THE MOLECULAR WEIGHTS OF SOME CRYSTALLINE ENZYMES FROM MUSCLE AND YEAST

I. ALDOLASE AND D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

by

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The crystallization of aldolase and D-glyceraldehyde-3-phosphate (GAP) dehydrogenase from rabbit skeletal muscle[1,2], each in a yield of about 10% of the soluble muscle protein, led to studies of their physical properties[1,2,3], composition[4,5], and biosynthesis[6], of the relation of aldolase to myogen A[7], and the combination of the dehydrogenase with diphosphopyridine nucleotide (DPN)[8,9]. In addition, muscle GAP dehydrogenase has been extensively compared with GAP dehydrogenase crystallized from yeast[10,11], *e.g.*, with respect to composition[12], antigenic behavior[13], and interaction with DPN[14]. As part of this program we undertook to determine the molecular weights of these proteins; we have measured the sedimentation and diffusion constants of all three enzymes and the partial specific volumes of the two from muscle. Preliminary accounts of this work have been presented[15,16].

MATERIALS AND METHODS

Enzymes

Crystalline aldolase was prepared and recrystallized 3 to 6 times, as previously described[1]. We are indebted to Dr. T. BARANOWSKI for a sample of aldolase that was shown to have maximal activity and a sample of crystalline myogen A that had an aldolase activity 35% of the maximum[7].

Crystalline muscle GAP dehydrogenase was prepared and recrystallized several times by Dr. G. T. CORI[2], who generously provided several different samples.

Crystalline yeast GAP dehydrogenase, prepared and recrystallized several times by the method of WARBURG AND CHRISTIAN[10,13], was generously provided by Dr. E. G. KREBS.

Sedimentation constant

Sedimentation constant, s, was determined in the Spinco analytical ultracentrifuge with the procedure and calibrations which have been described in detail[17]. Most of the experiments, however, were carried out at low temperatures in order to minimize thermal denaturation of the enzyme proteins. The ultracentrifuge rotor and cell were chilled to 2 to 4° and the protein solution was introduced into the cell in the cold room. The rotor was then encased in a close-fitting zippered bag of felt about 1 cm thick (covered with waterproof plastic) and carried to the centrifuge. The interior of the vacuum chamber had also been cooled to about 2°. The chamber was opened and the rotor was attached while still in the felt bag, through which the thermocouple

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could be inserted for the initial temperature reading. The bag was then removed, the chamber closed and the run carried out in the usual manner, with the refrigerating system in operation to minimize heat loss by radiation. At the end of the run the bag was rapidly replaced before the final temperature was recorded. With this procedure, which minimized condensation as well as temperature change, it was felt that temperature control was essentially as satisfactory as in runs carried out at room temperature[17].

Concentrated enzyme solutions were dialyzed to remove ammonium sulfate and diluted so as to contain the desired concentrations of protein, neutral salt and buffer. To repress the primary charge effect[8], 0.5 M NaCl or KCl was added to a 2.5% protein solution and proportionately less was present with lower protein concentrations. The dehydrogenase solutions also contained phosphate buffer, $\Gamma/2 = 0.1$, pH = 7.4.

The sedimentation constant was computed as previous described[17] and was corrected in the usual way[18] for the viscosity and density of the solvent medium to give $s_{20,w}$, the sedimentation constant in water at 20°, in Svedberg units (1 $S = 1 \cdot 10^{-13}$ sec).

Diffusion constant

The diffusion constant, D, was measured at 2°, either in the electrophoresis cell as previously described[1] or in a CLAESSON type cell[19] (constructed by Mr. OTTO RETZLOFF in the Physics Department shops). Enzyme solutions containing about 1 g/100 ml were dialyzed against phosphate buffers, $\Gamma/2 = 0.1$ or 0.2, pH = 7.1. The results were computed from enlarged tracings of schlieren scanning photographs, according to the relations $D_A = A^2/4\pi t H_m^2$ and $D_\mu = \mu^2/2t$, where A is the area under the diffusion diagram in sq. cm, t is the time in seconds, H_m is the maximum height of the curve in cm, and μ is one-half the breadth of the curve, in cm, at the inflection point $H_\mu = H_m/\sqrt{e}$. The diffusion constant was corrected for the temperature and for the viscosity of the solvent medium to give $D_{20,w}$.

