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Molecular Weight and Amino Acid Composition of Five-Times-Crystallized Phosphoglucose Isomerase from Rabbit Skeletal Muscle*

Ning G. Pon, Klaus D. Schnackerz, Michael N. Blackburn, Gora C. Chatterjee,† and Ernst A. Noltmann

ABSTRACT: Five-times-crystallized phosphoglucose isomerase from rabbit skeletal muscle has been subjected to physical and chemical characterization. The following physical parameters have been determined: $s^0_{20,w} = 7.19 \times 10^{-13}$ sec, $D^0_{20,w} = 5.15 \times 10^{-7}$ cm^2 sec^{-1}, and $\bar{V} = 0.740$ ml g^{-1}. On the basis of these values and those from independent equilibrium sedimentation experiments, a molecular weight of 132,000 g mole^{-1} has been assigned. Data on the amino acid composition of the enzyme have been obtained from chromatographic analysis of acid protein hydrolysates prepared as a function of time by employing a technique that allows virtually complete exclusion of oxygen during the process of acid hydrolysis. Under this condition, only tryptophan, threonine, and serine showed a significant extent of degradation during hydrolysis of phosphoglucose isomerase in 6 N HCl at 110° for up to 140 hr.

The same technique of trace-oxygen removal applied to alkaline hydrolysis has been found to make hydrolysis in 4 N barium hydroxide, followed by ion-exchange chromatography, a reliable and simple method for the direct analysis of tryptophan. Independent measurements have been made of the total nitrogen content, the biuret factor, the absorptivity coefficient at 280 mμ, as well as the contents of cysteine, tryptophan, and amide ammonia.

Phosphoglucose isomerase (D-glucose 6-phosphate ketol isomerase, EC 5.3.1.9) has been isolated in crystalline form from rabbit skeletal muscle several years ago (Noltmann, 1963). The enzyme has since been subjected to a detailed physical and chemical characterization in order to establish its molecular properties and to provide reference data for further analysis of its protein structure as well as for the elucidation of catalytically critical amino acid residues, and for studies designed to correlate structural and functional parameters basic to an understanding of its mechanism of action. The present paper reports data on the molecular weight, the overall amino acid composition, and some ancillary properties.

Materials and Methods

Preparation of the Enzyme. Phosphoglucose isomerase was isolated from rabbit skeletal muscle as described previously (Noltmann, 1964, 1966). To improve yield and purification, the ammonium sulfate saturation during crystallization was raised more slowly than described for the original procedure, such that the time for each crystallization step was extended over 2–4 weeks. Each preparation was routinely carried through five crystallizations resulting in final specific activities of approximately 850 μmoles of substrate turnover per min per mg at 30°, measured in the reverse reaction by coupling to the glucose 6-phosphate dehydrogenase system (Noltmann, 1964, 1966).

Prior to use in any of the physical measurements, an aliquot of the crystalline suspension was centrifuged, and the dissolved pellet was dialyzed at 2–4° for at least 48 hr against several changes of the desired buffer (usually 0.1 M sodium phosphate (pH 7.06, at 10°), 0.188 M Tris-chloride (pH 8.29, at 10°), or 0.147 M imidazole chloride (pH 7.01, at 10°)).

For the amino acid analyses, enzyme aliquots were dialyzed exhaustively first against 0.01 M KCl (to displace possibly protein-bound free ammonium ion—which would interfere with the amide ammonia analysis—by potassium ion) and then against multiple changes of deionized, glass-distilled water. The same precautions were taken in preparing the enzyme samples as reported for previous work on the amino acid analysis of phosphoryl transfer enzymes (Mahowald *et al.*, 1962). Protein concentrations were measured either spectrophotometrically or by the biuret procedure of Gornall *et al.* (1949). For extinction coefficients, refer to Results and Discussion.

Ultracentrifugal Analyses. Sedimentation experiments were performed at 10° in a Spinco Model E analytical ultracentrifuge equipped with a RTIC unit, with a phase plate for the schlieren and interference optical systems, and with an ultraviolet absorption optical system. Schlieren and interference patterns were measured with a Nikon Model 6C optical comparator, allowing direct measurements with a precision of 0.002 mm. Photographs obtained with the ultraviolet absorption optical system were scanned in a Beckman Model RB Analytrol equipped with a film densitometer attachment. The pen height travel was linearly proportional to the initial protein concentration used in all of the ultraviolet runs.

Velocity Sedimentation. Two concentration ranges were

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investigated: 1–12 mg ml^{-1} with the schlieren optical system and 0.05–1.00 mg ml^{-1} with the ultraviolet absorption optics. Single or double-sector cells with 12- or 30-mm aluminum centerpieces were employed in either the An-D or An-E rotor, respectively, to obtain overlapping enzyme concentration ranges for the two optical systems. Sedimentation velocity experiments were performed at 59,780 (An-D rotor) or 50,740 rpm (An-E rotor) with the running rotor temperature controlled at 10.4° for the schlieren runs and 10.0° for the ultraviolet runs. Apparent sedimentation coefficients were calculated by the usual procedures (Schachman, 1957) from the rate of boundary migration, and corrected to standard conditions (at 20°).

Diffusion Measurements. Photographs taken during sedimentation velocity runs performed with the ultraviolet optical system were also utilized to obtain values for the diffusion coefficient according to the procedure outlined by Schumaker and Schachman (1957). In addition, independent sets of data for the diffusion coefficients were obtained from the rate of boundary spreading (Schachman, 1957) during synthetic boundary runs made in connection with the low-speed sedimentation equilibrium centrifugation described in the next paragraph.

