difficult to define in detail. With its marked effect on the dielectric constant of the medium, dioxane could exert its effect solely through modification of ionic interactions in the protein molecule, by virtue of changing the pK of ionizing groups. This has been proposed as a mechanism for the effect of dioxane on the conformation of bovine serum albumin in weakly acidic solutions (Van Holde and Sun, 1962). On the other hand, dioxane presumably could operate also by direct action on hydrophobic regions of interaction in the protein.

In the case of GDH it is doubtful that regions of ionic interactions could be the sole sites of dioxane effect. The pH of the present experiments is probably too far removed from the critical pH at which dissociation sets in (Frieden, 1962) for the dielectric effect to be that important. Furthermore, the fact that 10% *sec*-butanol with a much higher dielectric constant has been found to be effective in dissociating GDH ($s_{20,w}$ in 10% *sec*-butanol = 14.4 S) favors the hypothesis that hydrophobic interactions are involved.

In a very simple picture, the 13 S GDH unit can be visualized as a molecule with a weakly hydrophobic surface region, the properties of which are such that it can be satisfied almost equally well by solvent (water) or by another GDH molecule, with some preference for the latter at high concentration where the probability of solute-solute interaction is sufficiently high. As this probability decreases on dilution, solute-solvent interaction becomes favored. This equilibrium between solute-solvent and solute-solute interaction should also be very sensitive to slight changes in the surface properties of the protein (electrostatic repulsions on either side of the isoelectric range, ionic or hydrophobic character change through specific interaction with coenzymes and other reagents) and to changes in the properties of the solvent (addition of dioxane and other organic solvents).

The findings that the rotatory dispersion of GDH did not change in the dissociation process and that several properties of the 13 S units were not affected by dioxane are both consistent with this picture.

An interesting feature of the dissociation in dioxane is the question whether an intermediate 16 S unit really exists in 5% dioxane. Gilbert (1959) has demonstrated that it is possible to observe two schlieren peaks when a monomer-hexamer mixture in rapid equilibrium was sedimenting in the ultracentrifuge. In spite of this, one cannot exclude the possibility of a nonresolvable, rapid equilibrium monomer-tetramer system. It is, however, tempting in view of the data in Figures 1 and 2 to propose that the dissociation of the 24 S GDH to the 13 S GDH goes through an intermediate 16 S (500×10^3 mw) unit.

To turn now to the next question: What is the molecular weight of the enzymatically active GDH? The experiments leading to the answer to this question involve a long extrapolation from hydrodynamic experimental conditions (0.1–1% enzyme concentration) to the conditions for measuring enzymatic activity (about 10^{-4}% enzyme concentration). The hazards involved in such extrapolations have been discussed by the other workers in this field, and are quite obvious. Perhaps the most serious drawback in the present experiment is the large change in the ratio of enzyme to dissociating reagent. Most of the "specific" reagents studied (thyroxine, DPN, DPNH, and steroids) have been used at a level of 1–10 $\times 10^{-5}$ M. In the activity assay the enzyme is about 10^{-4}% or, assuming a molecular weight of 10^6, 10^{-9} M. Thus, on the outside, the enzymatic assay is carried out at a molar ratio of reagent to enzyme of about 10^4. The corresponding hydrodynamic experiments are carried out with 10^3–10^4 times more concentrated enzyme, or at a corresponding molar ratio of 1:1. It would appear that this procedure, operating in the ultracentrifuge with a "low" reagent concentration (ratio 1:1), would give what must be referred to as "high" reagent concentration (ratio 10^4:1) in the enzyme assay.

Since DPN was known to cause association of GDH, it was felt of importance to test the hydrodynamic properties at a GDH–DPN ratio as similar as possible to that of the enzymatic assay; but even under these conditions the dissociation of GDH on dilution was observable in the ultracentrifuge. Since the 13 S unit shows full activity, and furthermore appears to be the only form of the enzyme existing in dilute solution, it must be safe to conclude that the 13 S unit is the active form of GDH *in vitro*.

This, of course, does not give any information about the possibility of higher molecular weight aggregates being important *in vivo* where high local concentrations or special environmental conditions could favor either form of the enzyme.

The mechanism of the effect of the coenzymes in stabilizing either 13 S or 24 S units remains obscure. The very strong DPNH inhibition enhanced in dioxane may be connected with the presence of DPNH-inhibitor (Dalziel, 1961). The recent report of Dolin's (1962), on an apparent connection between inhibitor and peroxide formation in DPNH solutions, may be of special significance here, since the presence of peroxides even in freshly purified dioxane cannot be disregarded.

One final question must be raised. In all this work it has been assumed that the same 13 S units arise from the 24 S unit regardless of the method of dissociation. Although there is no direct evidence for this assumption, it does appear to lead to a reasonable working hypothesis.

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