Dissociation of Native Octameric Brain Glutamine Synthetase to a Tetramer by Treatment with *N*-Acetylimidazole*

Sherwin Wilk, Alton Meister, and Rudy H. Haschemeyer

ABSTRACT: Native octameric glutamine synthetase from sheep brain ($s_{20,w}$ = 15 S) was partially acetylated when treated with the selective reagent *N*-acetylimidazole, and the products formed were studied in the analytical ultracentrifuge. A two-step dissociation process was observed in which a 9S component was formed first followed by formation of a 3.8S component. Enzymatic activity declined with progressive acetylation. The 9S component could be reassociated to the 15S form by increasing ionic strength. Approximately 12 to 16 acetyl groups per subunit were introduced as determined with 1-[^{14}C]-*N*-acetylimidazole. Incubation of the acetylated enzyme with hydroxylamine led to (a) reassociation of the 9S species to yield the octamer, and (b) the removal of about half of the acetyl groups. Acetylation in the presence of adenosine triphosphate (ATP) and Mg^{2+} did not lead to dissociation; however, subsequent removal of ATP and Mg^{2+} by gel filtration induced dissociation, although no 3.8S form appeared under these conditions. Addition of ATP and Mg^{2+} at concentrations equal to or greater than the K_m for ATP in the catalytic reaction led to reassociation. The sedimentation coefficients of the products formed are in excellent agreement with expected values for tetramer ($s_{20,w}$ = 9.0 S) and monomer ($s_{20,w}$ = 3.7 S). The two-step dissociation process is in accord with a model possessing D_4 symmetry.

The results suggest the participation of tyrosyl residues in the maintenance of the structural integrity of the enzyme and show that an additional degree of stabilization is conferred by nucleotide and metal ion. The data also indicate that the octamer is the active form of the enzyme.

Previous studies on the subunit structure of sheep brain glutamine synthetase have demonstrated that the native enzyme ($s_{20,w}$ = 15 S) is an octamer composed of apparently identical subunits (Ronzio *et al.*, 1969b; Haschemeyer, 1968; Wilk *et al.*, 1969). A model for the enzyme in which the subunits are arranged as two stacked tetrameric rings and which possesses D_4 symmetry has been proposed on the basis of electron microscope studies and considerations of symmetry (Haschemeyer, 1968, 1970).

In the presence of a number of perturbants the enzyme undergoes reversible dissociation to a new form ($s_{20,w}$ = 8.6 S), presumably a tetramer; such dissociation is prevented by ATP (or ADP) and Mg^{2+} (or Mn^{2+}) (Wilk *et al.*, 1969). Neither nucleotide nor metal ion was effective alone. Addition of nucleotide and metal ion to the 8.6S form results in reassociation to the octamer. It was impossible on the basis of previous findings to determine whether the presumed tetramer is enzymatically active because ATP and metal ion are required for determination of enzymatic activity. Moreover, identification of the 8.6S form as a tetramer by molecular weight determination was in some doubt because of continuing further dissociation during the several days required for attainment of equilibrium. In an attempt to circumvent these problems, a method of dissociation was sought in which introduction of blocking groups might lead to a time-independent, predominantly 8.6S form. Earlier work (Wilk *et al.*, 1969) revealed that reagents such as maleic anhydride and acetic anhydride dissociated the enzyme completely to an inactive, unfolded monomeric form. On the other hand, *N*-acetylimidazole has been shown by Riordan *et al.* (1965) to be a relatively selective acylating reagent, which preferentially acetylates exposed tyrosyl groups, although amino groups and cysteine sulfhydryl groups can also react. We have therefore investigated the effect of this reagent on glutamine synthetase. Treatment of the enzyme with *N*-acetylimidazole resulted in a stepwise dissociation of the 15S octamer to yield 9S and 3.8S forms; these values are in good agreement with the expected values for tetramer and monomer, respectively.