Partial specific volume

In order to compute partial specific volume, $\bar{V}$, the densities of protein solutions of known composition are required. The density gradient tube introduced by LINDERSTRØM-LANG AND LANZ[20] was found extremely satisfactory for these measurements, because only small amounts of protein are required and the observations can be conveniently carried out at low temperatures[15].

Kerosene-brombenzene gradients were prepared[20,21] in 500 ml graduated cylinders, with a density range of about 0.015 each. Several cylinders, needed to cover the desired density range, were placed in a water bath with glass walls, regulated to ± 0.01°. One such thermostat was operated at 20°; another, placed in a cold room, was set at 5°. The gradients were calibrated with standard KCl solutions, carefully prepared with calibrated apparatus, from recrystallized KCl. The densities of these solutions were measured pycnometrically and found to agree with published values[22]. Uniform sized droplets, about 1 mm³ in volume, were added to the gradient tubes from micropipets and the positions of the drops were determined with reference to the cylinder graduations.

Concentrated protein solutions were thoroughly dialyzed against the desired solvent. Weighed portions of these solutions were carefully dried to constant weight at 100° *in vacuo* over P_2O_5, with correction for salts when present, in order to determine the protein concentration. A series of solutions was prepared by dilution of the stock solution with the same solvent and the density of each solution was then measured in the appropriate gradient tube, with standard solutions present at the same time. The density of the protein solutions could be estimated within ± 0.00004 by careful interpolation.

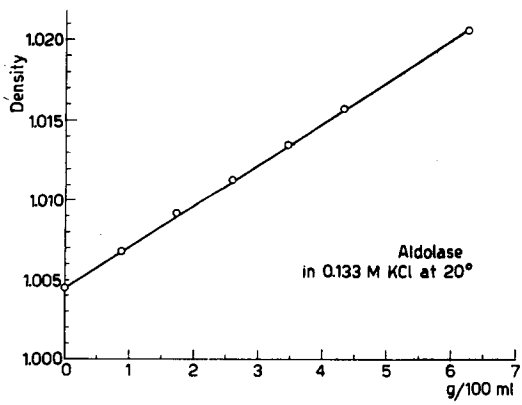

Fig. 1. Density of aldolase solutions, in 0.133 M KCl at 20°, as a linear function of aldolase concentration in g/100 ml.

The partial specific volume, $\bar{V}$, was computed from the slope of a plot of density, ϱ, against protein concentration, c. As shown in Fig. 1, such plots are linear, passing through the point representing $\varrho°$, the density of the solvent, at $c = 0$, with small random deviations. Therefore $\bar{V}$ can be calculated from the relation

$$\bar{V} = [1 - 100(\varrho - \varrho_0)/c]/\varrho_0$$

For the experiment plotted in Fig. 1, ϱ_0

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was 1.0045 and the density of an aldolase solution containing 6.28 g/100 ml was 1.02062, at 20°, from which $\bar{V}_{20}$ is found to be 0.740.

RESULTS AND DISCUSSION

Sedimentation

All samples of aldolase that were examined, including several with full enzymic activity and one sample of myogen A with "35%" aldolase activity, showed only one sharp, well defined peak in the ultracentrifugal schlieren diagram.

The values of $s_{20,w}$ obtained in four different series of determinations with aldolase are plotted against protein concentration, c, in Fig. 2. A few experiments performed before the details of operation had been well worked out, as well as occasional runs in which there was evidence of leakage from the cell, have been eliminated. It will be seen in Fig. 2 that the scatter of the results is appreciable. The dashed line in Fig. 2 represents the line of regression which has been fitted to all the data by the method of least squares. The constants of the equation, $s_{20,w} = s_{20,w}^{\circ} - k\,c$, for the regression line are given in Table I. The results of Experiment 43, carried out after much experience in the operation of the ultracentrifuge had been acquired,