Equilibrium Sedimentation. For low-speed equilibrium experiments, the An-J rotor was used throughout. Except for initial protein concentrations of 0.5 mg ml^{-1}, for which a 30-mm centerpiece was used, all other runs with higher concentrations were performed with a 12-mm double-sector cell. The right-hand sector of the latter was filled with 0.10 ml of the protein solution (height of the liquid column was 0.26 cm) while the other sector was filled with 0.11 ml of the appropriate buffer mixed with sufficient 1,3-butanediol to increase the refractive index of the medium to that of the protein solution (Richards *et al.*, 1968). Tests for leakage during the runs, and for attainment of equilibrium, measurements of the positions of the interference fringes, application of corrections for variations in the base lines, location of the hinge point fringe, and determination of the initial concentration from synthetic boundary runs were all performed according to Richards *et al.* (1968). The molecular weights at any level r in the liquid column, were calculated from

$$M_{w,r}^{app} = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{d \ln c(r)}{dr^2}$$

The high-speed sedimentation equilibrium experiments were performed according to Yphantis (1964), except that a standard Al-filled Epon double-sector cell was used. The conditions for these runs were calculated to achieve concentrations at the meniscus of no greater than 0.01 fringe (Teller, 1965) and no overspeeding was applied. All other procedures were similar to those used in the low-speed equilibrium technique. Both the vertical positions of a single fringe and the horizontal positions of separate fringes (all corrected for base-line distortions) were measured and used for calculating the molecular weights according to the formula given above.

Measurements of Densities and Viscosities. Both the parameters were determined at 10.00 ± 0.01°, with use respectively, of a Weld-type pycnometer (*ca.* 28 ml) and a Cannon-Fenske capillary viscometer, size no. 25, with an efflux time of approximately 400 sec for water. The following densities in g ml^{-1} and viscosities (relative to water at 20°), respectively, were found for 10.0°: imidazole-HCl, 1.0032, 1.320; Tris-HCl, 1.0109, 1.415; and sodium phosphate, 1.0128, 1.374. For composition and pH of these buffers see above under Preparation of the Enzyme. All weighings were done with a Mettler H-16 balance and were corrected for buoyancy changes due to moisture and pressure changes following procedures outlined by Bauer (1945), and by Macurdy (1965). The accuracy of the density measurements was estimated to be about 1 part in 100,000.

Determination of the Partial Specific Volume. Three independent methods were employed: (a) direct pycnometric density measurements of the enzyme in aqueous medium, (b) computation from the amino acid composition with the use of known values for $\bar{V}$ of the individual amino acid residues (Cohn and Edsall, 1943), and (c) sedimentation equilibrium ultracentrifugation in D_2O according to the method of Edelstein and Schachman (1967). The latter experiments were performed in 0.1 M phosphate (pH 7.0) that contained 89.7% D_2O; other conditions were those described above for high-speed equilibrium sedimentation.

For method a, a 1.7-ml capillary-type pycnometer equipped with a ground glass top (Type D, as described by Bauer (1945)) was used. Weighings were made as described above for the general density measurements and extrapolated to zero time to correct for evaporation. Protein concentrations were based on determination of dry weight (P_2O_5 at 110°), refractive index, and ultraviolet absorbance at 280 mμ.

Measurement of the Refractive Index Increment. Synthetic boundary experiments were made at 10° in the analytical ultracentrifuge with Rayleigh interference optics and with use of the An-J rotor running at 6166 rpm. In this manner, J, the difference in number of fringes between the protein solution and the solvent, was obtained and the refractive index increment was calculated from the relationship $dn/dc = J\lambda/ac$, where λ is the effective wavelength in cm of the light used, a the optical path length in cm, and c the protein concentration in g dl^{-1}. The complete system, including the 12-mm double-sector Al-filled Epon capillary-type synthetic boundary cell and the high-pressure mercury lamp, was calibrated with a standard sucrose solution according to Gosting and Morris (1949). The ratio of a/λ was found to be 2.198×10^4 which is in perfect agreement with the calculated value assuming that λ is exactly equal to 5.46×10^{-5} cm and a is exactly equal to 1.20 cm.

AMINO ACID ANALYSES. Acid Hydrolysis. Conditions for hydrolysis in 6 N HCl were generally the same as previously described (Mahowald *et al.*, 1962) with the exception that a 1:1 dilution of analytical grade concentrated HCl was employed (Moore and Stein, 1963) instead of the constant-boiling HCl. Aliquots containing approximately 1 mg of enzyme protein were hydrolyzed under nitrogen atmosphere[1] in sealed Carius microcombustion tubes (Corning No. 8640, 10 mm od, 200 mm length), kept at 110 ± 0.5° in a constant-temperature silicone oil bath for the desired length of time. The handling of the samples after hydrolysis has been described in a recent paper (Chatterjee and Noltmann, 1967a). Amino acid analyses were performed according to Spackman

[1] Exact details of the analytical procedures have been omitted at the request of the editors in order to conserve space. They are available on request to E. A. N.

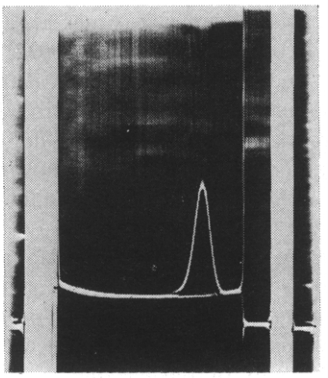

FIGURE 1: Sedimentation velocity ultracentrifugation of five-times-crystallized rabbit muscle phosphoglucose isomerase. Run 469: protein concentration, 9 mg ml^{-1}; 0.1 M sodium phosphate buffer (pH 7.0). Centrifugation was performed at 10.0° with a 12-mm, double-sector aluminum cell in an An-D rotor running at 50,740 rpm. The picture of the schlieren pattern was taken at a phase-plate angle of 65°, 40 min after the operating speed had been reached. Travel of the boundary is from right to left.

