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Monodispersity and Quaternary Structure of Glyceraldehyde 3-Phosphate Dehydrogenase*

R. Jaenicke,† D. Schmid, and S. Knof

ABSTRACT: The molecular weight of glyceraldehyde 3-phosphate dehydrogenase (GPD) has been reported to be in the range between 117,000 and 150,000 suggesting a dissociation–association equilibrium between the native enzyme and its protomers. To check this hypothesis the monodispersity of yeast and rabbit muscle GPD has been estimated comparing the mean values of M from sedimentation analysis, light scattering, and osmotic pressure. On the other hand, the influence of pH, ionic strength, solubilizing additives, etc., on the dissociation behavior has been investigated to analyze the interprotomer forces stabilizing the native quaternary structure.

The results confirm the molecular weight of 144,700 ± 3000 in accordance with a tetrameric quaternary structure. No anomalous dependence on protein concentration or temperature is observed in the entire pH range of enzymic activity. The values for the weight, number, and s,D average of M are identical within the limits of error. The enzyme, therefore, may be assumed to be strictly monodisperse. It is evident from ultraviolet differences pectra, opticalr otatory dispersion, and circular dichroism that dissociation into subunits in principle is accompanied by conformational changes. Therefore, no distinct indication regarding the interprotomer binding sites can be given. Qualitatively, the dissociation parameters lead to the conclusion that ion pairs and hydrogen bonds predominate as association sites while hydrophobic interactions seem to be of minor importance.

Glyceraldehyde 3-phosphate dehydrogenase (GPD,[1] EC 1.2.1.12) from rabbit skeletal muscle has been reported to have a molecular weight between 118,000 and *ca.* 150,000 (using sedimentation analysis and light scattering as analytical methods) (Dandliker and Fox, 1955; Fox and Dandliker, 1956; Taylor and Lowry, 1956; Elödi, 1958; Elias *et al.*, 1960).

Recently Jaenicke (1963) and Harrington and Karr (1965) found strong physicochemical evidence for an M value in the range of 140,000, verifying the figures of Dandliker and Fox (1955), Fox and Dandliker (1956), and Elödi (1958) and the "chemical" molecular weight of 140,000 following from amino acid analysis and end-group analysis as well as peptide mapping (Harris and Perham, 1965). The broad range of variability even under similar conditions of the experiments suggests a solvent- or concentration-dependent equilibrium

* From the Institut für Physikalische Biochemie der Universität, Frankfurt, Main, Germany, and the Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213. *Received October 23, 1967.* This investigation was supported by the Deutsche Forschungsgemeinschaft and the Verband der Chemischen Industrie and by a short-term fellowship from the European Molecular Biology Organization for one of us (R. J.). Part of the data presented in this paper is taken from the Diplomarbeit of D. S. and S. K., Universität Frankfurt, Dec 1966 and June 1967. A preliminary report of this work was presented at the 4th Meeting of the Federation of the European Biochemical Societies, Oslo, Norway, July 1967.

† Present address: Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pa. 15213. Wishes to thank Dr. Max A. Lauffer for supporting this work by a grant from the U. S. Public Health Service (GM 10403).

[1] Abbreviations used: BSA, bovine serum albumin; Y-GPD, yeast glyceraldehyde 3-phosphate dehydrogenase; R-GPD, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase; SDS, sodium dodecyl sulfate; NAD, nicotinamide–adenine dinucleotide.

TABLE I: Characterization of GPD and the Standard Proteins (Pyrophosphate pH 8 (GPD); Phosphate pH 7, 20°).

	RNase	BSA	Hb	Y-GPD	R-GPD
ϵ_{280} (ml/mg cm)	0.72	0.69	1.73 (274 mμ)	0.86	1.03
$\epsilon_{280}/\epsilon_{260}$	1.75	1.64	1.05	2.14	1.60
$(\partial n/\partial c)_\lambda$ 20° λ 436		0.189		0.189	0.1885
$(\partial n/\partial c)_\lambda$ 20° λ 546	0.187	0.185		0.184	0.1825
$[\alpha]°$, λ 546	−90	−76.6		−53	−38
b_0[a]		−330		−142	−150
a_0		−350		−225	−240
λ_c (mμ)	234	268		252	247
Specific activity (IU/mg)	30			155[b]	1860[c]

[a] $\lambda_0 \equiv 212$ mμ. [b] Activity decreases during storage; the number gives the maximum value. [c] Compare Biochemical Informationen (Boehringer).

according to $M_{native} \rightleftharpoons n \cdot M_{protomer}$ (n = number of subunits), leading to mean values of the apparent molecular weight $\overline{M} < M_{native}$. To test this hypothesis the monodispersity of GPD has been investigated comparing the different mean values of the particle weight resulting from sedimentation velocity ($M_{s,D}$), sedimentation equilibrium (M_w), light scattering (M_w), and osmometry (M_n).