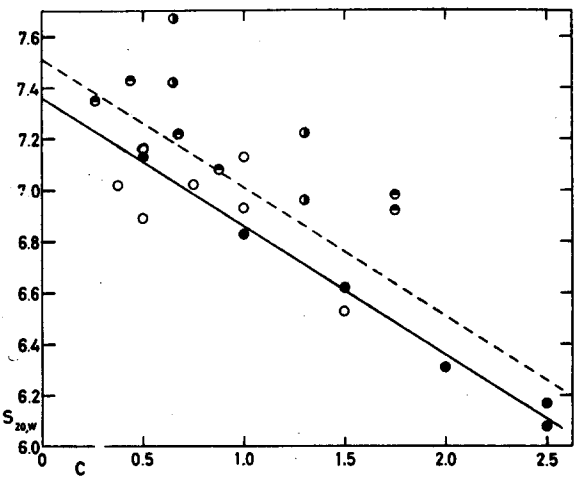

Fig. 2. Sedimentation constant, $s_{20,w}$ in Svedberg units, of aldolase and myogen A at different protein concentrations, c, in g/100 ml. Circles represent separate determinations. Aldolase ◑ Expt. 19, ◓ Expt. 22, ● Expt. 43; Myogen A (aldolase activity 35%) ○ Expt. 24. ----- line of regression fitted to all points; ——— line of regression fitted to Expt. 43. For equation and constants of lines see text and Table I.

TABLE I

SEDIMENTATION CONSTANTS OF CRYSTALLINE PROTEINS

Numerical constants for the general equation, $s_{20,w} = s_{20,w}^{\circ} + k\,c$, for the lines of regression calculated from the experimental data by the method of least squares.

Protein	*Number of runs*	$s_{20,w}^{\circ}$ *Svedberg units, S*	k	*Standard error of estimate*	*Correlation coefficient*
Aldolase					
Complete data	23	7.51	—0.50	0.07	—0.82
Experiment 43	6	7.35	—0.50	0.03	—0.995
GAP dehydrogenase					
Muscle	17	7.01	—0.38	0.06	—0.96
Yeast	11	6.80	—0.35	0.07	—0.95
Bovine plasma albumin[17]	14	4.32	—0.24	0.04	—0.96

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are believed to represent more nearly the best results that can be obtained with this instrument[17]. The solid line in Fig. 2 has been computed for these data only. It can be seen in Table I that the deviations are within satisfactory limits. The difference between the two values of $s_{20,w}^\circ$ is 0.16 S, about 2% of the value of $s_{20,w}^\circ$. Because the results of Experiment 43 are believed to be comparable with those obtained in the standardization of this ultracentrifuge with bovine plasma albumin[17] and with those reported below for other enzymes, we have adopted the value $s_{20,w}^\circ = 7.35\ S$, from this experiment for the calculation of the molecular weight of aldolase (see Table III).

One series of determinations (Experiment 24) was carried out with the sample of crystalline myogen A prepared by Dr. T. BARANOWSKI, which had an aldolase activity only 35% of that found for fully active aldolase. The results, included in Fig. 2, are in satisfactory agreement with the rest of the measurements. The aldolase used in experiments 19 and 22 was shown to have maximal enzymic activity; the sample used in experiment 43 was not tested but was prepared by the usual procedure and recrystallized several times.

GRALÉN[23] has previously reported a value of $s_{20,w} = 7.86\ S$ (not extrapolated to zero concentration) for a sample of myogen A crystallized by BARANOWSKI[24]; our value of $s_{20,w}^\circ$ for aldolase is about 7% lower. As a result of thorough examination of the entire experimental procedure, we have previously attributed this discrepancy, observed with other proteins[17], to a real difference in the method of estimation of rotor temperature. (The effect of adiabetic expansion of the rotor, discussed below, could account for only about one-third of the discrepancy.) All the values of $s_{20,w}$ reported in this paper have been measured by the procedure previously described for the calibration of our Spinco ultracentrifuge; our standard value of $s_{20,w}^\circ = 4.32\ S$ for bovine plasma albumin[17] is included in Table I.

GAP dehydrogenase, both from muscle and from yeast, showed only a single peak in the ultracentrifugal schlieren diagram. SHUGAR[25] has reported that his preparations of the muscle dehydrogenase showed two components in the ultracentrifuge, but he did not mention the rotor temperature. In preliminary experiments we found that samples of the muscle enzyme appear quite inhomogeneous in the ultracentrifuge at temperatures near 20° and that much of the protein precipitates during a run. KREBS[11] has also reported that the yeast enzyme appears to be homogeneous.