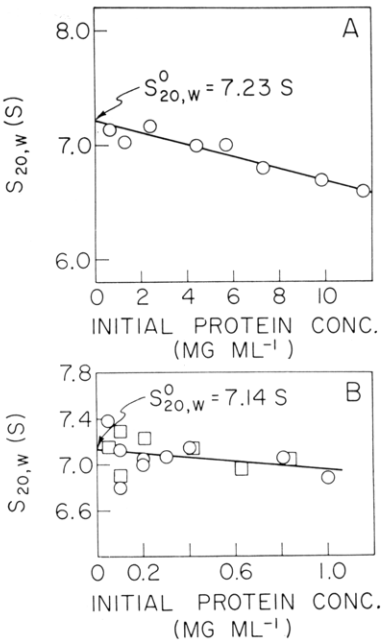

FIGURE 2: Sedimentation coefficient of five-times-crystallized rabbit muscle phosphoglucose isomerase as a function of protein concentration. (A) Data obtained with schlieren optics. Analyses were performed at 10.4° in 0.1 M sodium phosphate (pH 6.8) with an An-D rotor (12-mm cell) at 59,780 rpm or, for the two lowest enzyme concentrations, with an An-E rotor (30-mm cell) at 50,740 rpm. Data are corrected to standard conditions. (B) Data obtained with ultraviolet optics. Analyses were performed at 10.0° in 0.1 M sodium phosphate (pH 7.1) (designated by the circles) or in 0.15 ionic strength Tris-chloride (pH 8.3) (designated by the squares). An An-D rotor (12-mm cell) at 59,780 rpm was used for enzyme concentrations between 0.1 and 1.0 mg ml^{-1}, an An-E rotor (30-mm cell) at 50,740 rpm for concentrations between 0.05 and 0.3 mg ml^{-1}.

et al. (1958) on Beckman-Spinco automatic amino acid analyzers Models 120B and 120C with attention to experimental details elaborated upon in the past (Mahowald *et al.*, 1962; Noltmann *et al.*, 1962; Moore and Stein, 1963; Tristram and Smith, 1963).

Determination of Cysteine or Half-cystine. The following methods, each based on a different analytical principle, were employed: conversion into cysteic acid by oxidation with performic acid (Moore, 1963); conversion into *S*-carboxymethylcysteine by alkylation with iodoacetic acid after denaturation and reduction (Cole *et al.*, 1958; Noltmann *et al.*, 1962); air oxidation at pH 6.8 after acid hydrolysis (Hirs *et al.*, 1954); titration with *p*-mercuribenzoate (Boyer, 1954); and amperometric titration of sulfhydryl groups as the Tris–silver complexes (Benesch *et al.*, 1955).

Determination of Tryptophan. Spectrophotometric analysis of the ultraviolet absorption. In accordance with a suggestion by Wetlaufer (1962), the absorption spectra were determined in 8 M urea–0.1 N sodium hydroxide. In the blank cuvet, an equal volume of water was substituted for the enzyme solution in the sample cuvet. Absorbance measurements were made with a Beckman DUR monochromator attached to a Gilford Model 2000 absorbance recorder. Due to the expanded linear output of this instrument over an absorbance range of 3.0, values of 1.5 total absorbance were reproducible within ±0.001. For all of the measurements a correction for extraneous absorption was employed as suggested by Beaven and Holiday (1952). In addition, several analyses were performed in a Cary Model 15 spectrophotometer according to the recent method by Edelhoch (1967), which is based on absorbance measurements at 280 and 288 mμ in the presence of 6 M guanidinium chloride, at pH 6.5.

Colorimetric estimations with *p*-dimethylaminobenzaldehyde. These were made by "Procedure K" of Spies and Chambers (1949) on the unhydrolyzed protein.

Chromatographic determination by ion-exchange chromatography after alkaline hydrolysis with barium hydroxide. Two methods of hydrolysis were compared: (a) the procedure of Ray and Koshland (1962); (b) a modification[1] of an earlier method (Noltmann *et al.*, 1962), consisting of hydrolysis in 4 N barium hydroxide carried out in alkali-resistant digestion tubes[2] sealed under vacuum after complete substitution of air by prepurified nitrogen (containing less than 5 ppb of oxygen). Tryptophan was determined by chromatography on the short column of the amino acid analyzer after removal of the barium as barium sulfate.

Determination of Amide Ammonia. Although an approximate value for the amide ammonia content of a protein can be obtained by extrapolation to zero hydrolysis time of the ammonia values found in the standard acid hydrolysates obtained at various time intervals, it is desirable to have a second, independent analytical method. The following procedure was used for the quantitation of amide ammonia, after mild alkaline hydrolysis and diffusion into sulfuric acid, by chromatography on the short column of the Spinco amino acid analyzer. Aliquots of 20–100 μl of dialyzed enzyme solution containing approximately 15–90 μg of protein were added to 1.0 ml of 2 N NaOH in the outer well of Conway microdiffusion dishes (Corning No. 3135), which contained 0.5 ml of 0.8 N H_2SO_4 in the center well, and diffusion was allowed to take place at room temperature (25 ± 2°). Under these conditions, the ammonia of an ammonium sulfate solution was completely diffused within 48 hr, and no differ-

[2] Bellco Glass, Inc., Vineland, N. J. 08360, No. 4022 SPL ampoules made from noncorrosive borosilicate glass, capacity 5 ml.