Experimental Section

Materials. Glutamine synthetase was isolated from sheep brain by the method of Ronzio *et al.* (1969b). The preparations used here exhibited specific activities in the range of 170–200 units/mg and were stored in dilute solution (0.1 mg/ml) in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA at 4° for periods not longer than 2 months. The enzyme was brought to the appropriate concentration by using the small volume ultrafiltration cell described by Bowers and Haschemeyer (1968) employing XM-50 or XM-100 membranes (Amicon Co., Cambridge, Mass.).

Benzene was dried over sodium and then fractionally distilled. *N*-Acetylimidazole (Cyclo Chemical Co.) was recrystallized from dry benzene and stored in a vacuum desiccator over P_2O_5. Adenosine 5′-triphosphate was obtained from Sigma Chemical Co., and 1-[^{14}C]-*N*-acetylimidazole was obtained from CalBiochem. Co. Sephadex G-50 was purchased from Pharmacia Chemicals. The gels were suspended in 0.01 M potassium phosphate buffer (pH 7.2) for

* From the Department of Biochemistry, Cornell University Medical College, New York, New York. *Received January 19, 1970.* Supported in part by the National Institutes of Health, U. S. Public Health Service, the John A. Hartford Foundation, and the National Science Foundation.

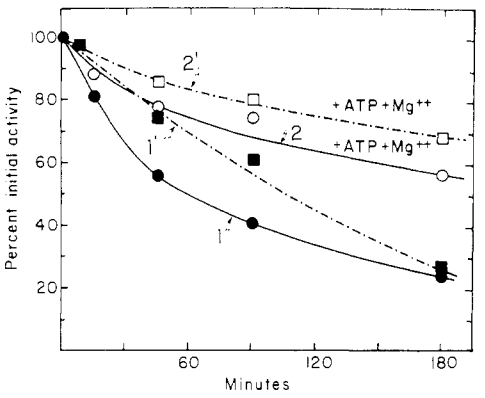

FIGURE 1: Inactivation of glutamine synthetase by *N*-acetylimidazole in the presence (open symbols) and absence (closed symbols) of ATP and Mg^{2+}. Curve 1: the enzyme (0.1 mg in 1.0 ml of 0.01 M Tris-HCl buffer (pH 7.5) containing 0.002 M EDTA) was treated with 1 mg of *N*-acetylimidazole; 25°. Curve 2: the conditions were the same as for curve 1, except that the enzyme was preincubated with 0.01 M ATP and 0.02 M $MgCl_2$ for 15 min at 37° prior to treatment with *N*-acetylimidazole. Curves 1′ and 2′: the conditions were the same as given for curves 1 and 2, respectively, except that 0.5 mg of *N*-acetylimidazole was used.

24 hr prior to use. Visking dialysis tubing was treated as previously described (Wilk *et al.*, 1969) in order to remove material exhibiting absorbance at 280 mμ.

Methods. Glutamine synthetase activity was determined by measuring the amount of inorganic phosphate formed in the synthesis of glutamine (Wellner and Meister, 1966).[1] Phosphate was determined by the method of Fiske and Subbarow (1925). Protein was determined by the method of Lowry *et al.* (1951) as previously described (Wilk *et al.*, 1969). Gel filtration was carried out as previously described (Wilk *et al.*, 1969). Treatment of the enzyme with *N*-acetylimidazole was performed after dialyzing the enzyme against 0.01 M Tris-HCl buffer (pH 7.5) containing 0.002 M EDTA. A solution containing 0.1–0.6 mg of protein in 0.5–1 ml of buffer was mixed with 1–5 mg of *N*-acetylimidazole, and the mixture was allowed to stand at 25° for 5 min–3 hr. The mixture was then placed on a 0.2 × 16 cm column of Sephadex G-50 and the peak which eluted in the void volume was examined in the analytical ultracentrifuge. The techniques used in the analytical ultracentrifugation studies were those previously described (Wilk *et al.*, 1969).