On the other hand, the intermolecular forces stabilizing the native quaternary structure of the enzyme have been examined using the specific conditions of dissociation of the enzyme into subunits as indirect criteria. The weight of evidence from this kind of argument depends on the conservation of the native subunit conformation in the dissociation process. This proposition, therefore, had to be tested experimentally, combining analysis of particle weight ($M_{s,D}$ or M_w) and molecular conformation (optical rotatory dispersion, circular dichroism, and ultraviolet difference spectra).

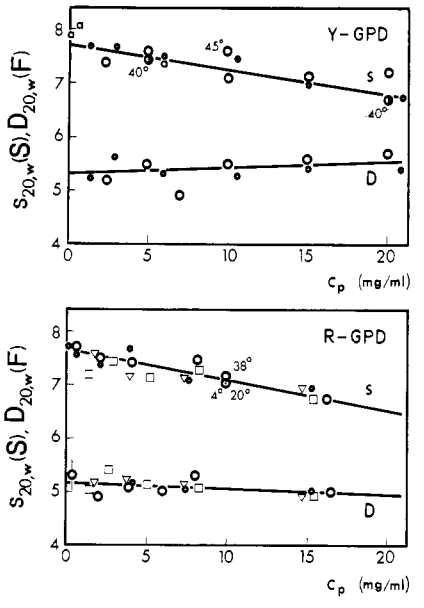

FIGURE 1: Concentration dependence of the sedimentation and diffusion coefficients of Y-GPD and R-GPD. s (S) and D (F), reduced to water viscosity and 20°; different temperatures are given at the respective concentrations. For $c_p < 2$ mg/ml: ultraviolet absorption optics (50,740 rpm) and activity transport (59,780 rpm, a). (○) Pyrophosphate–EDTA, pH 8.5, $I = 0.2$. (○, □, ▽) Sørensen phosphate, pH 7.0, $I = 0.2$, pH 7.0, $I = 0.15$, plus 5×10^{-3} M mercaptoethanol, pH 7.5, $I = 0.2$.

Materials and Methods

To check possible systematic errors of the methods applied, RNase, BSA, and hemoglobin have been used as standard systems. Y-GPD was purified according to Krebs (1952). R-GPD, as well as RNase (from pancreas), were purchased from Boehringer, Mannheim; BSA (dry, highest purity, Op. Nr.62 and 302) from Behring-Werke, Marburg/L.; and bovine hemoglobin from Pentex, Kankakee, Ill. The three-times-recrystallized systems were used without further purification. Solutions (1–2%) in the appropriate buffers were subjected to equilibrium dialysis for *ca.* 40 hr at 2°.

Protein concentration (c_p) was determined from optical density at 280 mμ, refractive index increment (546 mμ, 20°), and specific rotation $[\alpha]_{546\,m\mu}$ based on the dry weight at 106° (Table I).

Guanidine·HCl (Fluka, Basel and Schuchardt, München) was two-times recrystallized according to Y. Nozaki (personal communication). All reagents were substances of highest purity; quartz-distilled water was used as the solvent throughout.

Sedimentation analyses were performed in a Spinco ultracentrifuge (Model E) at 20° using absorption and schlieren optics with a phase plate or a thin wire as a diaphragm. The following cells were applied: filled-Epon or Kel-F normal and wedge cells (12 and 30 mm), filled-Epon synthetic boundary (capillary type) and double-sector (12 mm), Kel-F multichannel (Yphantis, 1960), and fixed partition. In general minimum rotor speed was applied to reduce the corrections due to ω^2 and dilution. To determine diffusion coefficients (D) runs without significant sedimentation have been performed; for evaluation the height–area method was

TABLE II: Sedimentation Analysis of GPD.

Medium[a]				Y-GPD			R-GPD		
	pH	I	T(°C)	$s^0_{20,w}$ (S)	$D^0_{20,w}$ (F)	$M \times 10^{-3}$[b]	$s^0_{20,w}$ (S)	$D^0_{20,w}$ (F)	$M \times 10^{-3}$[b]
Pyrophosphate–EDTA	6.2	0.2	20	7.77	5.0	148.5			
Phosphate	7.0	0.15	4				7.01	4.5	140.0
			20	7.80	5.25	142.0	7.65	5.05	144.8
			40				7.20	4.9	141.0
Pyrophosphate–EDTA	8.5	0.2	20	7.70	5.30	139.3	7.78	4.85	153.4
Borate	10	0.2	20				8.0	5.5	139
Phosphate	7.5	0.15	20	7.5	5.0	143.0	7.63	5.15	141.5
		0.50					7.60	5.05	150
		1.5		5.9	5.3	<125>[c]	5.4	5.5	<123>[c]
		2.0		5.3	5.8	<115>[c]			
		3.0					2.4	4.4	<88>[c]
Glycine–NaOH	12.5			2.10	5.2	37.5	2.00	5.4	35.5
Succinylated	8.0						2.25	4.8	39.2[d]
6 M guanidine·HCl (phosphate pH 8)				0.92	2.5	37.8[e]			35.8[f]
10 M urea (phosphate pH 8)							1.34	3.5	39.3
Oxidized (performic acid) (glycine–NaOH pH 12)							1.4	3.8	34.1[d]

