A Comparison of the Association of Yeast Phosphoglycerate Mutase (EC 2.7.5.3) with that of Haemoglobin

AN ULTRACENTRIFUGE STUDY

By S. PETER SPRAGG, JOHN J. ROCHE and JOHN WILCOX

Department of Chemistry, University of Birmingham, Birmingham B15 2TT, U.K.

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1. Previous work showed that yeast phosphoglycerate mutase (EC 2.7.5.3) has a mol.wt. of between 107000 and 110000. Preliminary examination showed that at dilutions less than 0.1 g/l the enzyme dissociated into its subunits. 2. This dissociation was quantitatively examined by both equilibrium and velocity centrifugation. 3. The mathematical analysis of the equilibrium records was tested against oxyhaemoglobin in a variety of ionic strengths and at two temperatures. 4. The estimated $L_{2,4}$ (interaction coefficient) for oxyhaemoglobin generally agreed with published values except at 6°C in 0.9M-NaCl, when it was 2.5 times larger than the published value. 5. Statistical analysis of ultracentrifugal-equilibrium experiments showed that the predominant reaction for phosphoglycerate mutase was monomer $\rightleftharpoons$ tetramer, to give an $L_{1,4}$ of 40.3 ± 23.4 (s.d.) $l^3 \cdot g^{-3}$ at 20°C. Decreasing the temperature decreased the association to give an enthalpy of between 40 and 60 kJ/mol. 6. Analysis of velocity experiments carried out with concentrations varying from 0.3 to 17 g/l gave an $L_{1,4}$ of $311 l^3 \cdot g^{-3}$. Incorporating errors from estimating $s_{20,w}$ into the analysis showed that this estimate could range from 893 to $1421 l^3 \cdot g^{-3}$. 7. The concentration-dependence of $s_{20,w}$ was $0.95 \text{ litre} \cdot g^{-1}$ and $s_{20,w}^0$ for the tetramer was 66.9 ps. 8. These results are discussed in relation to the activity of the enzyme.

Phosphoglycerate mutase (EC 2.7.5.3) converts 3-phosphoglycerate into 2-phosphoglycerate and appears to be distributed throughout cells. There are, however, variations between cells which affect both molecular size and requirements for a cofactor in the catalysis (reviewed by Grisolia, 1962). Preparations from yeast can be crystallized as a tetramer (Edelhoc *et al.*, 1957; Campbell *et al.*, 1972) having a mol.wt. of approx. 110000 (Sasaki *et al.*, 1970). Preparations from muscle yield a dimeric molecule which also crystallizes (Edelhoc *et al.*, 1957) and has a mol.wt. of approx. 65000. Both enzymes require 2,3-diphosphoglycerate as a cofactor in the reaction. A monomeric version of the enzyme from plants is said to exist which has a mol.wt. of about 35000 and does not require a cofactor for activity (Grisolia, 1962).

The oligomeric molecules appear to be assembled from identical subunits (Sasaki *et al.*, 1970; Campbell *et al.*, 1972) linked through low-energy bonds. The natural occurrence of three active independent molecules in various stages of aggregation makes it possible to study the relation of the biological activity to subunit interaction. If the oligomeric enzymes dissociate readily in relatively high concentrations (above 0.1 mg/l) compared with those where enzymic activity occurs (below 0.01 g/l) then one can question

the importance of the polymerization in the enzymic role.

Dissociation of polymeric proteins on dilution is not uncommon, the best example being haemoglobin (Kellett, 1971). Detection and measurement of the distribution is usually achieved by following the progressive decrease in molecular weight with increasing dilution of enzymic solutions. When oxyhaemoglobin solutions are diluted, it is found that a solution containing 0.5 g/l contains approx. 50% dimers (Kellett, 1971). The procedure of measuring molecular parameters with the ultracentrifuge over a range of concentrations has been used in the present study.

The ultracentrifuge can be used in two modes, velocity centrifugation, and equilibrium centrifugation, where all the components in the solution are simultaneously at chemical and mechanical equilibrium. Analysing the records resulting from velocity-centrifugation of interacting systems (Gilbert & Gilbert, 1973) is less contentious than the methods used for equilibrium centrifugation. To give confidence that our chosen procedure is reliable, we tested the whole experimental operation with oxyhaemoglobin. These results are presented here, together with our analyses on the dissociation of phosphoglycerate mutase.

Materials and Methods

Materials

Crystalline samples of phosphoglycerate mutase stored in $3\text{M}-(\text{NH}_4)_2\text{SO}_4$ were kindly donated by Dr. H. Watson and Dr. G. Hodgson (Department of Biochemistry, University of Bristol) and taken from the sample used in the X-ray-crystallographic study (Campbell *et al.*, 1972). Human oxyhaemoglobin was donated by Professor G. A. Gilbert (Department of Biochemistry, University of Birmingham) and was prepared from fresh citrated blood by the method of Rossi-Fanelli *et al.* (1961). Small samples of the concentrated purified haemoglobin were stored in buffer at liquid- N_2 temperatures and thawed before use.

Preparation of solutions

Phosphoglycerate mutase. Approx. 5 mg of crystals was separated from their mother liquor by centrifugation. They were then dissolved in 2 ml of the appropriate buffer and the solution was dialysed at 4°C for about 6 h against approx. 500 ml of the buffer. The concentrations of the enzyme at the end of this treatment varied between 0.8 and 1.2 mg/ml. Because tests showed that storage for 10 days at 4°C was accompanied by indefinite aggregation and degeneration of the protein, the solutions were analysed immediately after preparation.

Haemoglobin. The purified samples of oxyhaemoglobin were diluted with buffer to the required concentration (measured at A_{415}), needing a dilution of about 1:100. This solution was dialysed at 4°C against a large excess of buffer for about 4 h before being examined.

Buffers for phosphoglycerate mutase experiments. For most experiments a phosphate buffer (pH 7.0) contained, per litre, 1.52 g of KH_2PO_4 , 4.62 g of $\text{Na}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$, 0.37 g of EDTA (disodium salt) and 2.92 g of NaCl; this was equal to 0.024 M-phosphate ion, 0.076 M- Na^+ and 0.05 M- Cl^- , giving I 0.12 (approx.). In one experiment with phosphoglycerate mutase, 0.05 M- $(\text{NH}_4)_2\text{SO}_4$ replaced NaCl, giving I 0.22 (approx.).

Buffers for haemoglobin experiments. These were made to conform where possible with those used by previous workers (Kellett, 1971; Chiancone *et al.*, 1968). One buffer contained 1.85 mM- $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 4.07 mM- $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1 mM-EDTA (disodium salt) and 0.2 M-NaCl, giving I 0.22 and 0.5 M- Na^+ . Another contained 0.09 M-NaCl, 0.01 M-phosphate and 1 mM-EDTA, giving I 0.1 (approx.) (Chiancone *et al.*, 1968).