1-[^{14}C]-*N*-Acetylimidazole was diluted with freshly recrystallized *N*-acetylimidazole in dry benzene to give a product of final specific activity equal to 5 μCi/mg. The required amount of [^{14}C]-*N*-acetylimidazole was then transferred to a conical test tube and the benzene was removed *in vacuo* in a desiccator containing P_2O_5. The dry residue was mixed with 0.5 ml of enzyme solution containing 0.25 mg of protein. The reaction mixture was allowed to stand at 25° for 3 hr and the reaction was terminated by placing the mixture on a column of Sephadex G-50. The protein-bound radioactivity, cleanly separated from unreacted [^{14}C]-*N*-acetylimidazole, was determined by liquid scintillation counting.

TABLE I: Dissociation of Glutamine Synthetase by Treatment With *N*-Acetylimidazole.[a]

Experiment	% Undissociated	% 9S	% 3.8S
1 A Control (0.01 M Tris-HCl)	100	0	0
B After 5-min exposure to *N*-acetylimidazole	75	25	0
C After 2.5-hr exposure to *N*-acetylimidazole	20	40	40
2 A Control (0.05 M Tris-HCl)	100	0	0
B After 3-hr exposure to *N*-acetylimidazole	50	50	0
3 A Control (0.05 M borate)	100	0	0
B After 1.5-hr exposure to *N*-acetylimidazole	22	35	43

[a] In expt 1, 1 ml of solution containing 0.5 mg of enzyme in 0.01 M Tris-HCl buffer (pH 7.5) containing 0.002 M EDTA was treated with 1 mg of *N*-acetylimidazole. The same conditions were used in expt 2, except that the concentration of Tris was 0.05 M. In expt 3, 1 ml of solution containing 0.6 mg of enzyme in 0.05 M sodium borate buffer (pH 7.5) was used.

Results

When glutamine synthetase was mixed with *N*-acetylimidazole there was a progressive loss of enzymatic activity (Figure 1). The degree of inactivation[2] increased when the concentration of *N*-acetylimidazole was increased from 0.5 to 1 mg per ml. When the enzyme was preincubated with ATP and Mg^{2+} and then treated with *N*-acetylimidazole much less activity was lost.

When the treated enzyme was subjected to gel filtration and then examined in the analytical ultracentrifuge, it was found to be partially dissociated. The degree of dissociation increased with further exposure to *N*-acetylimidazole (Table I, expt 1). The pattern of dissociation was such that a 9S component was formed first; continued treatment with *N*-acetylimidazole led to formation of a 3.8S component. Riordan *et al.* (1965) have reported that the presence of 0.05 M Tris buffer reduced the acetylation of carboxypeptidase by *N*-acetylimidazole as compared with the results obtained in 0.01 M Tris buffer or borate buffer; a similar effect was observed in the present studies. Thus, less dissociation was observed in the presence of 0.05 M Tris buffer than with 0.01 M Tris buffer (Table I). The ultraviolet-scanning trace obtained in studies on the treated enzyme exhibited a positive slope in the plateau region separating the 15S and 9S forms indicating interaction between these compo-

[1] The usual assay method based on the synthesis of γ-glutamylhydroxamate was not used because hydroxylamine can react with *N*-acetylimidazole to yield acetylhydroxamate; furthermore, hydroxylamine readily deacylates acetyltyrosyl residues (Riordan *et al.*, 1965).

[2] This terminology is used rather than "rate of inactivation" since the observed kinetic behavior is complicated by the continual hydrolysis of *N*-acetylimidazole. Thus, if continual additions of the reagent are made, total inactivation results.