[a] In addition to the given components the medium contains 10^{-3} M EDTA plus 5×10^{-3} M mercaptoethanol. [b] The partial specific volume in the native state is $V_p^{20°} = 0.737 \pm 0.002$ ml/g; $V_p^{4°} = 0.730$; $V_p^{40°} = 0.747$. [c] Polydisperse. [d] $V_p = 0.730$ ml/g; M corrected for substitution of Lys and Cys, respectively. [e] $\rho_0 = 1.155$ g/ml; $V_p = 0.660$ ml/g. [f] M_w from Yphantis' run.

used. The zero time was taken from A^2/Z^2 *vs.* time plots. Sedimentation coefficients (s) were calculated from log r *vs.* t diagrams obtained from 20-fold magnifications ($f_xE = 20$) or from densitometer traces in the case of absorption patterns (Beckman Analytrol). D and s were extrapolated to zero concentration and corrected for 20° and water viscosity (Ostwald viscometer, 0.29- and 0.52-mm capillary). Archibald, Archibald–Trautman, and Yphantis runs were performed at 5,990 rpm, the solutions being layered over mercury or perfluorotributylamine; evaluation at 20-fold magnification.

Partial specific volumes were determned using the magnetic float method (Stauff and Rümmler, 1959) or 5-ml high-precision dilatometers. Relative light-scattering measurements at 436 mμ using BSA or benzene as standards have been performed in the apparatus described by Jaenicke and Stauff (1961). To remove dust the solutions were centrifuged at 26,000g for 40 min. To evaluate the refractive index increment a Brice-Phoenix differential refractometer was used.

Osmotic pressure was measured at 25° in a high-speed membrane osmometer (Mechrolab Model 501) using Schleicher & Schuell membranes B 19, respectively, Ultracella filters UC ff and UC af (Membranfilter-Gesellschaft, Göttingen). To minimize effects of surface tension in the capillary, detergents had to be added to the solution or to the solvent in the capillary (0.01% Tween 80, 0.2% Triton B1, 0.05–0.2% RBS, and 0.02–0.05% SDS).

Optical rotation and rotatory dispersion were measured with the following spectropolarimeters: LEP 0.002° (Zeiss, Oberkochen), Polarmatic 62 (Bellingham & Stanley, London), and Cary 60; circular dichroism was determined in the Bellingham & Stanley instrument. Temperature-jacketed cells (1, 10, and 100 mm) were used throughout.

Ultraviolet difference spectra with pH, ionic strength, concentration, and additional components as parameters were carried out in a Beckman DK 2A (prism) and an Optica CF 4 (grating) spectrophotometer in order to eliminate artifacts caused by half-width variation, high absorbancy, etc.

Experimental Results

Sedimentation Analysis. The results of the sedimentation velocity experiments in different media and the determination of the diffusion coefficients are given in Table II and Figure 1. The freshly prepared native enzymes migrate as single symmetric peaks without any detectable amount of aggregates or dissociation products. This rough indication for homogeneity is confirmed by the result of the Archibald method calculated for meniscus and cell bottom giving

$c_p = 16$ mg/ml $\quad \delta_m = 0.59 \pm 0.02 \quad \delta_b = 0.58 \pm 0.02$
$c_p = 8$ mg/ml $\quad \delta_m = 0.62 \pm 0.02 \quad \delta_b = 0.57 \pm 0.02$
$\bar{M}_{app} = 141{,}500 \pm 5000$

TABLE III: Activity Transport of Y-GPD in a Fixed Partition Cell.[a]

Initial c_p (mg/ml)	Solution (ml) in Test		$(\Delta OD/\Delta t)_{rel}$[c]		$s_{20,w}$ (S)[d]
	Blank[b]	Supernatant	Blank[b]	Supernatant	
6.0	0.2 (1:100)	0.2 (1:50)	≡100	86	≡7.4
0.6	0.2 (1:10)	0.04	101	79	8.1
0.06	0.2	0.4	100 ± 5	81	7.9