The values of $s_{20,w}$ obtained in several experiments with the two dehydrogenases are plotted against protein concentration in Fig. 3 A and B. The average rotor temperature was less than 10.5° in every run. (A few runs with obvious technical difficulties have been eliminated.) Table I summarizes the numerical constants of the equations of the regression lines, shown in Fig. 3, that have been fitted to the data. The standard error of estimate is approximately one per cent of the value of $s_{20,w}$ in each instance and the correlation appears to be satisfactory.

Although it appears probable that the sedimentation constant can be measured with a precision of about ± 1%, one source of uncertainty in the absolute value of $s_{20,w}^\circ$ must be mentioned. WAUGH AND YPHANTIS[26] have shown that the rotor temperature appears to fall slightly in the vacuum type ultracentrifuge when it is running at high speed, probably as the result of adiabatic expansion of the rotor. The true temperature during rotation is lower than the temperature interpolated

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from measurements of the resting rotor at the beginning and end of the run. The difference is thought to be 0.9 to 1.0° at a speed of about 60,000 r.p.m. which was used in all of our experiments. A correction for this effect would raise all of our values of $s_{20,w}$ by about 2.5%. The increase would bring the values of sedimentation constants measured with Spinco ultracentrifuges quite close to those recently determined with oil turbine ultracentrifuges but would not account for the larger differences that have been pointed out elsewhere[17]. (For a fuller discussion see EDSALL[27].) For example, as mentioned above, correction for this effect does not bring our value for $s_{20,w}^{\circ}$ of aldolase into agreement with GRALÉN's value for myogen A. Inasmuch as the sedimentation constants hitherto measured with vacuum-type ultracentrifuges have not been corrected for the effect of adiabatic expansion[27] and further work may be done on the precise magnitude under different conditions, we prefer to leave our results uncorrected until the time is ripe for the revision of published values and the establishment of a uniform procedure for future work.

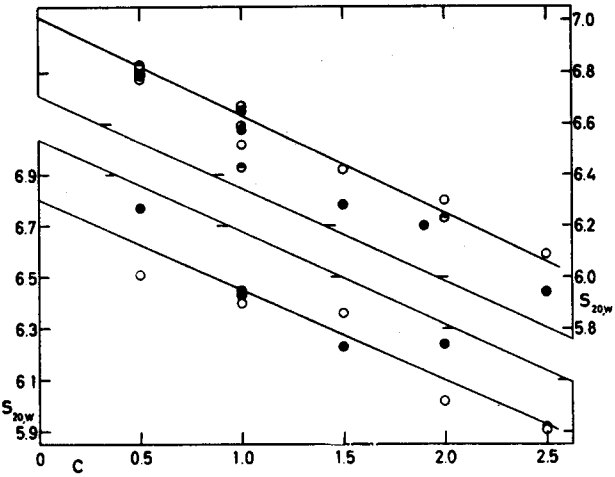

Fig. 3. Sedimentation constant, $s_{20,w}$ in Svedberg units of D-glyceraldehyde-3-phosphate dehydrogenases at different protein concentrations, c, in g/100 ml. For equation and constants of lines of regression see text and Table I. A Upper half. Muscle GAP dehydrogenase. ◑ Expt. 33, ◓ Expt. 35, ○ Expt. 37, ● Expt. 38. B. Lower half. Yeast GAP dehydrogenase. ● Expt. 42, ○ Expt. 44.

Diffusion

Two determinations of the diffusion constant of muscle GAP dehydrogenase were carried out. No turbidity was observed and the value of D remained essentially constant for 150 hours, except for a slight fall during the early part of one experiment; the observations during this period were rejected. The average values in water at 20° were for D_A 5.41, 5.65 and for D_μ 5.26, 5.51·10^{-7} cm^2 sec^{-1}; final average, $D_{20,w} = 5.46 \cdot 10^{-7}$ cm^2 sec^{-1}. One experiment was carried out with the yeast dehydrogenase. The value of D_A was 5.03, of D_μ 5.35; average value, $D_{20,w} = 5.19 \cdot 10^{-7}$ cm^2 sec^{-1}. The diffusion constant of aldolase was determined previously[1].