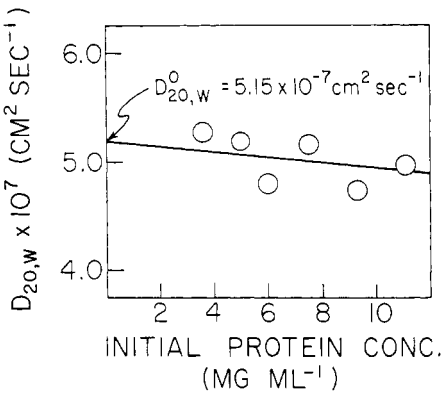

FIGURE 3: Diffusion coefficient of five-times-crystallized rabbit muscle phosphoglucose isomerase as a function of the initial protein concentration. Data, corrected to standard conditions, were obtained from synthetic boundary runs performed at 10.4° in 0.1 M sodium phosphate (pH 6.8) at a rotor speed of 15,220 rpm.

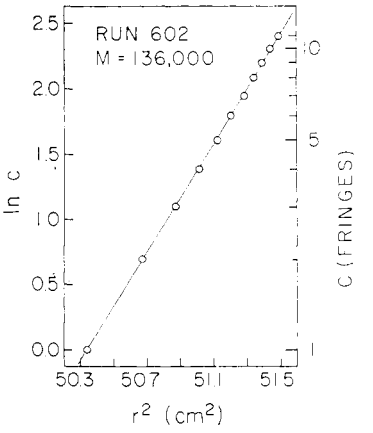

FIGURE 4: High-speed sedimentation equilibrium ultracentrifugation of five-times-crystallized rabbit muscle phosphoglucose isomerase. Ln c *vs.* r^2 plot of data obtained at 10.0° in 0.1 M sodium phosphate (pH 7.1) at an initial protein concentration of 0.49 mg ml^{-1} (rotor speed, 16,200 rpm).

ence of amide ammonia values for the enzyme was observed for diffusion times of 120 and 180 hr. After the diffusion the contents of the outer well were quantitatively transferred to the short column of the amino acid analyzer and pH 5.28 citrate buffer was employed for elution.

The chromatographic method was standardized against solutions made from twice-recrystallized analytical grade ammonium sulfate dried to constant weight over P_2O_5. All the data were corrected for ammonia blank values in the sulfuric acid, in the distilled water, and in the citrate buffer used to transfer the sample from the Conway dish to the ion-exchange column.

Results and Discussion

Physical Parameters. SEDIMENTATION COEFFICIENT. A typical schlieren pattern of a sedimentation velocity experiment is shown in Figure 1. Sedimentation velocity studies as a function of the concentration gave $s^0_{20,w}$ values (extrapolated to zero protein concentration) of 7.23 ± 0.04 and 7.14 ± 0.06 S from Figure 2A,B, respectively, for experiments with schlieren optics and those with ultraviolet absorption optics. The most probable $s^0_{20,w}$ is thus considered to be 7.19 S, representing the average between the two values.

DIFFUSION COEFFICIENT. Apparent diffusion coefficients were also measured as a function of protein concentration; the results together with data derived from schlieren patterns of synthetic boundary runs are shown in Figure 3. Although the points are scattered, the $D^0_{20,w}$ agrees very closely with that obtained from synthetic boundary experiments utilizing interference optics: 5.15×10^{-7} and 5.14×10^{-7} cm^2 sec^{-1}, respectively. Since $D^0_{20,w}$ from the rate of spreading of the boundary observed during sedimentation velocity experiments using ultraviolet optics was $5.2 \pm 0.9 \times 10^{-7}$ cm^2 sec^{-1} (average of 12 determinations plus and minus mean error), the higher of the two numbers, *i.e.*, 5.15×10^{-7} cm^2 sec^{-1}, is considered the most consistent value for $D^0_{20,w}$.

PARTIAL SPECIFIC VOLUME. Calculation of the partial specific volume from the weight percentages of the amino acid residues, derived from the total amino acid composition (*vide infra*), and their respective specific volumes (Cohn and Edsall, 1943) resulted in a $\bar{V}$ of 0.739 ml g^{-1}. Equilibrium sedimentation in D_2O and H_2O (Edelstein and Schachman, 1967), utilizing data from a total of 13 runs, yielded a partial specific volume of 0.743 ml g^{-1}. Pycnometric measurements were performed over a range of protein concentrations (10–30 mg ml^{-1}), the latter determined by three independent means: dry weight, refractive index increment, and absorbance at 280 mμ. A total of 14 calculated points[3] was plotted in the form of $(\rho - \rho_0)/\rho_0$ (ρ, density of the protein solution at each concentration; ρ_0, density of the solvent) as a function of the protein concentration. From the slope of the straight line in this plot, which is equal to $1/\rho_0 - \bar{V}$ (Sia and Horecker, 1968), $\bar{V}$ was computed to be 0.740 ml g^{-1}. This value, on the basis of reliability of the method and number of data available, is considered the best estimate for the partial specific volume; it has been used in all calculations of this report in which this parameter is required.