[a] Test according to Biochemica Informationen (Boehringer). Duration of sedimentation 50 min at 59,780 rpm, temperature = 20°; pyrophosphate–EDTA, pH 8.5, I = 0.2. [b] To eliminate inactivation of GPD at high dilution during centrifugation an aliquot of the initial dilution was measured without centrifugation. [c] $(\Delta OD/\Delta t)$ of the blank for 6 mg/ml ≡ 100. [d] The s value for c_p = 6 mg/ml is taken from Figure 1.

in the case of R-GPD as an example (phosphate (pH 7), I = 0.15, 20°, and Philpot angle 85°). The Trautman modification of the Archibald method (Trautman, 1956; Elias, 1961) under equal conditions of the experiment (c_p = 7.3 mg/ml) gives M_{app} = 141,000 ± 4200. The short-column sedimentation equilibrium (Yphantis, 1960) establishes the linear M_{app} *vs.* c_p relationship following from the extrapolations in Figure 1. The numbers are Y-GPD: M_w = 146,000 ± 6000 and R-GPD: M_w = 142,500 ± 4500 using phosphate (pH 7.5), I = 0.15, plus 10^{-3} M EDTA plus 5×10^{-3} mercaptoethanol at 20°; c_p = 12, 9, 6, and 3 mg/ml. Extension of the c_p range which can be measured by schlieren and absorption optics under the given conditions of experiment ($20 \geqq c_p \geqq 0.5$ mg/ml) may be provided by the transport of activity in a fixed partition cell. Using this method the linear relationship between s and c_p is confirmed at concentrations as low as 0.06 mg/ml (Table III and Figure 1). The temperature in a range between 2 and 40° has no influence on the reduced hydrodynamic parameters (Figure 1). The limiting value of the particle weight under dissociating conditions is the 36,000 protomer observed by Harrington and Karr (1965) in the case of the pig muscle enzyme. It is found in strongly alkaline media at high dilution as well as in 8 M guanidine·HCl or after succinylation (Table II; *cf.* Figure 3). In strongly acidic media (glycine–HCl (pH 2), I = 0.4; formic acid–soldium formate (pH 2.4), I = 0.2;[1] acetic acid plus 0.4 M NaCl; pyridine–water–acetic acid; acetic acid, formic acid, and phosphoric acid (pH <1)) experiments are complicated by a secondary aggregation reaction which may be suppressed, but not eliminated, by high dilution or application of strongly protic solvents.[2]

Normally this aggregation leads to a system containing a certain amount (≦50%) of protomer besides the polydisperse aggregates. Evaluating s and D from the mixture gives approximately half the protomer weight, caused by the Johnston–Ogston effect (Johnston and Ogston, 1946) as well as by the anomalous flattening of the gradient curve in the heterodisperse system. Separating the slow peak using a fixed partition cell and repeating the sedimentation analysis of the nonaggregated protein lead to the exact protomer weight. Equivalent values are obtained after dialysis of the polydisperse system against 6 M guanidine·HCl. In all cases mentioned before the low sedimentation coefficients ($s_{20,w}$ = 1.3 ± 0.3 S) are caused by dissociation and strong conformational changes including alterations of the gross shape of the subunits (*cf.* Figure 6).

High ionic strength leads to partial dissociation of both different forms of the enzyme (Table II). The coenzyme (NAD) seems to have no influence on the particle weight: The concentration dependence of s and D in the presence of NAD remains unchanged. Both coefficients are somewhat increased, the binding process being weakly cooperative under certain conditions of the medium (R. Jaenicke and W. B. Gratzer, in preparation).

Light Scattering. Relative light-scattering experiments were carried out at protein concentrations down to 0.05 mg/ml using

$$M_x = M_{st} \frac{\left(\lim_{c\to 0} \frac{Kc}{R_{90}}\right)_{st} \left(\frac{\partial n}{\partial c}\right)^2_{st}}{\left(\lim_{c\to 0} \frac{Kc}{R_{90}}\right)_{x} \left(\frac{\partial n}{\partial c}\right)^2_{x}}$$

and

$$R_x = R_{st} \frac{\sigma_{solution} - \sigma_{solvent}}{\sigma_{standard}}$$

for evaluation (*cf.* Doty and Edsall, 1951). The refractive index increments $\partial n/\partial c$ of GPD and BSA are identical within the limits of error (Table I). The results represent the weight average of the molecular weight (M_w). As BSA turns out to be partially aggregated

[2] In the case of formic acid (*e.g.*, for performic acid oxidation) the lyophilized protein was used to maintain a water-free solvent. The presence of water causes splitting of covalent bonds as a consequence of the extreme low pH of the solvent under these conditions; BSA as a single-chain protein under these conditions is split into "subunits" while GPD or lactic dehydrogenase simulate "subsubunits." In gel chromatography the systems show asymmetric peaks anomalously smeared on the low molecular weight shoulder.