Partial specific volume

Values of $\bar{V}$, determined at 20° and 5° by the gradient tube method, for aldolase and muscle GAP dehydrogenase, are presented in Table II. The results of two determinations of $\bar{V}$ for bovine plasma albumin are also presented in order to show that results obtained by the present method agree satisfactorily with values of $\bar{V}$ determined by others[28,29].

In Table II there are also given values of $\bar{V}$ calculated from the amino acid

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TABLE II

PARTIAL SPECIFIC VOLUMES OF CRYSTALLINE PROTEINS

Protein	$\bar{V}_{20}$ *Observed*	$\bar{V}_{20}$ *Calculated*	$\bar{V}_5$	$\bar{V}_{20}-\bar{V}_5$
Aldolase	0.739		0.722	0.017
	0.746		0.729	0.017
	0.740			
Average	0.742	0.742		
Myogen A (GRALÉN[23])	0.735			
Muscle GAP dehydrogenase	0.740		0.729	0.011
	0.737			
Average	0.739	0.742*		
Bovine plasma albumin	0.728		0.713	0.013
	0.734		0.717	0.015
	0.734			
Average	0.732	0.734		
KOENIG[28]	0.730			
DAYHOFF *et al.*[29]	0.734**			

* Ignoring contribution of DPN.
** Determined at 25°.

composition of these three proteins[4,30] and the partial specific volumes of the amino acid residues[31,32] according to the relation[31]

$$\bar{V} = \frac{\sum w_i \bar{v}_i}{\sum w_i}$$

where the w_i's represent the weight fractions of the amino acid residues and the $\bar{v}_i$'s the partial specific volumes of the respective residues. The validity of this method has now been established for a number of proteins[32]. The agreement between the measured and calculated values for the two enzymes and albumin appears to be satisfactory.

$\bar{V}$ was not determined for yeast GAP dehydrogenase and cannot be calculated because the complete amino acid composition has not yet been reported[12].

The values of $\bar{V}$ at 5°, which are presented in Table II for reference, cannot be compared with other values because none have yet been noticed in the literature. The difference, $\bar{V}_{20}-\bar{V}_5$, is somewhat larger than that estimated by extrapolation from published values of $\bar{V}_{20}$ and $\bar{V}_{30}$[18].

Molecular weight

Table III summarizes the results of the measurements of $s_{20,w}°$, $D_{20,w}$ and $\bar{V}_{20}$ described above, together with the molecular weights of aldolase and the GAP dehydrogenases calculated from the relation[18]

$$M = \frac{RT\,s}{(1-\bar{V})\,D}$$

The molecular weight of aldolase differs slightly from that previously reported[15,16] as a result of slight differences in the extrapolation of $s_{20,w}$ to zero concentration.

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It will be noted that our value for the molecular weight of aldolase is quite close to GRALÉN's value for myogen A, in spite of the difference in $s_{20,w}$ previously mentioned. None of the values reported by GLIKINA AND FINOGENOV[33] are very close either to ours or GRALÉN's, however; no explanation for this discrepancy can be offered.

TABLE III

$s_{20,w}^{\circ}$, $D_{20,w}$, $\bar{V}_{20}$ AND MOLECULAR WEIGHTS OF CRYSTALLINE PROTEINS

Protein	$s_{20,w}^{\circ}$	$D_{20,w}$	$\bar{V}_{20}$	*Molecular weight*
Aldolase				
This investigation	7.35	4.63*	0.742	149,000
GLIKINA AND FINOGENOV[33]	8.27**	4.29	—	180,000
Myogen A GRALÉN[23]	7.86§	4.78	0.735	150,000
Muscle GAP dehydrogenase				
This investigation	7.01	5.46	0.741	120,000
DANDLIKER AND FOX[34]	—	—	—	140,000§§
Yeast GAP dehydrogenase	6.80	5.19	(0.74)***	122,000
Phosphoglucomutase				
This investigation, Part II	3.69	4.83	(0.75)***	74,000

* Previously reported[1].
** Extrapolation not indicated.
*** Assumed value.
§ Not extrapolated to zero concentration.
§§ Determined by light scattering method.