TABLE II: Reassociation of Acetylated Glutamine Synthetase.[a]

Experiment	% Undissociated	% 9 S	% 3.8 S
1 A Acetylated enzyme in 0.01 M phosphate buffer (pH 7.2)	23	77	0
B After dialysis *vs.* same buffer containing 0.15 M KCl	90	10	0
2 A Acetylated enzyme in 0.01 M phosphate buffer (pH 7.2)	25	69	6
B After dialysis *vs.* 0.05 M phosphate buffer (pH 7.2)	86	12	2
3 A Acetylated enzyme	22	35	43
B After incubation with NH_2OH	50	14	36
4 A Acetylated enzyme	12	50	38
B After incubation with NH_2OH	42	29	29

[a] In expt 1 and 2, 1 mg of acetylated enzyme in 1 ml of 0.01 M potassium phosphate buffer (pH 7.2) was dialyzed against 0.01 M buffer containing 0.15 M KCl, and against 0.05 M buffer, respectively. In expt 3, the enzyme obtained in expt 3 (Table I) was incubated with 0.125 M NH_2OH for 5 hr at 25°. In expt 4, 0.5 mg of enzyme in 0.5 ml of 0.01 M Tris-HCl buffer (pH 7.5) containing 0.002 M EDTA was treated with 2 mg of *N*-acetylimidazole for 3 hr at 25°; then, (expt 4 B) the acetylated enzyme was incubated with 0.125 M NH_2OH for 30 min at 37°.

TABLE III: Effect of ATP and Mg^{2+} on Acetylated Glutamine Synthetase.[a]

Experiment	% Undissociated	% 9 S	% 3.8 S
1 A Acetylated enzyme	12	63	25
B Above incubated with 0.01 M ATP and 0.02 M Mg^{2+}	60	0	40
2 A Acetylated enzyme	22	78	0
B Above incubated with 0.001 M ATP and 0.002 M Mg^{2+}	64	14	22
C Enzyme (2 A) incubated with 0.0001 M ATP and 0.0002 M Mg^{2+}	16	58	26
3 A Acetylated enzyme	22	61	17
B Above incubated with 10×10^{-6} M ATP and 0.002 M Mn^{2+}	77	6	17
4 A Enzyme + 0.01 M ATP and 0.02 M Mg^{2+}; + 1 mg of *N*-acetylimidazole	100	0	0
B Above mixture passed through Sephadex G-50	22	78	0

[a] In expt 1–3 the incubations with ATP and $MgCl_2$ (or $MnCl_2$) were carried out for 15 min at 37°. In expt 4 A, the enzyme (0.6 mg in 0.8 ml of 0.01 M Tris-HCl buffer (pH 7.5) containing 0.002 M EDTA) was incubated with ATP and $MgCl_2$ for 15 min at 37° and then treated with 1 mg of *N*-acetylimidazole for 3 hr at 25°; this solution was examined in the ultracentrifuge at 294 mμ.

nents. When the ionic strength of the solution was increased by addition of KCl or by increasing the concentration of potassium phosphate buffer, reassociation of the 15S from the 9S component occurred (Table II, expt 1 and 2).

When the acetylated enzyme was incubated with hydroxylamine there was a substantial increase in the amount of undissociated enzyme and a corresponding decrease in the percentage of the 9S component. The amount of 3.8S component decreased to a very small extent under these conditions (Table II, expt 3 and 4). When the acetylated enzyme was incubated with 0.01 M ATP and 0.02 M Mg^{2+} the amount of the 15S component increased at the expense of the 9S component (Table III, expt 1). About 50% reassociation occurred at a concentration of ATP (0.001 M) not far from the K_m value for ATP in the catalytic reaction (1.1×10^{-3} M); see Table III, expt 2B. When a concentration of ATP that was considerably below the K_m value was used, reassociation was not observed (Table III, expt 2C); indeed, under these conditions the amount of 3.8S material increased during incubation suggesting that the acetylated enzyme is relatively labile. When Mn^{2+} was substituted for Mg^{2+} a much lower concentration of ATP was capable of promoting reassociation (Table III, expt 3).