TABLE IV: Osmotic Molecular Weight of GPD and Some Standard Proteins (Sørensen Phosphate pH 7, I = 0.15, 25°).

Protein	Detergent (%)	B^{*a}	$\lim_{c\to 0} \frac{\Pi^a}{c_p}$	M_n^b
RNase	RBS 25 (0.01)	+0.026	1.86 ± 0.06	13,600 ± 450 (2)
Hemoglobin	Tween 80 (0.01)	±0.0010	0.379 ± 0.015[c]	66,300 ± 2,500 (4)
BSA	SDS (0.017) or Tween 80 (0.01)	+0.0031	0.305 ± 0.020	83,700 ± 7,800 (4)
Y-GPD[d]	SDS (0.01)	+0.0009	0.177 ± 0.011[c]	143,300 ± 9,000 (2)
R-GPD	Tween 80 (0.01) + 10^{-2} M MEt	+0.0006	0.167 ± 0.008[c]	151,200 ± 7,400 (5)
	RBS 25 (0.2)	+0.0011	0.182 ± 0.015[c]	138,800 ± 12,000 (1)

[a] [II]: cm (H_2O); [c_p]:g $l.^{-1}$; [B*]: cm $l.^2$ g^{-2}. [b] Number of measurements in parentheses. [c] Nonlinear Π/c_p *vs.* c_p relationship at low c_p. [d] Pyrophosphate–EDTA (pH 8.5) plus 5×10^{-3} M mercaptoethanol (MEt).

highly purified benzene (Merck, p.a., sodium potassium distilled and glass filtered) was used as a standard ($R_{90°,u}$ = 48.5 × 10^{-6}; λ 436 mμ; 25°). Taking into account the effective particle weight of the given BSA sample following from sedimentation equilibrium, gel chromatography, and osmosis ($M_w \approx$ 91,000, Yphantis method; $M_n \approx$ 84,000) good agreement for M_{GPD} was found from the different reference systems (Figure 2). The resulting limiting value, $\lim_{c=0} (Kc_p/R_{90})$, gives molecular weights M_w = 147,500 ± 10,000 (Y-GPD) and M_w = 142,500 ± 6000 (R-GPD), independent of the buffer system and the presence or absence of the coenzyme.

The limiting pH value of dissociation is established by light-scattering titration taking into account the influence of ionization of the protein on the scattering intensity ($I \geqq 0.15$, $c_p \leqq 0.5$ mg/ml) (Edsall *et al.*, 1950). Results are shown in Figure 3 which also gives the result of the spectrophotometric titration proving the coincidence of dissociation and liberation of anomalous tyrosyl residues masked in the native tetrameric state (*cf.* Libor *et al.*, 1965).

Osmotic Pressure. Osmotic measurements represent an absolute method to determine the number-average particle weight. In the case of the high-speed osmometer this fact had to be checked because of the necessity of adding detergents to the solutions and/or the solvent. Therefore, hemoglobin, BSA, and RNase have been measured as standard systems (Table IV). In most experiments with dissociable proteins and detergents the Π/c_p *vs.* c_p curves show deviations from linearity in the range of low protein concentration, *i.e.*, high relative molar concentrations [detergent]/[enzyme]. The curva-

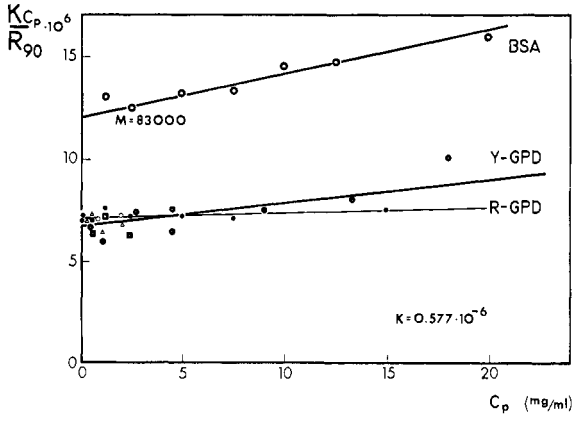

FIGURE 2: Relative light scattering of GPD with benzene and BSA as standard. 436 mμ, T = 25°, R_{90}: reduced scattered intensity at 90°; c_p: protein concentration (grams per milliliter). (●) R-GPD (phosphate, pH 7, 10^{-3} M EDTA, and 5×10^{-3} M mercaptoethanol) $M_{BSA} \cong$ 83,000 as standard (*cf.* osmosis and sedimentation analysis). (△heavy, □, ○) Y-GPD (pyrophosphate–H_2SO_4, –HCl, and –EDTA and 3×10^{-3} M NAD; Veronal–HCl, pH 8.5, I = 0.2). Benzene as standard.