MOLECULAR WEIGHT. High-speed sedimentation equilibrium runs (Yphantis, 1964) gave straight line ln c *vs.* r^2 plots, one of which is shown in Figure 4. This linearity may be taken as an indication that phosphoglucose isomerase is homogeneous, at least under the conditions of the Yphantis-type runs (1-mg ml^{-1} initial protein concentration, or less). When higher concentrations were studied by the low-speed method, the ln c *vs.* r^2 plots seemed to display two slightly different slopes, suggesting the possibility of a second molecular species. In a single low-speed equilibrium experiment, however, performed at a very low concentration (0.5 mg ml), the ln c *vs.* r^2 plot was linear. In the other low-speed runs, the molecular weights at the meniscus averaged 119,000, those at the bottom of the cell 137,000; the respective higher values were used in the plot of M_{app} *vs.* initial protein concentration, combining the data from both high- and low-speed runs (Figure 5), on the assumption that a very minor component of a molecular weight lower than 132,000 would possibly not be observed in the high-speed sedimentation equilibrium experiments. As Figure 5 shows, the apparent molecular weight is somewhat

[3] Representation of the experimental data has been omitted at the request of the editors.

TABLE I: Physical Parameters of Five-Times-Crystallized Rabbit Muscle Phosphoglucose Isomerase.

Parameter	Method	Value
$s^0_{10°}$, phosphate	Velocity sedimentation, pH 7.1, ultraviolet optics	4.98×10^{-13} sec
$s^0_{20,w}$	Velocity sedimentation, pH 7.1 or 8.3, ultraviolet optics	7.14×10^{-13} sec
$s^0_{20,w}$	Velocity sedimentation, pH 6.8, schlieren optics	7.23×10^{-13} sec
$s^0_{20,w}$	Average, *best value*	7.19×10^{-13} sec
$D^0_{10°}$, phosphate	Synthetic boundary sedimentation, pH 7.1, interference optics	3.63×10^{-7} cm^2 sec^{-1}
$D^0_{20,w}$	Synthetic boundary sedimentation, pH 7.0 or 7.1, interference optics	5.14×10^{-7} cm^2 sec^{-1}
$D^0_{20,w}$	Synthetic boundary sedimentation, pH 6.8, schlieren optics (*best value*)	5.15×10^{-7} cm^2 sec^{-1}
$D^0_{20,w}$	Boundary spreading during velocity sedimentation, pH 7.1, or 8.3, ultraviolet optics	5.2×10^{-7} cm^2 sec^{-1}
$M^0_{s,D}$	From best values for $s^0_{20,w}$ and $D^0_{20,w}$	130,100 g mole^{-1}
M^0_{eq}	Molecular weight plus and minus standard deviation from equilibrium sedimentation (*best value*)	132,400 ± 1,200 g mole^{-1}
$\bar{v}$	Computed from amino acid composition	0.739 ml g^{-1}
$\bar{v}$	Equilibrium sedimentation in D_2O	0.743 ml g^{-1}
$\bar{v}$	Pycnometry (*best value*)	0.740 ml g^{-1}
$(dn/dc)_{10°}$	Refractive index increment	1.80×10^{-3} dl g^{-1}
f/f_0	Molar frictional ratio	1.23

dependent upon the initial protein concentration. Extrapolation to zero protein concentration yields 132,000, which is considered the most probable, true molecular weight of rabbit muscle phosphoglucose isomerase. Calculation of the molecular weight from sedimentation and diffusion coefficients gives values between 129,000 and 131,000, in good agreement with the figure from equilibrium sedimentation. A summary of the available physical parameters is given in Table I. It may be worthwhile to mention that the refractive index increment (1.80×10^{-3} dl g^{-1}) appears to be slightly low for 10° (Perlmann and Longsworth, 1948) and the molar frictional ratio (approximately 1.23) slightly high, indicating that phosphoglucose isomerase may not be a "typical" protein.

Amino Acid Composition. REFERENCE FACTORS. Concomitant with the determination of the amino acid composition of phosphoglucose isomerase, its nitrogen content, biuret factor, and ultraviolet absorption at 280 and 260 mμ were determined, since their numerical values are required as standard reference parameters for all future work on the enzyme's protein structure. Analyses[4] for total nitrogen (Dumas method) were performed on two different enzyme preparations and yielded a value of 16.7 ± 0.2%. Calibration of the biuret procedure (Gornall *et al.*, 1949) against the nitrogen content, resulted in a biuret factor of 31.6 mg/1.0 absorbance at 540 mμ for a 10-mm light path and a 10.0-ml total biuret reaction volume. The absorbance at 280 mμ (10-mm light path) of 1 mg of enzyme/ml of 0.01 M phosphate (pH 7.0) was found to be equal to 1.32; thus, an absorbance at 280 mμ of 1.0 corresponds to a concentration of 0.76 mg/ml. Furthermore, a molar absorptivity coefficient of 1.74×10^5 M^{-1} cm^{-1} may be calculated for a molecular weight of 132,000. Finally, the ratio of the absorbancies at 280 and 260 mμ was determined to be 1.79.

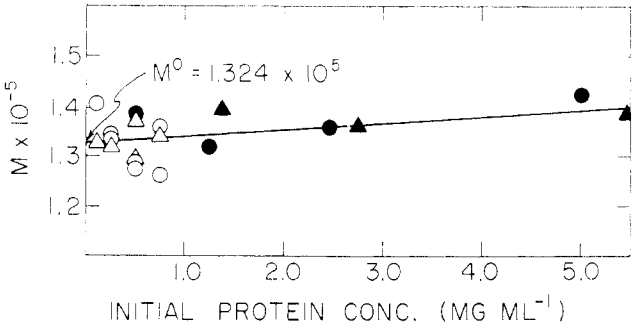

FIGURE 5: Apparent molecular weight of five-times-crystallized rabbit muscle phosphoglucose isomerase as function of the initial protein concentration. (○, ●) 0.1 M sodium phosphate buffer (pH 7.1); (△, ▲) 0.15 M imidazole chloride (pH 7.0). Open symbols represent runs made by the high-speed sedimentation equilibrium method of Yphantis (1964), closed symbols those by the low-speed technique of Richards *et al.* (1968). For additional information refer to Methods.