When the enzyme was preincubated with 0.01 M ATP and 0.02 M Mg^{2+} followed by reaction with *N*-acetylimidazole, no dissociation was observed when the reaction mixture was immediately examined in the ultracentrifuge (Table III, expt 4 A). (In these studies, the ultraviolet-scanning system was used at 294 mμ as previously described (Wilk *et al.*, 1969)). When the reaction mixture was freed of nucleotide by passing it through a column of Sephadex G-50, the protein found in the void volume of the column was 22% undissociated and the remainder was present as 9S material; no 3.8S component was observed (Table III, expt 4 B). When the enzyme was preincubated with ATP and Mg^{2+} and then treated with relatively large amounts of *N*-acetylimidazole (5 mg/ml), extensive precipitation of the protein was observed. After this reaction mixture was freed of nucleotide by gel filtration, the usual pattern of dissociation, *i.e.*, a mixture of 15S, 9S, and 3.8S components, was found.

Experiments were carried out with [^{14}C]-*N*-acetylimidazole in order to determine the extent of acetylation of the enzyme;

TABLE IV: Experiments with [^{14}C]-*N*-Acetylimidazole.[a]

Expt	Conditions	Moles of ^{14}C/60,000 g of Enzyme with *N*-Acetylimidazole: 1 mg	0.5 mg	0.25 mg
1	Enzyme	16	12	13
2	Enzyme + ATP + Mg^{2+}	14	4	5
3	Expt 1 + NH_2OH	8.5	6.5	5

[a] The enzyme (0.5 mg in 0.5 ml of 0.01 M Tris-HCl buffer (pH 7.5) containing 0.002 M EDTA) was treated with the amount of [^{14}C]-*N*-acetylimidazole indicated for 3 hr at 25° and then subjected to gel filtration as described under methods. In expt 2, the enzyme was incubated with 0.01 M ATP and 0.02 M $MgCl_2$ for 15 min at 37° prior to treatment with [^{14}C]-*N*-acetylimidazole. In expt 3, acetylated enzyme (after gel filtration) was incubated with 0.125 M NH_2OH for 30 min at 37° and then subjected again to gel filtration.

the results (Table IV) are expressed in moles of ^{14}C/60,000 g of enzyme. In these studies the enzyme was treated with varying amounts of [^{14}C]-*N*-acetylimidazole for 3 hr at 25° and the solution was subjected to gel filtration; the bound radioactivity was then determined. Under these conditions, 12–16 moles of ^{14}C was bound per 60,000 g of enzyme. Similar studies carried out after brief preincubation with ATP and Mg^{2+} revealed substantially lower amounts of ^{14}C bound in the experiments with 0.5 and 0.25 mg of *N*-acetylimidazole. When the enzyme acetylated in the absence of ATP and Mg^{2+} was then treated with hydroxylamine, about half of the bound radioactivity was released.

Discussion

The present studies extend the earlier work (Wilk *et al.*, 1969) in which it was shown that the octameric enzyme undergoes reversible dissociation to a form (presumably a tetramer) exhibiting a sedimentation coefficient of 8.6 S. Thus, treatment of the octamer with *N*-acetylimidazole yields a species exhibiting a sedimentation coefficient of 9 S, a value which is in excellent agreement with the "theoretical" sedimentation coefficient (9 S) for a tetramer (Wilk *et al.*, 1969); similarly, the component exhibiting a sedimentation coefficient of 3.8 S agrees closely with the "theoretical" value (3.7 S) for the monomer. The observed two-step dissociation (15 S → 9 S → 3.8 S) is entirely consistent with the model proposed previously (Haschemeyer, 1968; Wilk *et al.*, 1969) according to which the native octameric enzyme is composed of identical subunits arranged in a structure possessing D_4 symmetry. Thus, the more sensitive isologous bonds are cleaved first to yield a tetramer; the heterologously bonded tetramer, which has four identical bonding sets, yields only the monomer on further dissociation. Most of the monomer formed is not in equilibrium with the tetrameric form, presumably because of irreversible chemical modification of the heterologous bonding sets.

Although the present data indicate some lack of selectivity in the action of *N*-acetylimidazole on the enzyme, this reagent is evidently much more specific than acetic anhydride which (at an equimolar concentration) gave only an unfolded inactive monomer. It is notable that when the *N*-acetylimidazole-treated enzyme was incubated with hydroxylamine there was restoration of the octameric structure and release of about half of the acetyl groups. Previous studies indicate the presence of only a single reactive sulfhydryl group per subunit of glutamine synthetase (Ronzio *et al.*, 1969b), and modification of this group does not lead to dissociation (S. Wilk, unpublished data, 1969). It therefore seems most likely at this time that the effect observed here is the result of stabilization of the octamer by tyrosyl residues.