Apparatus

An MSE analytical ultracentrifuge fitted with multi-hole rotors and 20 mm double-sector centre-

pieces was used. The temperature of the rotor was controlled during the experiments, and tests showed that the error was $\pm 0.05^\circ\text{C}$ in the measurement and about $\pm 0.1^\circ\text{C}$ in temperature control over a period of 12 h.

Equilibrium experiments

Small columns (between 1 and 2.5 mm long) of solution were floated on shorter columns of perfluorophenanthrene and run at speeds ranging from 10000 rev./min to 17000 rev./min. Generally the solutions were run overnight to ensure equilibration of the system (approx. 17 h) before collection of records of absorbance at fixed radial positions. A computerized system was used to collect records; this comprised a Data General Nova 1220 computer (Data General Ltd., Greenford, Middx. UB6 9BH, U.K.) to control the scanner and to average transmissions at each radius from up to nine individual sectors (the system is fully described in Spragg *et al.*, 1976). Between three and six scans of each solution were collected and analysed individually. Light was provided by a 150 W xenon arc filtered through a grating monochromator producing a bandwidth of approx. 5 nm. The wavelength was calibrated by using lines from a high-pressure mercury arc lamp. Radiation of 280 nm wavelength was used for concentrations of phosphoglycerate mutase less than 0.05 g/100 ml; for higher concentrations the wavelength was increased to 290 nm. A summary of the wavelengths used to examine haemoglobin is given in Table 1.

Velocity experiments

Schlieren records were collected on film for sedimentation at 20°C of phosphoglycerate mutase at concentrations of 0.34 g/l and 1.0 g/l at speeds of 55000 rev./min. The co-ordinates were measured from the photographs by using a semi-automatic system developed in the Department of Physics, University of Birmingham, for measuring records from bubble chambers. This equipment measured co-ordinates to an accuracy of $0.1\text{ }\mu\text{m}$, but after processing to give the sedimentation coefficient the errors were equivalent to an uncertainty of $\pm 1\text{ }\mu\text{m}$. The increase is caused by extra errors introduced from difficulties in locating the centre of the schlieren lines.

Extinction coefficient

Two estimates of the specific extinction coefficient of phosphoglycerate mutase are given in the literature. Sugimoto *et al.* (1963) report an $A_{280}^{1\%}$ of 14.2, and Edelhoch *et al.* (1957) quote 14.5. Repeating the estimates with the present samples, by using concentrations of protein estimated from schlieren

profiles in the ultracentrifuge and absorbances measured on a Unicam SP.500 spectrophotometer, gave a value 14.3 ± 0.2 . The error is the standard deviation calculated by compounding errors from the centrifuge (calibration and measurement) and four estimates from the spectrophotometer.

The assumed specific extinction coefficients of oxyhaemoglobin are listed in Table 1.

Estimation of density and viscosity of solvents

Conventional pycnometers and viscometers were used to measure these parameters in a water bath whose temperature was controlled to the temperature of the ultracentrifuge experiment with a precision of $\pm 0.01^\circ\text{C}$.

Analysis of records

Equilibrium data. Edited records from individual solutions were analysed and the results tested for consistency by fitting records to three mathematical models.

Model (1). A curvilinear regression analysis was used to fit $\ln A$ against r^2 (A , absorbance, and r , radius) and from the slope an apparent molecular weight (M_a) was calculated from eqn. (1):

$$\frac{d \ln A}{dr^2} = \frac{M_a(1 - \bar{v}\rho)\omega^2}{2RT} \quad (1)$$

Here $\bar{v}$ = partial specific volume (taken to be 0.74 ml/g for phosphoglycerate mutase and 0.75 ml/g for oxyhaemoglobin), ρ = density of solvent (for the low concentrations this is approximately equal to that of the solution), R and T are gas constant and temperature, and ω = angular rotor velocity. If the test for curvature in the regression analysis showed that the linear model (eqn. 1) was not significant at the 95% probability level, then a chord was fitted to the record and a molecular weight calculated from the slope. In all the analyses of phosphoglycerate mutase, covering 32 solutions, about 10% showed significant curvature by this test.

Model (2). This provided an estimate of the concentrations of the components in a mixture of ideal solutes at centrifugal equilibrium. This used eqn. (2), where the concentration at any radius $[c(r)]$ is equal to the sum of the individual concentrations defined by the integral of eqn. (1) between the appropriate limits:

$$c(r) = \sum_{i=1}^n c(i)_{\text{ref.}} \exp [\alpha_i M_i (r^2 - r_{\text{ref.}}^2)] \quad (2)$$

Here $\alpha_i = (1 - \bar{v}_i \rho) \omega^2 / 2RT$, $c(i)_{\text{ref.}}$ is the concentration of the i th component at $r_{\text{ref.}}$. At finite concentrations where thermodynamic activity is not equal to concentration, the true molecular weight (M_i) would not

equal the apparent molecular weight (M_a). These two are related through eqn. (3):

$$M = M_a(1 + Bc) \quad (3)$$

To assume that the virial coefficient $B \approx 0$, it is necessary to carry out experiments with concentrations of 0.1 g/l or above and $I \geq 0.1$ at least. These conditions are fulfilled in the present work.

Model (3). This is a more contentious model for analysing interacting species and is taken from the Rinde (1928) equation (eqn. 4). Here the integral solution to eqn. (1) is found by including conservation of mass at equilibrium as a boundary condition for n independent species:

$$c(\xi) = \sum_{i=1}^n \frac{c_i^0 \lambda_i M_i \exp(-\lambda_i M_i \xi)}{1 - \exp(-\lambda_i M_i)} \quad (4)$$

Here $\lambda_i = \alpha_i(r_b^2 - r_m^2)$ and $\xi = (r_b^2 - r^2)/(r_b^2 - r_m^2)$ (the suffixes m and b refer to the meniscus and base of the column of liquid). c_i^0 is the initial concentration of the i th species.

Eqn. (4) is to be preferred to eqn. (2) in analysing mixtures because accurate measurements of the initial total concentrations (c_i^0 , measured by absorbance) are available before putting the solution into the centrifugal cells, and it is possible to apply the constraint $c_i^0 = \sum_{i=1}^n c_i^0$, in the analysis. c_i^0 can be measured in the cell immediately after reaching speed or it can be measured statically. Measurement in the centrifuge is preferred because it minimizes uncertainties caused by unwanted large denatured aggregates in the solution, which contribute to absorbance determined statically, but are swept out of solution at low centrifugal speeds. We found that the static and dynamic measurements with both proteins agreed closely, showing that the solutions did not contain denatured aggregates.