AMINO ACID RECOVERIES AFTER ACID HYDROLYSIS. The recovery data presented in Table II as a function of hydrolysis time were obtained in 1963–1964. Since then, numerous 20-hr hydrolyses have been performed on control samples in the course of protein modification studies involving more than 50 different preparations of five-times-crystallized phosphoglucose isomerase. These analyses representing at the present time approximately 500 individually hydrolyzed samples, agree within 3–5% of the values given in the table, which is considered quite satisfactory.

It is noteworthy that the efforts expended to remove all traces of oxygen apparently have the desired effect. If both the absolute recoveries and the experimental deviations are taken into account, only tryptophan, threonine, and serine showed a significant continuous degradation in the course of hydrolysis. In particular, tyrosine, which is traditionally regarded as unstable to traces of oxygen present during acid hydrolysis

[4] The nitrogen analyses were performed by Elek Microanalytical Laboratories, Torrance, Calif. 90505.

TABLE II: Amino Acid Composition of Five-Times-Crystallized Rabbit Muscle Phosphoglucose Isomerase.

Amino Acid Residue	Recovery[a] after Acid Hydrolysis				Amino Acid Extrapolated to Zero Time Hydrolysis Or at Max. Recov[b]	Calcd No.[c] of Residues for a Mol Wt of 132,000
	20 hr	40 hr	74 hr	140 hr		
Lysine	0.624 ± 0.016 (5)	0.619 ± 0.002 (3)	0.610 ± 0.004 (4)	0.613 ± 0.002 (3)	0.168	81.6
Histidine	0.358 ± 0.006 (6)	0.360 ± 0.002 (3)	0.358 ± 0.004 (4)	0.363 ± 0.002 (3)	0.359	47.4
Arginine	0.321 ± 0.004 (6)	0.325 ± 0.002 (3)	0.324 ± 0.002 (3)	0.320 ± 0.003 (4)	0.322	42.5
Aspartic acid	0.875 ± 0.012 (5)	0.866 ± 0.003 (3)	0.868 ± 0.003 (4)	0.864 ± 0.013 (3)	0.877	115.8
Threonine	0.586 ± 0.018 (6)	0.559 ± 0.002 (3)	0.514 ± 0.004 (4)	0.428 ± 0.005 (3)	0.620	81.8
Serine	0.503 ± 0.017 (6)	0.456 ± 0.006 (3)	0.375 ± 0.007 (4)	0.245 ± 0.003 (4)	0.580	76.6
Glutamic acid	0.961 ± 0.011 (6)	0.949 ± 0.004 (4)	0.961 ± 0.003 (4)	0.948 ± 0.009 (4)	0.962	127.0
Proline	0.378 ± 0.010 (4)	0.370 ± 0.006 (4)	0.374 ± 0.006 (4)	0.370 ± 0.011 (4)	0.379	50.0
Glycine	0.666 ± 0.008 (5)	0.664 ± 0.003 (4)	0.664 ± 0.001 (3)	0.662 ± 0.005 (4)	0.666	87.9
Alanine	0.643 ± 0.012 (4)	0.637 ± 0.005 (4)	0.636 ± 0.001 (3)	0.621 ± 0.008 (4)	0.639	84.3
Valine	0.462 ± 0.015 (6)	0.491 ± 0.004 (4)	0.515 ± 0.002 (4)	0.515 ± 0.006 (4)	0.515	68.0
Methionine	0.216 ± 0.009 (6)	0.214 ± 0.004 (4)	0.217 ± 0.003 (4)	0.216 ± 0.004 (4)	0.216	28.5
Isoleucine	0.480 ± 0.021 (6)	0.498 ± 0.002 (4)	0.519 ± 0.002 (4)	0.508 ± 0.006 (4)	0.519[d]	70.5
Leucine	0.871 ± 0.006 (5)	0.864 ± 0.004 (3)	0.869 ± 0.006 (3)	0.861 ± 0.007 (4)	0.872	115.1
Tyrosine	0.185 ± 0.005 (5)	0.183 ± 0.002 (3)	0.180 ± 0.003 (4)	0.181 ± 0.003 (3)	0.185	24.4
Phenylalanine	0.459 ± 0.010 (5)	0.452 ± 0.002 (3)	0.453 ± 0.001 (3)	0.455 ± 0.001 (3)	0.459	60.6
Tryptophan					0.185[e]	24
Cysteine					0.093[f]	12
Amide ammonia					0.82[g]	108

[a] The values are expressed as μmoles of amino acid recovered per 1 mg of hydrolyzed enzyme applied to the column. The deviations given are the average values of the absolute deviations from the mean. Numbers included in parentheses indicate the number of determinations, each of which represents a single analysis of an individually hydrolyzed protein sample. All of the values are listed to three decimal places to illustrate the precision of the analyses. It is not implied that the overall accuracy is better than ±2%. [b] From the data in this column, a weight recovery of 101.7% and a nitrogen recovery of 103.9% may be calculated. [c] The data in this column are not rounded off to integral numbers in order to avoid an increase in computational error if fractions of these numbers need to be expressed in terms of integers in case the enzyme is composed of identical subunits. It is not implied that the accuracy is better than ±2%. [d] This value does not include alloisoleucine formed during hydrolysis. At maximum isoleucine recovery (74 hr), alloisoleucine was estimated as 0.015 μmole, thus bringing the total isoleucine to 0.534 μmole/mg of enzyme. [e] Based on a tryptophan content of 24 residues/molecule (Table IV). [f] Based on a cysteine content of 12 residues/molecule (Table III). [g] See text.