It is also apparent that ATP and Mg^{2+} serve to stabilize the octamer. Thus, enzyme preincubated with ATP and Mg^{2+} did not dissociate when treated with *N*-acetylimidazole (despite the occurrence of acetylation; Table IV); after gel filtration to remove ATP and Mg^{2+}, dissociation did occur. Furthermore, addition of ATP and Mg^{2+} to the tetrameric form in the present studies and in the earlier work (Wilk *et al.*, 1969) induced association. Reassociation of the 9S component was observed with 10 μM ATP in the presence of Mn^{2+} (Table III), a result of particular interest since under these conditions only 8 moles of ATP is bound to the enzyme (Ronzio *et al.*, 1969a). Thus, it seems probable that the same nucleotide involved in enzymatic activity is also capable of stabilizing the octamer, and it would appear that the octamer is the enzymatically active form of the enzyme.

When the enzyme was preincubated with ATP and Mg^{2+} and then treated with *N*-acetylimidazole, no dissociation occurred (Table III), expt 4 A); when this was followed by removal of ATP and Mg^{2+} by gel filtration, dissociation proceeded only to the 9S stage suggesting that ATP and Mg^{2+} prevent formation of the 9S form and acetylation of critical residues required for stability. This conclusion is also consistent with the lower incorporation of ^{14}C from [^{14}C]-*N*-acetylimidazole into the enzyme in the presence of ATP and Mg^{2+} (Table IV). The findings indicate that the binding of ATP and Mg^{2+} to the enzyme is sufficient to overcome the loss of intersubunit bonding associated with acetylation. Thus, a degree of stability is conferred upon the octamer by nucleotide and metal ion quite apart from the role of tyrosyl groups in maintaining structural integrity. The studies reported here differ significantly from our earlier work in that dissociation was produced by chemical modification and reversed merely by removal of blocking groups upon incubation with hydroxylamine. It may be anticipated that chemical modification of this type may selectively interfere with specific bonding sets in other oligomeric proteins and thus will provide a useful adjunct to other procedures for deciding between alternate symmetry models.

References

Bowers, W. F., and Haschemeyer, R. H. (1968), *Anal. Biochem. 25*, 549.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Haschemeyer, R. H. (1968), *Trans. N. Y. Acad. Sci. 30*, 875.

Haschemeyer, R. H. (1970), *Advan. Enzymol. 34* (in press).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall,

R. J. (1951), *J. Biol. Chem. 193*, 265.
Riordan, J. F., Wacker, W. E. C., and Vallee, B. L. (1965), *Biochemistry 4*, 1758.
Ronzio, R. A., Rowe, W. B., and Meister, A. (1969a), *Biochemistry 8*, 1066.
Ronzio, R. A., Rowe, W. B., Wilk, S., and Meister, A. (1969b), *Biochemistry 8*, 2670.
Wellner, V. P., and Meister, A. (1966), *Biochemistry 5*, 872.
Wilk, S., Meister, A., and Haschemeyer, R. H. (1969), *Biochemistry 8*, 3168.

Yeast Pyruvate Kinase. Native and Subunit Molecular Weight*

R. T. Kuczenski† and C. H. Suelter‡

ABSTRACT: Yeast pyruvate kinase was shown to have a molecular weight of 162,000 to 168,000 as calculated with the Svedberg equation from data obtained under two sets of solvent conditions given below, and by the high-speed equilibrium method at low protein concentration. The enzyme sediments as a single symmetrical peak with an $s^0_{20,w}$ of 8.85 S and a $D^0_{20,w}$ of 4.84×10^{-7} cm^2 sec^{-1} in 0.1 M tetramethylammonium cacodylate buffer, pH 6.2, containing 0.1 M KCl, 2.6×10^{-2} M $MgCl_2$, and 10^{-3} M fructose 1,6-diphosphate. In 0.1 Tris·HCl, pH 7.5, the values obtained are 8.34 S for $s^0_{20,w}$, and 4.52×10^{-7} cm^2 sec^{-1} for $D^0_{20,w}$.