Eqn. (4) has been applied to analyse heterogeneity in synthetic polymers (Scholte, 1968) that are not chemically interacting. However, its use for chemically analysing interacting systems is inexact (Adams, 1969). Reinhardt & Squire (1965) were aware of the problem when they used this method to analyse results for interacting ovine interstitial-cell-stimulating hormone. They derived a term that corrected for the mutual dependence of the individual species when at equilibrium. Provided the effects of pressure can be ignored, then chemical equilibrium at any point is maintained when a solution is at sedimentation equilibrium (Adams & Fujita, 1963). Thus, for self-association, the interaction coefficient at any radial parameter (ξ) is given by eqn. (5):

$$L(\xi) = \frac{c(\xi)_n}{[c(\xi)_i]^n} \quad (5)$$

Further, assuming the partial specific volumes of all interacting species to be equal ($\lambda_1 = \lambda_2 = \dots = \lambda_n$;

eqn. 4) and $M_i = iM_1$, then by inserting the appropriate expressions for $c(\xi)_i$ from eqn. (4) into eqn. (5) gives eqn. (6):

$$L(\xi) = \frac{c_n^0 \lambda \exp(-\lambda M_n) \cdot [1 - \exp(-\lambda M_1)]^n}{(c^0)^n [1 - \exp(-\lambda M_n)] [\lambda M_1 \exp(-\lambda M_1 \xi)]^n} \quad (6)$$

This expression can be used to calculate the true interaction coefficient from the apparent coefficient (L_a). L_a was calculated from the concentrations estimated from eqn. (4).

The general correction is given by eqn. (7):

$$L = L_a \frac{n[1 - \exp(-\lambda M_1)]^n}{\lambda^{(n-1)} M_1^{(n-1)} [1 - \exp(-\lambda n M_1)]} \quad (7)$$

Obviously many assumptions are made in reaching eqn. (7), and it is important that results calculated by this model are reliable. For this reason one of the tests for consistency was to calculate L from concentrations estimated from eqn. (2) and compare these with values calculated from eqn. (7).

Statistical analyses. The measured records can be fitted directly to eqns. (2) and (4) through a multi-linear regression procedure. This is expressed generally as:

$$y = \sum_{i=1}^n A_i X_i \quad (8)$$

where X_i equals either $\alpha_i M_i (r^2 - r_{ref}^2)$ (eqn. 2) or $\lambda_i M_i \exp(-\lambda_i M_i \xi) / [1 - \exp(-\lambda_i M_i)]$. In all our experiments the records from individual solutions were analysed separately, but it is possible to extend the fitting procedure to include all the records from one experiment as the single experimental set, as well as to collect records over a range of speeds of centrifugation in one experiment and include these in one set. Roarke (1976) has described a method for carrying out this type of experiment, including the formulae required for calculating the times taken to reach equilibrium after the change in speed.

The use of an on-line computer to record absorbances means that the multiple scans can readily be recorded and combined in a single fitting. However, combining the records masks any systematic errors that might occur, and that could be detected by trends of values with time. Hence we have treated each scan separately and combined the results from these analyses to provide mean values of interaction coefficients together with their standard deviations.

Velocity measurements. (a) Calculation of $\bar{s}_{20,w}$. Since this experiment involves transport of the interacting molecules under the influence of the centrifugal field, it is necessary to estimate a weight-average sedimentation coefficient ($\bar{s}$) from a series of protein-concentrations to measure L . Up to 25 co-ordinates (radius and schlieren-deflexion) were measured across the schlieren profile from each photograph, and from these the true centroid of the

boundary ($\bar{r}$) was calculated by the second moment (eqn. 9):

$$\bar{r}^2 = \frac{\int_{r_m}^{r_b} (dn/dr) r^2 dr}{\int_{r_m}^{r_b} (dn/dr) dr} \quad (9)$$

The integrals in eqn. (9) were evaluated by direct summation (Simpson's rule could not be used because the records were not collected at equal intervals in the radius). The integral calculated for the denominator was used as an estimate of protein concentration. In the experiments the boundaries moved a maximum of 3 mm during the experiment and no statistically significant decrease in area with time was detected (as expected by the squared law for dilution in a sector-shaped cell). The average area was used to calculate concentration. The $\bar{s}$ value was calculated from the linear regression of $\ln \bar{r}$ against time, and no significant curvature was detected in any of the analyses. These values were corrected for viscosity and density of the buffer relative to water at 20°C to give $\bar{s}_{20,w}$ values.

(b) Estimation of interaction coefficient ($L_{1,4}$). From the definition of a weight-average sedimentation coefficient ($\bar{s}_{20,w}$) and the definition of $L_{1,4}$ (eqn. 11) eqn. (10a) can be derived (w_i = concentration of i th component in w/v), and the empirical dependence of $\bar{s}_{20,w}$ on concentration is given by eqn. (10b) [g is the concentration-dependence coefficient given in units of inverse concentration (Gilbert & Gilbert, 1973)]:

$$\bar{s}_{20,w} = \frac{s_1^0 w_1 + s_4^0 w_1^4 L_{1,4}}{w_1 + w_4} \quad (10a)$$

$$\bar{s}_{20,w}(i) = s_i^0 (1 - g_i c_i) \quad (10b)$$

We have assumed a constant g for all species, so leaving three unknowns ($L_{1,4}$, g , s_4^0) in these equations to be estimated (s_4^0 is the sedimentation constant for the tetramer). Non-linear statistical methods are required to fit the sedimentation coefficients to the concentrations to this model by minimizing the sum of squares of the residuals between calculated and experimental $\bar{s}_{20,w}$ values, and several procedures have been described to perform this calculation. We used the simplex method described by Nelder & Mead (1965) to minimize the errors caused by combining results from the present study, from that of Sasaki *et al.* (1970) (supplied as photographs, which we measured) and three values derived from Fig. 1 in Edelhoch *et al.* (1957).

Interconversion of units for calculating equilibrium constants

To calculate interaction coefficients, it is necessary to convert absorbance measurements into concentrations (w/v); these interaction coefficients can then

be converted into equilibrium constants (assuming no concentration-dependence). With phosphoglycerate mutase, a monomer $\rightleftharpoons$ tetramer reaction appears to predominate and $L_{1,4}$ is defined by eqn. (11):

$$L_{1,4} = \frac{w_4}{w_1^4} (l^3 \cdot g^{-3}) \quad (11)$$

The equilibrium constant $K_{1,4}$ was calculated by assuming that the monomer mol.wt. is 27500 (Sasaki *et al.*, 1970) to give the relationship in eqn. (12):

$$K_{1,4} = 5.1992 \times 10^6 L_{1,4} (l^3 \cdot M^{-3}) \quad (12)$$

For oxyhaemoglobin the dimer $\rightleftharpoons$ tetramer reaction predominates (Kellett, 1971), and $K_{2,4}$ was calculated by using a conversion factor in eqn. (12) of 1612.7, assuming a mol.wt. for the tetramer of 64500.