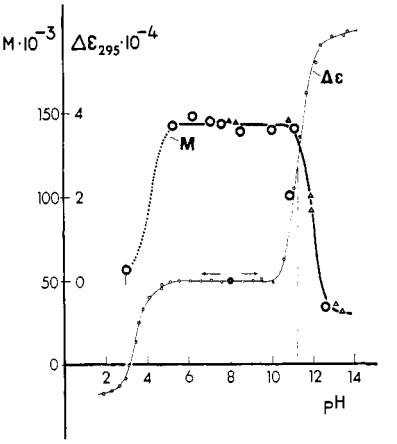

FIGURE 3: pH-dependent dissociation (light-scattering titration) and spectrophotometric titration of R-GPD. M, M_w, and $M_{s,D}$ molecular weights, respectively; Δε, molar extinction difference at 295 mμ. (○) Sedimentation data (*cf.* Table II). (△) Relative light scattering with benzene as standard; measurements at $c_p \cong$ 0.8 mg/ml; I = 0.5; $B^* \equiv 0$ (o—o) forward titration, start at pH 8.0.

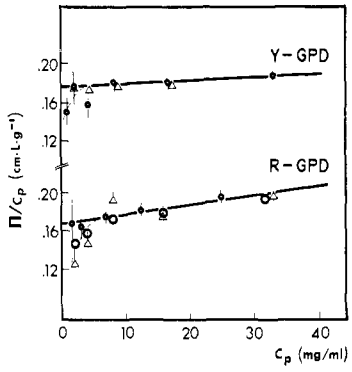

FIGURE 4: Reduced osmotic pressure (Π/c_p) *vs.* protein concentration (c_p) for GPD at 25°. Y-GPD: pyrophosphate–EDTA and 5×10^{-3} M mercaptoethanol, pH 8, $I = 0.2$. R-GPD: phosphate and 10^{-2} M mercaptoethanol, pH 7, $I = 0.15$.

tures may be positive or negative depending on the detergent as well as the protein (*cf.* Jaenicke, 1965b,c). To evaluate the initial molecular weight, eliminating the dissociating or aggregating effect of the detergent, the linear part of the curves in the Π/c_p *vs.* c_p diagrams was used for the extrapolation. The results for RNase and hemoglobin are in good agreement with the known M values; on the other hand, BSA shows remarkable aggregation establishing the discrepancies in the relative light scattering discussed before.

The results for GPD are given in Table IV and Figure 4. The error represents the mean deviation from mean values of two to five measurements.

Conformation. The conformation of the native apoenzyme under optimal conditions of enzymic activity has been investigated by several authors (Jirgensons, 1962; Jaenicke, 1963; Listowsky *et al.*, 1965; Havsteen, 1965; Bolotina *et al.*, 1966; Shibata and Kronman, 1967). The results in terms of the parameters of the Doty–Yang and Moffitt–Yang equations (Urnes and Doty, 1961) differ slightly because of differences in the provenance of the enzyme as well as in the medium.

In connection with possible alterations of the quaternary structure only relative changes under variation of the conditions of the medium are of interest. Figure 5 gives the optical rotatory dispersion and circular dichroism spectra of the native apoenzyme showing very similar behavior of both enzymes, especially in the characteristic inflection in the range of the absorption of the aromatic amino acid residues. It is still more pronounced in the circular dichroism spectrum and might be due to the anomalous tyrosyl residues mentioned by Libor *et al.* (1965). Dissociation of the native molecules at $3 > \text{pH} > 11$ leads to a decrease of the depth of the trough and to a flattening of the shoulder at 280 mμ (*cf.* Shibata and Kronman, 1967). To a lesser extent the same holds true in the case of high ionic strength (Figure 6). Comparing the pH and ionic strength ultraviolet difference spectra (220–360 mμ) the same analogy is observed (Figure 7). A strong maximum at ~295 mμ in the range of dissociation into subunits (pH 11.5 *vs.* 12.5) appears very much reduced