DANDLIKER AND FOX[34] have recently determined the molecular weight of muscle GAP dehydrogenase by means of the light-scattering method and have reported that the value is not less than 140,000. Correction for adiabatic expansion could raise our value to 123,000 while consideration of the fall in rate of diffusion, observed in one experiment at the start, could only lead to a smaller molecular weight. It was suggested[34] that the difference might have arisen from the use of ethylene diamine tetra-acetate which was added in their preparation of the enzyme. It may also be suggested, however, that a slight amount of aggregation of the enzyme protein at room temperature, which would tend to increase the molecular weight measured by light scattering, might not be entirely prevented by ethylenediaminetetraacetate, although the latter is known to enhance greatly the stability of the enzyme[35,*].

II. PHOSPHOGLUCOMUTASE

by

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Phosphoglucomutase was crystallized from rabbit skeletal muscle by NAJJAR[36] and some of its properties have been studied[36,37]. We have carried out preliminary measurements of the sedimentation and diffusion constants of this enzyme, from which its molecular weight has been calculated.

* We have now made preliminary measurements of the sedimentation constant of muscle GAP dehydrogenase prepared in 0.002 *M* ethylenediamine tetra-acetate. Duplicate values of $s_{20,w}$ determined at a concentration of 1 g protein/100 ml agree closely with the results shown in Fig. 3A.

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MATERIALS AND METHODS

The crystalline phosphoglucomutase was prepared and recrystallized by NAJJAR's method[36]. The determination of sedimentation and diffusion constants were carried out as described in Part I of this paper.

RESULTS AND DISCUSSION

Sedimentation

Phosphoglucomutase showed only a single peak in the ultracentrifugal schlieren diagram. The sedimentation constants obtained in seven runs at different protein concentrations are given in Table IV. The equation of the line of regression, fitted to the data by the method of least squares, is $s_{20,w} = 3.69 - 0.12\,c$. The correlation coefficient (—0.60) is low and the standard error of estimate (0.07) is somewhat high. A better fit could be obtained with 5 of the 7 points, giving a value of $s_{20,w}^{\circ}$ about 3.5% higher, but there is no apparent reason to reject the two low points. It must be concluded that these results establish the value of $s_{20,w}^{\circ}$ within only $\pm 2\%$.

TABLE IV

SEDIMENTATION CONSTANT OF PHOSPHOGLUCOMUTASE

Concentration of enzyme, g/100 ml	0.28	0.57	0.42	0.84	1.24	1.68	2.10
$s_{20,w}$, Svedberg units	3.73	3.76	3.53	3.41	3.60	3.49	3.43

Diffusion

One experiment was carried out, in the electrophoresis cell at 2°, with a 0.65% solution of the enzyme in acetate buffer, $\Gamma/2 = 0.2$, pH = 5.0. The values of D_μ calculated separately for the two limbs of the cell at different times were almost identical, yielding an average value of $D_{20,w} = 4.83 \cdot 10^{-7}$ cm^2 sec^{-1}.

Molecular weight

From these data the molecular weight is calculated to be 74,000, with the assumption that $\bar{V}_{20} = 0.75$. ($\bar{V}$ was not measured and the amino acid composition of the enzyme has not yet been determined.) This molecular weight is lower than the value 77,000 previously quoted[37,38] because the extrapolation of $s_{20,w}$ to zero concentration, discussed above, leads to a slightly lower value of $s_{20,w}^{\circ}$ than that previously estimated. Inasmuch as the value of $s_{20,w}^{\circ}$ may be uncertain by $\pm 2\%$ and the value of $\bar{V}$ was not determined, the molecular weight of phosphoglucomutase is probably uncertain by at least $\pm 5\%$ and should therefore be accepted and used with caution.

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SUMMARY

The molecular weights of four crystalline enzymes have been determined from measurements of the sedimentation constant in the Spinco ultracentrifuge, of the diffusion constant, and of the partial specific volume by a gradient tube method.

For three enzymes from rabbit skeletal muscle, the molecular weights are, of aldolase 149,000, of D-glyceraldehyde-3-phosphate dehydrogenase 120,000, and of phosphoglucomutase 74,000.

The molecular weight of D-glyceraldehyde-3-phosphate dehydrogenase from yeast is 122,000.

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