TABLE III: Determination of the Cysteine Content of Rabbit Muscle Phosphoglucose Isomerase.

Method	Residues/Molecule of Enzyme[a]
Performic acid oxidation	11.5 (12.2)[b]
Carboxymethylation after denaturation and reduction	9.5 (10.4)[c]
Air oxidation after acid hydrolysis	9.9 (12.3)[d]
PMB titration according to Boyer	12.5[e]
Amperometric silver titration[f]	9–10

[a] Calculated for a molecular weight of 132,000. [b] The value in parentheses is calculated by assuming a recovery of 94% (Moore, 1963). [c] The value in parentheses is calculated assuming a recovery of 91% (Cole *et al.*, 1958). The data are based on carboxymethylation experiments performed in 8 M urea at pH 9 after reduction with mercaptoethanol. In addition, similar alkylation experiments were conducted in 1% sodium dodecyl sulfate; however, difficulty was experienced in achieving reproducible extents of carboxymethylation. [d] The value in parentheses is calculated by assuming a recovery of 80% (Gundlach *et al.*, 1959; Noltmann *et al.*, 1962). [e] Compare Chatterjee and Noltmann (1967b). [f] These analyses were kindly performed by J. E. Dyson.

TABLE IV: Tryptophan and Tyrosine Contents of Rabbit Muscle Phosphoglucose Isomerase.

Method	μmoles/mg of Protein: Tryptophan	Tyrosine	Trp Residues/Molecule of Enzyme[a]
Ultraviolet absorption at alkaline pH			
Absolute values[b]	0.192	0.196	25.3
Tyr:Trp with independent value for Tyr[b]	0.181	(0.185)[c]	23.9
Molar absorbance formulas[d] for 280 and 294 mμ	0.162	0.247	21.4
Ultraviolet absorption at pH 6.5[e]	0.191	0.182	25.2
p-Dimethylaminobenzaldehyde	0.179		23.6
Ion-exchange chromatography after alkaline hydrolysis	0.178[f]	0.184	23.5

[a] Calculated for a molecular weight of 132,000. [b] Calculated according to Bencze and Schmid (1957). [c] From ion-exchange chromatography after hydrolysis in 6 N HCl (Table II). [d] Calculated by the method of Goodwin and Morton (1946) as described by Beaven and Holiday (1952). [e] According to the method of Edelhoch (1967). [f] Average of eight determinations.

(Carpenter and Chrambach, 1962), was not significantly affected. When the hydrolysis in 6 N HCl was performed in the presence of a phenol crystal, a technique which was empirically found to stabilize tyrosine (Sanger and Thompson, 1963), equal tyrosine recoveries were obtained as in its absence. Also, about 60% of the tryptophan was recovered after 20-hr acid hydrolysis at 110° which is high for a protein containing a substantial number of free sulfhydryl groups. Although the stability of each amino acid species is obviously dependent on the inherent structure of each individual protein, the good recoveries observed under the conditions of essentially complete oxygen removal appear to be more than accidental.

The degradation of threonine and serine appears to follow closely pseudo-first-order kinetics,[5] analogous to observations previously made with two other enzymes from rabbit skeletal muscle (Mahowald *et al.*, 1962; Noltmann *et al.*, 1962) which were hydrolyzed under similar conditions. It should be noted, however, that the pseudo-first-order rate constants for degradation, $k_{Thr}^{1} = 2.7 \times 10^{-3}$ hr^{-1} and $k_{Ser}^{1} = 6.2 \times 10^{-3}$ hr^{-1}, are different from those for myokinase ($k_{Thr}^{1} = 1.8 \times 10^{-3}$ hr^{-1}, $k_{Ser}^{1} = 4.6 \times 10^{-3}$ hr^{-1}) and creatine kinase ($k_{Thr}^{1} = 2.0 \times 10^{-3}$ hr^{-1}, $k_{Ser}^{1} = 4.5 \times 10^{-3}$ hr^{-1}) (Noltmann *et al.*, 1962). This finding once more exemplifies the necessity of performing protein hydrolysis as a function of time (*e.g.*, Tristram and Smith, 1963), and emphasizes that choosing an arbitrary value for the threonine or serine recoveries after 20- or 22-hr hydrolysis is not a substitute for a complete hydrolysis time study in arriving at reliable values for these two amino acids.

[5] Figure deleted at the request of the editors.

CYSTEINE. Data from various methods for the determination of cysteine are summarized in Table III. The results, after correction for incomplete recoveries, are most in agreement with a content of 12 cysteine residues/phosphoglucose isomerase molecule of 132,000 mol wt. The conditions of amperometric silver titration appear to permit some oxidation of sulfhydryl groups, thereby reducing the number of those available for titration. Thus, it was recently concluded by Yue *et al.* (1967), on the basis of extensive chemical and physical studies, that the presence of a disulfide group in creatine kinase—previously postulated because only approximately 80% of the total of eight half-cystine residues could be titrated with silver in 8 M urea (Benesch *et al.*, 1955; Noltmann *et al.*, 1962)—is incompatible with the experimentally proven dimeric structure of the enzyme. It is notable that not more than 10 sulfhydryl groups could be alkylated by iodoacetate under various conditions of reduction and denaturation of phosphoglucose isomerase.