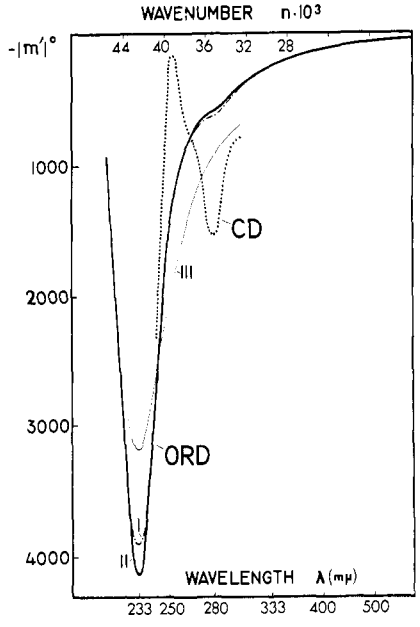

FIGURE 5: Optical rotatory dispersion and circular dichroism of GPD (apoenzyme) in the visible and near-ultraviolet spectrum. [m'] mean residue rotation based on mean residue weights (MRW) = 111.8 and 108.0 for Y-GPD and R-GPD, respectively (*cf.* Alison and Kaplan, 1964). $c_p = 10^{-4}$, 10^{-5}, and 5×10^{-6} M; $T = 20$ and 27°. I, R-GPD (phosphate and 10^{-3} M EDTA, pH 7.6, $I = 0.3$), II, Y-GPD (pyrophosphate–EDTA, pH 8.5, $I = 0.2$), and III, Y-GPD (glycine–NaOH, pH 12.5, $I = 0.2$).

(5%) in the difference spectrum with $I = 0.2$ *vs.* 2.0 at constant protein concentration. The extinction of the different components of the solvent has been corrected in these experiments using the "tandem cell" method according to Herskovits and Laskowski (1962).

Detergents and apolar solvents lead to drastic changes in the tertiary structure (increase of f/f_0) without marked alteration of helicity ($b_0 \cong$ constant) (Elödi *et al.*, 1963; Jaenicke, 1965b, 1967; Bolotina *et al.*, 1966). The ultraviolet difference spectra in this case show strong alterations in the tryptophan and tyrosine absorption pointing to a complete change in the intramolecular interactions. They confirm completely the results recently given by Libor *et al.* (1965).

Discussion

The data for the molecular weight of the native GPD following from sedimentation analysis, light scattering, and osmotic pressure represent the weight, number, and z average of M as well as the less well-defined $M_{s,D}$ mean value. As shown in the preceding results these different mean values are identical within the limits of error (Δ) (Table V). No species-specific differences can be detected.

Concerning the influence of the components of the medium no corrections have been made other than in the cases of oxidation and succinylation where substitution takes place (SO_3H instead of SH; $C_4H_4O_3N$ instead of NH_2). Since sufficiently high ionic strength with the various ions leads to equivalent M values,

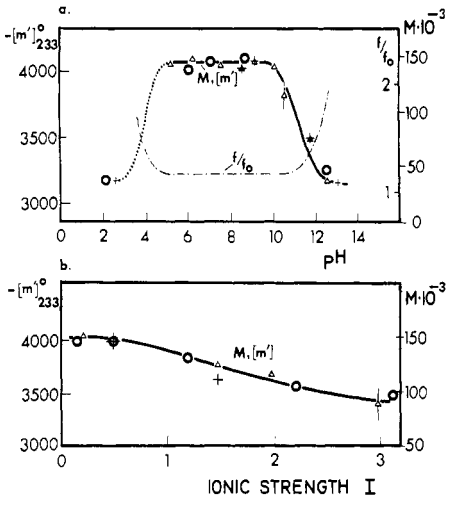

FIGURE 6: Conformational changes during dissociation of Y-GPD into subunits. (a) Influence of the pH on the depth of the trough at 233 mμ ($[m']_{233}^0$, ○), on the molecular weight (M) from sedimentation (Δ) and light scattering (+), and on the frictional ratio (f/f_0). $0.15 \leqq I \leqq 0.5$, $T = 20°$. (b) Influence of ionic strength on $[m']_{233}^0$ (○), and M from sedimentation (Δ) and light scattering (+), pH 8.5, $T = 20°$.

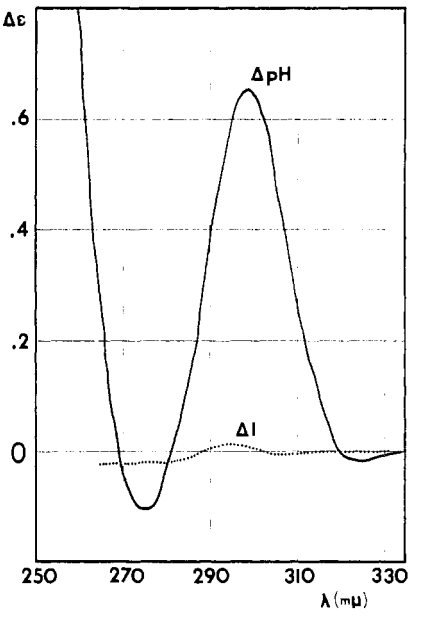

FIGURE 7: Ultraviolet difference spectra of R-GPD with pH and ionic strength as parameters. $c_p = 4$ mg/ml, path length 10 mm (four cuvets). ΔpH: pH 12.5 *vs.* 11.5, $I = 0.2$; ΔI: $I = 0.2$ *vs.* 2.0, pH 7.5.