The enzyme was shown to be a tetramer, each polypeptide chain having a molecular weight of 42,000 to 45,000. Complete dissociation was obtained in 6 M guanidine hydrochloride–0.15 M 2-mercaptoethanol. Utilizing sedimentation equilibrium under these solvent conditions, similar values for $M_w{}^0$ and $M_z{}^0$ were obtained indicating the subunits had approximately equal molecular weights.

Dissociation was also obtained by extensive treatment of the enzyme with maleic anhydride, resulting in a symmetrically sedimenting peak with $M_w{}^0(s/D)$ of 42,200 excluding bound maleyl groups. Some physical characteristics of yeast, rabbit muscle, and rat liver pyruvate kinases are compared.

Investigations in this laboratory have been directed toward a clearer understanding of the conformational changes of yeast and muscle pyruvate kinase associated with the binding of substrates and effectors. Fundamental for an analysis of such changes is a characterization of the molecular weight of the enzymes and determination of the size and number of subunits associated therewith. We report the results of an investigation of the native and subunit molecular weights of yeast pyruvate kinase. These results are compared with those obtained with other preparations.

Methods

Preparation of Enzyme. Pyruvate kinase was isolated from fresh "Budweiser" Baker's yeast (Anheuser-Busch, Inc.) according to the procedure of Hunsley and Suelter (1969a) and stock enzyme was stored as a suspension in 90% saturated (3.6 M) $(NH_4)_2SO_4$. Protein concentrations were estimated from the absorbance at 280 nm ($E^{0.1\%}_{1\ cm}$ 0.653) (Hunsley and Suelter, 1969a). Kinetic assays were performed under the conditions and with reagents described by Hunsley and Suelter (1969b). All enzyme preparations had a minimum specific activity of 210 μmoles/min per mg at 30°. Stock enzyme was equilibrated with appropriate buffers by chromatography on Sephadex G-25 (coarse). Aliquots of the protein were tested for complete removal of $(NH_4)_2SO_4$ with saturated $BaCl_2$. Unless otherwise noted, the enzyme, after passage over Sephadex, was allowed to stand at room temperature for at least 3 hr before initiation of ultracentrifugal studies (Kuczenski and Suelter, 1970).

Ultracentrifugal Analysis. A Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics and an RTIC unit was used for all sedimentation experiments. Sedimentation velocity experiments were run at 59,780 rpm near 20°. Diffusion coefficient experiments were performed in double-sector synthetic boundary cells at 4908 rpm at 20° and the coefficients were calculated using height-to-area analysis (Schachman, 1957).

A molecular weight for native enzyme was also determined using the meniscus depletion technique of Yphantis (1964). Runs were performed at 20° with a rotor speed of 15,200 rpm using Rayleigh interference optics and the six-channel Kel-F centerpiece designed by Yphantis (1964). Rayleigh patterns were recorded on Kodak 11-G photographic plates.

The short-column sedimentation equilibrium technique of Van Holde and Baldwin (1958) was used for molecular weight determinations in 6 M guanidine hydrochloride. These experiments were run for 30 hr (results remained constant from 30 to 40 hr) near 20° with a solution column depth of 1.7 mm. Enzyme for these experiments was prepared by extensive dialysis (48 hr) against the appropriate guanidine

* From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823. *Received January 20, 1970.* Supported in part by Grant GB-7780, National Science Foundation, and the Michigan State Agricultural Experiment Station, Michigan State Journal No. 4767.

† National Institutes of Health Predoctoral Fellow (5 F01 GM-37, 540-02).

‡ Research Career Development Awardee 1-K3-GM-9725 of the National Institutes of Health.