TRYPTOPHAN. Table IV summarizes the data for the tryptophan content of phosphoglucose isomerase obtained by several procedures. (1) The spectrophotometric evaluation of the ultraviolet absorption measured at alkaline pH gave different values depending on the calculation method that was used to convert the experimental data into concentrations

of tryptophan. This might be considered somewhat disturbing and it also emphasizes the problems involved in calculating tryptophan concentrations within the protein molecule with the use of molar absorbance indices obtained for free tryptophan in solution. Nevertheless, it is believed that the most reliable of the three calculations is the one by Bencze and Schmid (1957) in which the spectrophotometric data are used only to establish the molar ratio of tyrosine to tryptophan. An accurate value for tyrosine is available from chromatography on the amino acid analyzer following acid hydrolysis. Concerning the equations of Beaven and Holiday (1952), the authors themselves point out that, although the molar ratios appear to be characteristic for a certain protein, the tyrosine estimate will tend to be high and that of tryptophan low, a finding which is confirmed here for phosphoglucose isomerase. A comparatively high value for tryptophan was estimated with Edelhoch's method by which the absorbance measurements are made at pH 6.5 after unfolding of the protein in 6 M guanidinium chloride (Edelhoch, 1967). (2) Data from the colorimetric analysis with the "Procedure K" of Spies and Chambers employing Ehrlich's reagent are in good agreement with data from the chromatographic analysis after alkaline hydrolysis and from the spectrophotometric ratio method. (3) The conditions of alkaline hydrolysis described by Ray and Koshland (1962), that is, where the hydrolysis is carried out in polyethylene tubes under nitrogen but without sealing the hydrolysis vessel, did not completely prevent[3] a degradation of tryptophan during the hydrolysis. In contrast, when total replacement of all traces of oxygen in the enzyme solution and in the barium hydroxide crystals was achieved by repeated alternate evacuation and flushing of the tube with nitrogen, then tryptophan was apparently stable[3] even for prolonged periods of hydrolysis at 110°. It is concluded from the results presented in Table IV that the most probable value for the tryptophan content is 24 moles/132,000 g of enzyme.

AMIDE AMMONIA. Values for the amide ammonia were arrived at by two means: extrapolation to zero time acid hydrolysis yielded 0.84 and diffusion after mild alkaline hydrolysis 0.82 μmole of ammonia/mg of enzyme. The latter value is considered more accurate since some uncertainties are involved in assuming that the liberation of ammonia during acid hydrolysis is a linear function of the hydrolysis time. A value of 0.82 μmole of amide ammonia/mg of protein corresponds to 108 amide groups/phosphoglucose isomerase molecule of 132,000 mol wt.

The final data for the amino acid composition of rabbit muscle phosphoglucose isomerase,[6] as derived from the various analytical methods, are summarized in the last column of Table II. The calculated recovery in terms of per cent of total weight is 101.4, in per cent of total nitrogen 103.9; these values are slightly high but compare favorably with data obtained on over-all amino acid analysis of similar size proteins (Tristram and Smith, 1963). A possible source of error may originate from the total nitrogen analysis which is inherently difficult to relate to the absolute dry weight of a protein. The essentially complete recovery on a weight basis may be taken as indicating that the enzyme does not contain a nonprotein moiety.

[6] While the manuscript of this paper was in the process of revision, Carter and Yoshida (1969) have published an amino acid analysis of human muscle phosphoglucose isomerase. With the exception of arginine, the contents in the human muscle enzyme of practically all of those amino acids that can be reliably determined from a single-time acid hydrolysis, appear to agree (within reasonable experimental error) with those reported in this paper for the rabbit muscle enzyme, which may be taken to suggest a remarkable similarity between human and rabbit muscle phosphoglucose isomerase.

Added in Proof

While this manscript was in galley stage, a further paper was published by Yoshida and Carter (1969) in which the resolution of a commercial preparation of crystalline rabbit muscle phosphoglucose isomerase into three isoenzyme fractions is described. Two of the three fractions were claimed to have different amino acid compositions and different structural genes were suggested to be involved in producing them. Interestingly, these reported amino acid compositions for the two isoenzymes show differences that are larger than those existing between the values given by Carter and Yoshida (1969) for the human muscle enzyme and the analyses reported here for five-times-crystallized rabbit muscle phosphoglucose isomerase.

Repeated attempts in this laboratory[7] to reproduce the chromatographic separation pattern on carboxymethyl-Sephadex as described by Yoshida and Carter (1969) under their conditions (0.01 M phosphate, pH 6.8; linear gradient, 0 to 0.1 M NaCl) have failed to separate fractions corresponding to their isoenzymes I and II. However, elution of the enzyme with a phosphate gradient without NaCl in a total concentration range (6 to 12 mM) one order of magnitude below that of Yoshida and Carter (1969) has yielded multiple forms of rabbit muscle phosphoglucose isomerase.[7] In contrast to the findings by Yoshida and Carter (1969), these various forms do not remain single species on rechromatography under the same conditions and show amino acid compositions indistinguishable within the precision of the method and essentially equal to that reported in Table II. Furthermore, addition of dithiothreitol has been found to prevent formation of the multiple forms from previously separated single peaks. A detailed account of these data will be reported elsewhere.

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