Results

Haemoglobin

Four separate experiments were carried out (Table 1), covering a range of protein concentrations at three temperatures. In three of these experiments the time allowed for centrifugal equilibrium was 24 h, in another it extended to 72 h, and for the lowest concentration (1.4 mg/100 ml) a shortened experi-

ment was carried out in which equilibration was hastened by the overspeeding procedure of Richards *et al.* (1968). Static experiments showed that a maximum of 6% methaemoglobin formed at 20°C in 72 h. Replicates of solutions were run and at least four scans for each solution were collected. These were analysed separately by using eqns. (2), (4) and (7), and Table 2 shows representative results. In all experiments the interaction coefficient calculated from eqns. (4) and (7) agreed within three and often four significant figures with those calculated from the same record by using eqn. (2). This correspondence gave confidence that the correction of Reinhardt & Squire (1965) was sufficiently accurate for most purposes.

Having established that eqns. (4) and (7) can be applied correctly to results from interacting systems, we then showed that the total absorbance formed by summing the individuals (calculated from eqn. 4) was not significantly different from that measured in a conventional spectrometer (Table 3). As the results show, there was a noticeable trend of decreasing estimated concentration with increasing speed of rotor, although the differences were not statistically significant. The effect arises because the model (eqn. 4) does not allow for interaction between the macromolecular species. Thus an additional term should be added to eqn. (4) which gives the cross-

Table 1. *Experimental conditions used to examine oxyhaemoglobin*

Expt. 1 included three speeds; recordings were made after reaching each new equilibrium. The whole experiment extended over 72 h.

Expt.	Concn. (mg/100 ml)	Temperature (°C)	Speed (rev./min)	$A_{1cm}^{1\%}$ (litre $\cdot$ g $^{-1}$ $\cdot$ cm $^{-1}$)	Wavelength (nm)	Duration of experiment (h)
1 (a)	19-13	20	14710	16.0	440	24
(b)			15940			48
(c)			17900			72
2	12	21	13700	21	436	24
3	4.6	6	14390	65.0	420	24
4	1.4	6	14150	76.5	415	8

Table 2. *Examples showing the correspondence between interaction coefficients estimated by eqn. (4) (corrected by using eqn. 7) with those estimated at three positions by using eqn. (2)*

The coefficients are quoted in units of absorption, except for the last column, where the appropriate $L_{2,4}$ value is quoted. r_m , r_{mid} and r_b are the reference radii (eqn. 2) for meniscus, mid-point and bottom of the cell.

Speed (rev./min)	Temperature (°C)	Correction term	$L_{2,4}$ (absorption units)				$L_{2,4}$ (l/g)
			Eqn. (4)	Eqn. (2)			
				r_m	r_{mid}	r_b	
14390	6	0.9097	0.32788	0.32788	0.32785	0.32185	5.36
14710	20.0	0.8813	5.0446	5.0438	5.0443	5.0438	18.88
17900	20.0	0.6629	2.3595	2.3594	2.3594	2.3593	15.15
17900	20.0	0.7933	2.5030	2.5030	2.5030	2.5030	11.91

Table 3. Comparison of the absorbance calculated from eqn. (4) with those estimated by using a conventional spectrophotometer (Unicam SP. 500)

The measurements were made at 440nm. Sectors 1, 2 and 3 had absorbances of 0.31 and that for sectors 4 and 5 was 0.21 with 10mm-light-path cuvettes in the Unicam SP. 500 instrument. The estimated values from the ultracentrifuge (u.c.) were corrected by using a previously determined calibration curve for 410nm ($A_{u.c.} = A_{SP.500} \times 3.0$). Values are means $\pm$ S.D. with the numbers of scans in parentheses.

Sector	Ultracentrifuge speed (rev./min)	Absorbance		
		Dimer	Tetramer	Total
	14710			
1		0.092 $\pm$ 0.029 (6)	0.145 $\pm$ 0.044	0.237 $\pm$ 0.050
2		0.075 $\pm$ 0.015 (6)	0.235 $\pm$ 0.080	0.310 $\pm$ 0.080
3		0.088 $\pm$ 0.010 (6)	0.260 $\pm$ 0.050	0.348 $\pm$ 0.075
4		0.105 $\pm$ 0.015 (5)	0.131 $\pm$ 0.040	0.236 $\pm$ 0.064
5		0.115 $\pm$ 0.025 (5)	0.167 $\pm$ 0.064	0.282 $\pm$ 0.095
	15940			
1		0.102 $\pm$ 0.051 (3)	0.165 $\pm$ 0.072	0.267 $\pm$ 0.100
2		0.085 (2)	0.184	0.269
3		0.068 $\pm$ 0.040 (3)	0.252 $\pm$ 0.061	0.320 $\pm$ 0.11
4		0.054 $\pm$ 0.035 (3)	0.164 $\pm$ 0.054	0.218 $\pm$ 0.090
5		0.130 (2)	0.139	0.269
	17900			
1		0.089 $\pm$ 0.011 (5)	0.166 $\pm$ 0.053	0.255 $\pm$ 0.071
2		0.092 $\pm$ 0.015 (5)	0.132 $\pm$ 0.040	0.224 $\pm$ 0.61
3		0.085 $\pm$ 0.020 (5)	0.23 $\pm$ 0.048	0.315 $\pm$ 0.072
4		0.064 $\pm$ 0.030 (4)	0.150 $\pm$ 0.045	0.214 $\pm$ 0.080
5		0.13 $\pm$ 0.030 (5)	0.081 $\pm$ 0.049	0.211 $\pm$ 0.082

interactions between the initial concentrations; the magnitude of this term equals the trend shown in Table 2. This factor is only important when examining the absolute concentrations of the interacting species; the calculated interaction coefficients are corrected by eqn. (7). Despite these limitations it was found that for conditions generally used in our experiments ($c_b/c_m \leq 5$ at 14710 rev./min) the correspondence between static and ultracentrifugal concentrations was reasonable.

The signal-to-noise ratio set for the experiments was approx. 20dB, which would produce an uncertainty in absorbance of about ± 0.04 (in units measured for the centrifuge), this being the normal amount of uncertainty between cell windows. Normally this was the residual standard deviation calculated after fitting eqn. (4), suggesting the source of the errors was random noise and not systematic deviations. Choosing between fitting multiple or individual scans is not a clear-cut decision. As the two representative results in Fig. 1 show, fitting 143 pairs of co-ordinates produced an average $L_{2,4}$ value that matched the data from any of the individual scans (only one is shown in Fig. 1b, containing 36 pairs of co-ordinates). However, the residual variance was always larger from fits with combined scans than with individual scans, showing that systematic errors between scans are important

in the analysis. These errors arise largely from uncertainty in the radial dimensions of the meniscus and the bottom of the column (although the final calculation of c_0^0 is not very sensitive to this error). Throughout the present study we have chosen the method of calculating interaction coefficients from individual scans and averaging across scans and between replicated solutions. The representative fitted curve showed no obvious systematic deviations from the experimental record (Fig. 1).