specific binding may be neglected as far as buffer ions, EDTA, and mercaptoethanol are concerned. The second virial coefficient, B^*, under these conditions is near zero. It seems, therefore, justified to compare the data given in Table V without further corrections. The Δ values reflect the maximum uncertainty of the extrapolation to zero concentration. The systematic error in the case of $M_{s,D}$ in general causes too low M values because of the perturbation of the diffusion. Taking into account the zero-time correction this error does not exceed 5%. For the equilibrium methods the ambiguity of the position of meniscus or bottom does not allow predictions of the sign of the deviation. The absolute values from light scattering and osmosis are strongly influenced by the accuracy of the determination of protein concentration. In comparing $M_w(R_{90})$ and M_n this influence vanishes as a common factor. In general, the range of error for the individual measurement is increased with decreased c_p.

Considering these general features of the data there are two conclusions to be drawn. (1) Under the conditions of experiment no dissociation-association equilibria between the native enzyme and its protomers can be observed at concentrations as low as ~0.06 mg/ml and in the temperature range between 2 and 40°. Former determinations of M showing much lower values, therefore, cannot be due to dissociation-association phenomena. (2) As a consequence GPD may be considered as homogeneous regarding the molecular weight. This follows clearly from the coincidence of the M values in Table V as any kind of a multicomponent system would lead to $M_n < M_w < M_z$. The resulting mean value is $\overline{M} = 144{,}700\ (\pm 2.6\%)$.

As shown by light scattering and spectrophotometric titration as well as sedimentation analysis at different ionic strength, high electrostatic repulsion of the subunits, and/or screening of ion pairs lead to dissociation of the native enzyme. The monodisperse protomer of $M = 36{,}600 \pm 2500$ is produced at maximum negative net charge (pH > 11, succinylation) and by breaking hydrogen bonds (guanidine·HCl, urea in the presence of SH-protecting agents) while solubilization of hydrophobic areas does not necessarily lead to dissociation. Comparing the protomer weight to the value for the native enzyme the tetrameric quaternary structure of GPD is clearly established.

Regarding the interprotomer binding sites so far, no clear-cut answer can be given because dissociation of the GPD molecule in general seems to be accompanied by conformational changes. Contrary to this, analysis of the binding sites necessarily presupposes the unchanged "native" conformation of the protomers.

The behavior of Y-GPD at high net charge has been recently discussed by Shibata and Kronman (1967); it may be generalized for R-GPD. In both cases dissociation as shown by s and R_{90} parallels inactivation and (partial) unfolding as shown by spectrophotometric titration and optical rotatory dispersion. The coincidence of the dissociation and the liberation of anoma-

TABLE V: Molecular Weight of Native GPD.[a]

	Ultracentrifugation		Light Scattering	Osmosis
	$M_{s,D}$	M_w	M_w	M_n
Y-GPD	143,600	146,000	147,500	143,000
R-GPD	144,700	141,700	142,500	149,000

[a] Ranges of error ±Δ: $M_{s,D}$, 3%; M_w (sedimentation), 3.6%; $M_w(R_{90})$, 6%; M_n, 6%.

lous tyrosyl residues (decrease of the circular dichroism band at 280 mμ) may either be due to the specific position of some of the tyrosines in the tangent plane between the subunits or to the general unfolding reaction mentioned before; a decision so far cannot be given.

The effect of succinylation is analogous to the dissociation at pH >11 because of the large increase of net charge coupled with the reaction at the given pH. In the case of solubilization by detergents or apolar solvents as well as with urea or guanidine·HCl the structural change refers to conformation and shape. In the case of the ionic strength dependent dissociation these changes are small but detectable. b_0 nearly remains constant, the depth of the trough at 233 mμ is weakly decreased, and the difference spectra show small peaks in the near-ultraviolet spectrum.

Finally as dilution does not cause dissociation at concentrations as low as 0.06 mg/ml, it seems impossible to dissociate the GPD molecule without at least slightly modifying the "native" conformation. Therefore the interprotomer bonds cannot be determined unambiguously from the given experimental data. The dissociating effects of urea and guanidine·HCl as well as high net charge and ionic strength suggest electrostatic forces and hydrogen bonds as association sites. Hydrophobic interactions on the other hand seem to be of minor importance as shown by the influence of solubilizing agents and high ionic strength (Kauzmann, 1959; Jaenicke, 1965a). A more precise definition of the association sites responsible for the quaternary structure is impossible at the present stage.

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