The estimates of $L_{2,4}$ and $K_{2,4}$ obtained for oxy-haemoglobin are summarized in Table 4. Any trends of $L_{2,4}$ with increasing speed (reflecting the results given in Table 3) were removed by the correction factor and no effect of speed was detected in the final values. The $L_{2,4}$ values estimated for 20 and 21°C were significantly different neither from one another nor from the generally accepted value for a similar buffer (Kellett, 1971). Similarly, results at 6°C for 0.2M-NaCl agreed with those of Guidiotti (1967) but differed from those of Johnson & Perrella (1971b), who used sheep carboxyhaemoglobin. In a buffer of lower ionic strength and $[Na^+]$ we found nearly 2.5 times more association than that reported by Chiancone *et al.* (1971). It should be pointed out that even this difference may be low because the concentration (1.4mg/100ml) is close to the limiting concentration for stability of the equilibrated profile when using the

present systems to control temperatures of the rotor. Small convective currents would decrease $L_{2,4}$, as would partial conversion of oxyhaemoglobin into

metoxyhaemoglobin. We are reasonably confident, however, that our estimate of the $L_{2,4}$ for human oxyhaemoglobin at 6°C in 0.2M-NaCl is correct (within the errors).

Before leaving these tests, some discussion is required on what appear at first glance to be relatively large errors for $L_{2,4}$ and K . The standard deviation varied between 15% (Expt. 1c, Table 4) and 24% (Expt. 4, Table 4), yet the errors in the estimated values for concentrations of dimer and tetramer seldom exceeded 8%. The standard deviations for the coefficients were calculated from replicate solutions by means of replicated scans. Calculation of standard deviations by compounding the errors and covariances from the multilinear regression with eqn. (4) gave values of between 15 and 30% of the $L_{2,4}$ value. This range is similar to that found for variation between sectors, showing that random errors are the major source of uncertainty and not systematic errors arising from dirty cell windows.

Phosphoglycerate mutase

Sedimentation equilibrium. Initial experiments with concentrations less than 0.1 g/100 ml showed that, although no significant curvature could be detected from the logarithmic dependency of absorbance on r^2 (eqn. 1), the estimated molecular weights were considerably less than expected from previous work (Edelhoch *et al.*, 1957; Sasaki *et al.*, 1970). Measurements were then made covering a number of enzyme concentrations in the range 0.1–1.6 g/l, and before interaction coefficients could be calculated from these records it was necessary to determine whether the mixture contained monomers, dimers and tetramers, or was limited to monomer–tetramer. Even at relatively high concentrations (1.6 g/l, Table 5), where it might be thought dimer–tetramer could

Fig. 1. Comparison between fitting of combined records of oxyhaemoglobin (143 ordinates) to eqn. (4) (a) with results from fitting an individual scan (36 ordinates) (b)

The residual variance for the fitting was (a) 2.549×10^{-3} and (b) 1.884×10^{-3} . In (a), points from one of the combined scans are superimposed on the line calculated from $L_{1,4}$ obtained for all four scans.

Table 4. Summary of estimated interaction coefficients ($L_{2,4}$) and equilibrium constants (K) for the association of oxyhaemoglobin. Values are means $\pm$ S.D. for the numbers of estimates in parentheses. The coefficients were calculated from eqn. (4), corrected according to eqn. (7). For experimental details, see Table 1. Hb, haemoglobin.

Expt.	Temperature (°C)	$L_{2,4}$ (l/g)	K ($10^{-5} \times$ l/mol)	NaCl (M)	I	Recorded value ($L_{2,4}$) (l/g)
1 (a)	20	16.45 ± 4.24 (4)	2.65 ± 0.68	0.05	0.12	21.5*
(b)		24.53 ± 8.63 (4)	3.96 ± 1.39			
(c)		17.13 ± 2.56 (5)	2.76 ± 0.41			
Mean		19.74 ± 7.01 (13)	3.18 ± 1.13			
2	21	28.50 ± 5.50 (4)	1.60 ± 0.89	0.05	0.12	5.0†
3	6	5.11 ± 1.72 (7)	0.82 ± 0.28	0.20	0.22	19.0‡
4	6	46.28 ± 10.88 (6)	6.08 ± 1.75	0.09	0.1	20.0§
						62.0

* HbO₂ (Kellett, 1971).

† HbCO (Guidiotti, 1967).

‡ Sheep HbCO in 0.2M-NaCl (Johnson & Perrella, 1971b).

§ HbO₂ (Chiancone *et al.*, 1968).

|| Sheep HbCO in 0.2M-NaCl (Johnson & Perrella, 1971a).

Table 5. *Results of attempting to fit dimer-tetramer association for phosphoglycerate mutase to centrifuge equilibrium results*
 Values are means $\pm$ S.E.M. F is the ratio of residual variances, all for 20 degrees of freedom. The speed of the rotor was 10590 rev/min.

Concn. (g/100ml)	Fitted absorbances				F
	Dimer	Tetramer	Monomer	Tetramer	
0.16	0.270 $\pm$ 0.202	0.967 $\pm$ 0.225	0.184 $\pm$ 0.125	1.055 $\pm$ 0.146	2.82
0.15	0.260 $\pm$ 0.144	0.881 $\pm$ 0.155	0.179 $\pm$ 0.090	0.966 $\pm$ 0.100	1.15
0.16	0.392 $\pm$ 0.133	0.808 $\pm$ 0.148	0.256 $\pm$ 0.081	0.948 $\pm$ 0.095	1.35

predominate, this model only showed between 75 and 80% probability of being important compared with monomer-tetramer reaction (by using the F test to compare significances; Table 5). At lower concentrations, fitting the model monomer-dimer-tetramer to eqn. (4) usually produced negative concentrations for the dimer. Hence we have used the monomer-tetramer model for calculating interaction coefficients.

It is possible for erroneous interaction coefficients to be calculated that have relatively low standard deviations yet the records contain systematic errors. Further, these records could lead to false conclusions about the components of the system. It is difficult to detect systematic errors in records, but we hoped that, if the errors produced in experiments with phosphoglycerate mutase compared favourably with those found for oxyhaemoglobin after fitting to eqn. (4), then the amount of uncertainty would be comparable. This proved to be the case because, as with oxyhaemoglobin, no statistical differences were found between parameters obtained by fitting combined scans of a solution containing between 80 and 150 co-ordinates when compared with the mean calculated from the four or five individual scans of the experiment. The consistency can best be illustrated by comparing co-ordinates from a single scan with co-ordinates calculated from parameters estimated from the combined records of this sector (Fig. 2). The $L_{1,4}$ at 20°C for this solution estimated from the combined five scans was $25.9 \pm 12.9 \text{ l}^3 \cdot \text{g}^{-3}$ compared with $38.5 \text{ l}^3 \cdot \text{g}^{-3}$ estimated for the individual scan shown in Fig. 2. This difference is not significant, but the calculated absorbances from the combined $L_{1,4}$ (broken line, Fig. 2) were higher by about 0.04 than the curve for the individual scan (solid line, Fig. 2). This difference is within the expected error for the signal-to-noise ratio set for this experiment. The results given in Fig. 2 were typical of most experiments and showed that the fitted curves did follow the records with random perturbations of the points about the fitted line. Fig. 2 also illustrates the problem of measuring accurately interaction coefficients for a monomer-tetramer reaction. Small errors in the absorption caused by shifts in baselines of cells are considerably magnified in the final values. An average

Fig. 2. *Comparison between combined records of phosphoglycerate mutase (----) with results for an individual scan (—)*

The $L_{1,4}$ for 136 combined ordinates was $25.9 \pm 12.9 \text{ l}^3 \cdot \text{g}^{-3}$ and the individual scan (25 pairs) gave $38.5 \text{ l}^3 \cdot \text{g}^{-3}$ (concn. 0.23 mg/ml).

shift of about 0.04 moves the $L_{1,4}$ value by about 40%.

The standard deviation calculated from the estimated variances and covariances in the multilinear regression for the record in Fig. 2 was $\pm 15.5 \text{ l}^3 \cdot \text{g}^{-3}$. This compared well with the standard deviation calculated by averaging means for individual scans and ignoring the individual errors ($\pm 12.9 \text{ l}^3 \cdot \text{g}^{-3}$). These factors are similar to those noted with oxyhaemoglobin and suggest that they are reasonable estimates of the random error in the values for $L_{1,4}$.

Correction for bias in results introduced through use of an inexact equation gave acceptable results with oxyhaemoglobin. In the present reaction, where the monomer $\rightleftharpoons$ tetramer reaction predominates, the amount of the correction is considerably larger than for dimer $\rightleftharpoons$ tetramer, since it increases with the order of the reaction as well as with speed of centrifugation. Even at relatively low speeds (e.g. 13880 rev./min) a typical value for the correction factor was 0.69, and at

higher speeds (15870 rev./min) it was 0.57. By using these correction factors, the $L_{1,4}$ for the first speed was corrected from $95.1 \pm 6.7 \text{ l}^3 \cdot \text{g}^{-3}$ to give $65.5 \pm 4.1 \text{ l}^3 \cdot \text{g}^{-3}$. After increasing the centrifugal speed to the higher value and continuing the experiment for a further 24 h, the value for the $L_{1,4}$ before correction was $132 \pm 25 \text{ l}^3 \cdot \text{g}^{-3}$, which became $75.7 \pm 14.2 \text{ l}^3 \cdot \text{g}^{-3}$ after correction. These values show that the correction appears to be sufficient to minimize this source of systematic error.

Conversion of absorbances into concentration. Because of the wide bandwidth of the filtered light in the centrifuge compared with that in normal spectrophotometers, it was not possible to use the conventional specific extinction coefficient to calculate concentrations. A separate value must be estimated for each solution by recording the absorbance of the solution (averaged over many radial positions) immediately after reaching the desired centrifugal speed. This value was compared with the original absorbance measured with a spectrophotometer to produce a conversion factor for the centrifuge. This introduces a further systematic error in calculating interaction coefficients. The only evidence we have that the procedure yields reasonable values is that the static concentrations compare favourably with those calculated by summing the concentrations (calculated from eqn. 4) of the monomer and tetramer (Table 6). Except for the highest concentration, there was the expected agreement within the errors between the two estimates of concentration. The disagreement between the first entries in Table 6 probably arose from a larger-than-usual negative baseline at the end of the experiment in this sector. Because of uncertainties in the correct procedure for estimating baselines for extended experiments, no absorbances were corrected for baselines.

Estimated interaction coefficients. The discussion so far has been concerned with origins and extents of errors in the estimated $L_{1,4}$; now it is possible to

consider the average values obtained for $L_{1,4}$. Table 6 contains the results for one experiment where a range of concentrations was examined at 20°C. The $L_{1,4}$ spread over a tenfold range in this experiment and the mean and standard deviation for the five solutions was $40.3 \pm 23.4 \text{ l}^3 \cdot \text{g}^{-3}$. This was the largest error encountered for the three temperatures studied, and, although we repeated the experiment at 20°C, we were unable to decrease this error. Lowering the temperature decreased both the association and the proportional errors (Table 7). From these three temperatures an enthalpy of association of between 45 and 60 kJ/mol was calculated. Increasing I from 0.12 to 0.22 had no significant effect on the association either at 20 or 6°C.

Apparent molecular weights. The centrifugation speeds used in all the equilibrium experiments were arranged to ensure that $c_b/c_m \leq 5$, and generally under these conditions no curvature could be detected statistically when fitting eqn. (1) to records (Fig. 3). From these analyses, apparent molecular weights were estimated assuming partial specific volumes of 0.74 ml/g (Edelhoch *et al.*, 1957). These results are given in Fig. 4 for an experiment carried out at 20°C. The continuous line in Fig. 4 is the expected dependence calculated from eqn. (13) [w_t is the total concentration of enzyme and M_1 has been assumed to be 27 500 (Sasaki *et al.*, 1970)].

$$\bar{M} = \frac{M_1 w_1 + 4M_1 L_{1,4} w_1^4}{w_t} \quad (13a)$$

$$L_{1,4} w_1^4 + w_1 = w_t \quad (13b)$$

The concentrations of monomer in the mixture are calculated from eqn. (13b) by solving the quartic by a Newton-Raphson procedure. Molecular weights estimated from eqn. (1) give only approximate weight-average molecular weights ($\bar{M}$). Nevertheless, the correspondence between the estimated values and those calculated from an $L_{1,4}$ value of $40.3 \text{ l}^3 \cdot \text{g}^{-3}$ is satisfactory (Fig. 4). This gave further confidence that the estimated interaction coefficient contained no obvious systematic errors. Curves similar to those shown in Fig. 4 were obtained for the other two temperatures. From these calculations it was found that the $\bar{M}$ value for a concentration of 10 g/l would be 104 182, and increasing $L_{1,4}$ by 1 s.d. (Table 7) to give

Table 6. Summary of $L_{1,4}$ estimates from a typical centrifugal equilibrium experiment at 20°C

The concentrations are from the static measurements on a Unicam SP.500 (SP.500) compared with those calculated from the sums of the absorbances (eqn. 4). Values are means $\pm$ s.d. for the numbers of estimates shown.

Concn. (mg/ml)		$L_{1,4}$ ($\text{l}^3 \cdot \text{g}^{-3}$)	No. of estimates
SP.500	Eqn. (4)		
0.38	0.29 ± 0.02	47.6 ± 18.9	6
0.26	0.26 ± 0.02	6.5 ± 0.3	4
0.17	0.17 ± 0.02	77.3 ± 20.8	7
0.14	0.17 ± 0.03	27.6 ± 13.7	5
0.11	0.11 ± 0.03	49.5 ± 20.9	4
Mean		40.3 ± 23.4	5

Table 7. Summary of estimates of association constants for phosphoglycerate mutase

Values are means $\pm$ s.d. with the numbers of replicate solutions in parentheses.

Temperature (°C)	$L_{1,4}$ ($\text{l}^3 \cdot \text{g}^{-3}$)	$10^{-14} \times K$ (l^3/mol)
20 (5)	40.3 ± 23.4	2.08 ± 1.23
10 (6)	16.6 ± 6.7	0.86 ± 0.34
6 (4)	7.1 ± 3.0	0.37 ± 0.15

Fig. 3. Typical regression of eqn. (1) to a record for phosphoglycerate mutase (0.23 mg/ml) running at 13880 rev./min. The fitted line is superimposed on the experimental points and from its slope (0.316 ± 0.022) an apparent mol.wt. of 28600 ± 1990 was calculated.

$63.71^3 \cdot \text{g}^{-3}$ increased $\bar{M}$ to 104850. This shows how insensitive $\bar{M}$ is to association at high concentrations.

Velocity experiments. The original estimate of the molecular weight of phosphoglycerate mutase was based on separate estimates of $s_{20,w}$ and $D_{20,w}$ at concentrations greater than 1.5 g/l. Sasaki *et al.* (1970) have confirmed these estimates of $s_{20,w}$ but quote lower molecular weights. As far as we are aware there are no estimates of weight-average $s_{20,w}$ ($\bar{s}_{20,w}$) either at high or low concentrations (the previous work estimated $s_{20,w}$ from the velocity of peaks in schlieren profiles). We estimated $\bar{s}_{20,w}$ at 0.35 g/l and 1 g/l, and Dr. Sasaki and Dr. Sugimoto generously gave us photographic records from their experiments made with high concentrations of phosphoglycerate mutase. These we re-measured and the results combined with ours and three values extracted from Fig. 1 of Edelhoch *et al.* (1957). These estimates for $\bar{s}_{20,w}$ covered a range of concentrations extending from 0.3 g/l to 17.4 g/l, so making it possible to estimate $L_{1,4}$, g and $s_{20,w}^0$.

Obtaining a separate estimate of $L_{1,4}$ is important in proving that the equilibrium results were not artifacts; the results from velocity experiments include a separate set of assumptions compared with the equilibrium analyses. The most important of these new assumptions is accurate compensation for viscosity and density of buffers and the assumption that the concentration-dependence of $\bar{s}_{20,w}$ (eqn. 10b) is the same for monomer as for tetramer. Examination of eqn. (10a) shows that two sedimentation constants are required to calculate $\bar{s}_{20,w}$ at any concentration, one for the tetramer (s_4^0) and another for the monomer (s_1^0). In the present analysis it is assumed that the shape of the monomer is the same as that

Fig. 4. Comparison between calculated apparent molecular weights ($\bar{M}_w$) and total concentration for phosphoglycerate mutase

The continuous line was calculated by assuming that $L_{1,4}$ was $40.31^3 \cdot \text{g}^{-3}$, and the dotted line was calculated after increasing $L_{1,4}$ by one standard deviation to $621^3 \cdot \text{g}^{-1}$. The two symbols refer to separate experiments.

of the tetramer; hence the two can be related by $s_1^0/s_4^0 = 0.25^{\frac{1}{3}}$. In principle it is possible to release constraints on both sedimentation constants, but for this to be successful it is necessary to obtain accurate measurements of $\bar{s}_{20,w}$ at concentrations less than 0.1 g/l. We have not been able to achieve this with the present equipment.

Analysing the data (Fig. 5) by minimizing the squared differences between calculated and experimental $\bar{s}_{20,w}$ (see analysis section) produced the following parameters: $L_{1,4} = 3111^3 \cdot \text{g}^{-1}$, $s_4^0 = 0.669$ ps and $g = 0.951$ /g. Values for the errors on these parameters were calculated, but, since they only reflect the deviations produced from the estimated $\bar{s}_{20,w}$ and not the uncertainties in these estimates, it was considered that they do not represent the true uncertainty in the estimated parameters. Instead, the analysis was re-run, analysing data produced by displacing each $\bar{s}_{20,w}$ by one standard error [given

Fig. 5. Estimation of interaction and hydrodynamic coefficients for phosphoglycerate mutase from velocity experiments

$s_{20,w}$ values were obtained from our experiments (○), measurements on records loaned by Dr. Sasaki and Dr. Sugimoto (△, results reported in Sasaki *et al.*, 1970), and from Fig. 1 in Edelhoch *et al.* (1957) (■) (the bars represent ± 1 s.d.). The solid line was calculated from the coefficients $L_{1,4} = 3111^3 \cdot \text{g}^{-3}$, $s_{20,w}^0 = 0.6695$ ps and $g = 0.951/\text{g}$. T = tetramer $s_{20,w}^0$, M = monomer $s_{20,w}^0$.

from the original measurement of the coefficients; the extracted values from Edelhoch *et al.* (1957) were assumed to be exact]. Increasing the values for the coefficients produced the following parameters: $L_{1,4} = 8931^3 \cdot \text{g}^{-3}$, $g = 0.811/\text{g}$ and $s_4^0 = 0.660$ ps, and decreasing the coefficients gave $L_{1,4} = 1421^3 \cdot \text{g}^{-3}$, $g = 0.981/\text{g}$ and $s_4^0 = 0.668$ ps. These analyses show that not only are the estimates of $L_{1,4}$ relatively sensitive to errors in $s_{20,w}$, but also the distribution of errors is non-gaussian. As expected, these alterations produced only small deviations in g and s_4^0 because the results that determine these values have been obtained at high precision. The fitted curve calculated from the median parameters portrays the data satisfactorily (Fig. 5). The s_1^0 calculated from the estimated s_4^0 was 0.266 ps, which closely agreed with the $s_{20,w}^0$ value of 0.27 ps measured by Sasaki *et al.* (1970) for fully dissociated phosphoglycerate mutase.

Discussion

X-ray crystallography has shown that phosphoglycerate mutase has a unit cell composed of four

similar subunits (Campbell *et al.*, 1972) bound by non-covalent interaction. Previous work (Edelhoch *et al.*, 1957; Sasaki *et al.*, 1970) showed that the mol.wt. of the tetramer is approx. 110000, but no information existed on the energetics of the binding between the subunits. The present work shows that dissociation of the tetramer mainly into monomers readily occurs at concentrations less than 0.1 g/100 ml. The value for the interaction coefficient at 20°C lies between 40 and $3101^3 \cdot \text{g}^{-3}$, with more data suggesting that the lower value is nearer the truth. From the equilibrium results an approximate enthalpy of association of 45–60 kJ/mol was calculated with a free energy at 20°C of approx. -46 kJ/mol ; the entropy of association was relatively constant over the three temperatures studied (approx. $300 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). Despite the large uncertainties in these estimates, the low sensitivity of association to changing temperature makes it tempting to suggest that interaction between the subunits is largely through hydrophobic interactions. The low sensitivity to changes in ionic strength supports this idea and suggests that ionic interactions are not very important in the self-association.

Evidence that suggests these conclusions may be erroneous come from the electrophoretic observations of Edelhoch *et al.* (1957), where five unseparated ionic species were observed with purified yeast phosphoglycerate mutase but not with the muscle enzyme. At the enzyme concentration used (1.7 g/l) dissociation would not have produced significant distortion of the boundaries, and, even then, if protein-protein interactions were the only reaction occurring in the solution, less than five boundaries would be produced. Should dissociation be accompanied by shifts in the pK of ionizable groups or in the number of bound buffer ions, then it would be possible for more boundaries to form. If the changes in charge were small, then the relatively small changes in ionic strength we used would not be sufficient to change $L_{1,4}$ significantly. The results of Edelhoch *et al.* (1957) do emphasize, however, that the differences between muscle and yeast phosphoglycerate mutase extend beyond differences in size and may also include ill-defined charge differences. It is noteworthy that Cascales & Grisolia (1966) found that increasing ionic strengths up to 1.0 changes the apparent reaction mechanism of phosphoglycerate mutase. This they interpret as being possibly through a change in conformation of the protein until it resembles that of the natural monomeric molecules.

At the beginning of the present study we were concerned that the results were produced by experimental perturbations imparted to the relatively low concentrations of macromolecule in the solutions. Confidence in the results returned when we were able to reproduce results of previous workers for oxyhaemoglobin. These tests showed that

relatively simple numerical procedures gave acceptable estimates of equilibrium constants from ultracentrifugal-equilibrium records of absorbance. Previous methods for estimating interaction constants are largely designed for analysing interference records gathered at concentrations higher than those used in the present work. The two most notable procedures [moment analysis by Roarke & Yphantis (1969) and that by Adams (1969), which includes virial coefficients in the analysis] use numerical differentiation in the analysis. With errors in the records we were able to achieve, neither of these methods gave satisfactory analyses. Thus despite the criticism that in the present analysis we used inexact equations, the results are shown to be trustworthy. Our velocity results with low concentrations recorded on schlieren photographs show that, despite the application of advanced measuring methods, we were working at the limits of the optics. Scanning schlieren (where the displacement of the light-beam is measured as a decrease in light-transmission caused by cut-off at the schlieren knife-edge) can work at these limits, but this introduces further uncertainties. These arise from a non-uniform decrease in light-intensities after passage through the solution (e.g. caused by light-scattering) compared with the solvent. Fresnel aberrations at the knife-edge may also be important, but these can be calculated and allowed for during the collection of the record.

Efforts have been made to show that the errors in the equilibrium runs were random, whereas their magnitude was increased by calculating a ratio containing a fourth power of the denominator. Previous experiments have shown that the major source of these errors is unevenly distributed dirt on the cell windows, and attempts to decrease this 'noise' below an absorbance of 0.02 have been unsuccessful. A further error arises from uncertainties in the radial dimensions, amounting to approx. $40\mu\text{m}$ (Spragg *et al.*, 1976), produced by erroneous location of the two reference slits and giving a systematic error in any scan. It is thought that this accounts for the displacement of the fitted curve for combined scans compared with records from one scan within the combination (Fig. 2). In general, these uncertainties combine to produce random errors but they are not thought to be the main reason for the systematic difference between the value of $L_{1,4}$ calculated from velocity runs compared with that obtained from equilibrium runs.

The oxyhaemoglobin results show that it is possible to obtain acceptable results from equilibrium runs, and mixing caused by density inversions across the column of liquid can be excluded. The major difference between the haemoglobin and phosphoglycerate mutase experiments was in the order of the reaction, where oxyhaemoglobin undergoes dimerization whereas phosphoglycerate mutase apparently

undergoes tetramerization. Hence, the 1.4% error found in the present study for the specific extinction coefficient would result in a 4.2% error in $L_{1,4}$. In addition, we did not correct for any bias introduced through non-trivial baselines, but previous work suggests that the maximal error this could introduce is within the standard deviations calculated in the present paper. Hence these errors alone cannot account for the difference between the two types of experiment. Systematic errors can occur in the velocity experiments through incorrect normalization of the sedimentation coefficient by allowing for density and viscosity of the buffer relative to water. Here, incorrect measurement of the temperature of the rotor by 0.1°C would produce a 1% error in $s_{20,w}$. We calibrated the measuring system before the experiment, but cannot guarantee the absolute measurement to better than 0.1°C while the rotor is running. Further, the analyses showed that an error of about 6% in $s_{20,w}$ at 0.3mg/ml shifted $L_{1,4}$ from 311 to $142\text{l}^3\cdot\text{g}^{-3}$, illustrating the sensitivity of $L_{1,4}$ to relatively small errors in $s_{20,w}$ at this concentration. Hence it seems likely that the differences reflect the combination of all these systematic errors.

Biologically, it is noteworthy that enzymes from different sources exist in different extents of polymerization, yet they perform the same catalytic reaction. Phosphoglycerate mutase is not unique in this respect, but it may be significant when discussing the role of the polymerizing reaction with respect to their activities to note that only the muscle and yeast enzymes (that is the naturally occurring multisubunit forms) require 2,3-diphosphoglycerate as a cofactor in their enzymic role. Since yeast phosphoglycerate mutase is active at concentrations where the concentration of tetramer is virtually zero ($80\mu\text{g/litre}$; Grisolia & Cascales, 1966), this would suggest that monomer is active, whereas the tetrameric form is produced in response to solvent-protein interactions. Calculations of the average concentration of phosphoglycerate mutase in yeast showed that it exceeded 1g/litre [data were taken for this from Rodwell *et al.* (1957), Grisolia & Joyce (1959), and Grisolia (1962)]. Hence it seems probable that in the cell most of the enzymic molecules are condensed as tetramers.

The present work was carried out in the absence of cofactor, and this may be important when relating the self-association to biological activity. The addition of a ligand to a multisubunit protein can produce marked changes in the interaction between the subunits. The most striking of these occurs when oxygen is added to deoxyhaemoglobin, changing the $L_{2,4}$ by orders of magnitude. In the centrifuge it is possible to detect dissociation of deoxyhaemoglobin, but, as shown in the present and other papers, it is readily measured for oxyhaemoglobin. If the cofactor plus 3-phosphoglycerate (the sub-

strate) does produce an active tetramer with phosphoglycerate mutase by changing the $L_{1,4}$, then its effect is the reverse of that found for haemoglobin reacting with O_2 . A further possibility is that the inorganic phosphate present in the buffers substitutes in part for the cofactor to produce a much weaker association than with organic phosphates. These effects remain to be explored in the future, but it is noteworthy that the sign of the enthalpy of association for phosphoglycerate mutase is opposite to that found for oxyhaemoglobin.

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