Paik, W. K., and Benoiton, L. (1963), *Can. J. Biochem. Physiol. 41*, 1643.
Paik, W. K., Bloch-Frankenthal, L., Birnbaum, S. M., Winitz, M., and Greenstein, J. P. (1957), *Arch. Biochem. Biophys. 69*, 56.
Smith, E. L., and Hill, R. L. (1960), *Enzymes 4*, 37.
Theodoropoulos, D. (1958), *J. Org. Chem. 23*, 140.
Troll, W., and Cannan, R. K. (1953), *J. Biol. Chem. 200*, 803.
Work, E. (1957), *Biochem. J. 67*, 416.

Metabolic Control and Structure of Glycolytic Enzymes. III. Dissociation and Subunit Structure of Rabbit Muscle Pyruvate Kinase*

M. A. Steinmetz† and W. C. Deal, Jr.

ABSTRACT: Pyruvate kinase (mol wt 237,000) has been found to be a tetramer, each polypeptide chain having mol wt 57,200. The data further suggest that pyruvate kinase consists of two identical catalytic particles, or protomers (mol wt 115,000 each), with each protomer in turn consisting of two polypeptide chains (mol wt 57,000 each). With only one site each for Mn^{2+} and phosphoenolpyruvate per protomer, the two polypeptide chains within a protomer probably are not identical, despite their similarity in mass. Structural studies as a function of urea concentration revealed intermediates with $s^{0,6\%}_{20,w}$ = 7.3 S in 1.5 M urea, and 3.6 S in 3 M urea.

Complete dissociation ($s^{0,6\%}_{20,w}$ = 1.8 S) of the enzyme occurred in 4 M urea alone, indicating noncovalent subunit bonding. Characterization of the subunits in 7.4 M urea yielded values of $s^0_{20,w}$ = 2.01 S, $D^0_{20,w}$ = 3.46 × 10^{-7} cm^2/sec, and $M^0_w(s/D)$ = 56,300. Detailed subunit molecular weight analyses utilized sedimentation equilibrium in three separate dissociating systems; namely, (1) 7.4 M urea, (2) 7.4 M urea–0.12 M β-mercaptoethanol, and (3) 6.8 M guanidine–0.12 M β-mercaptoethanol. In the latter two systems, the subunits were more stable and M^0_w and M^0_z were essentially the same, indicating that the subunits had approximately equal molecular weights.

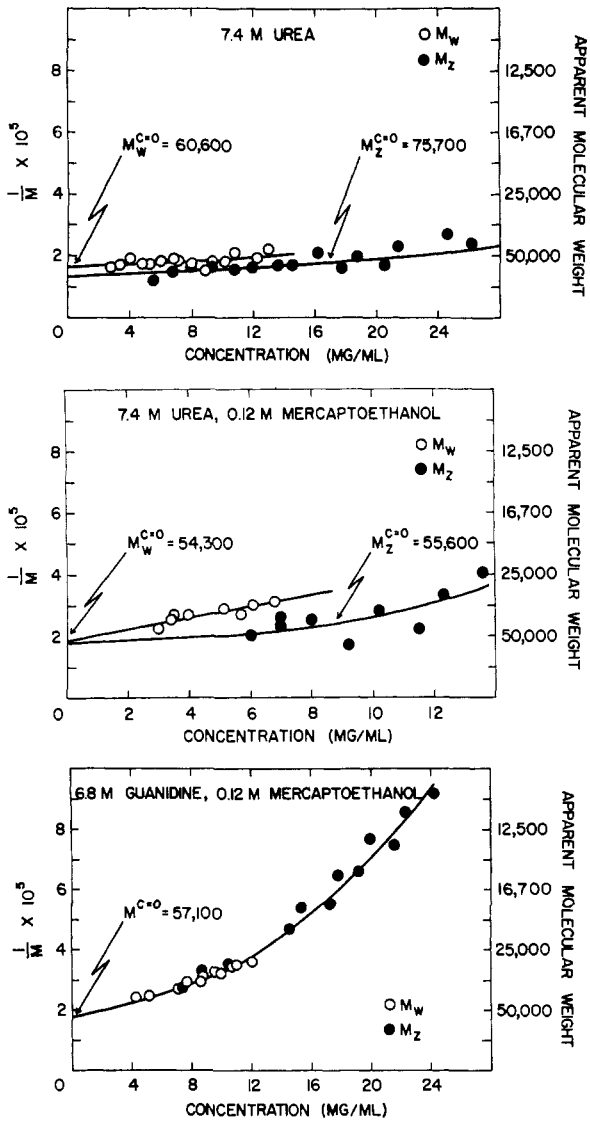
Recent years have seen the accumulation of a considerable amount of evidence indicating the presence of several polypeptide chains in the structure of many enzymes, especially in those enzymes with mol wt 65,000 or greater. Although most of the glycolytic enzymes of rabbit skeletal muscle are large enough that they would be expected to consist of several subunits, direct physical measurements of the subunits of only one such enzyme had been reported (Deal and Van Holde, 1962; Deal *et al.*, 1963b; Stellwagen and Schachman, 1962) when this study was undertaken. It seemed clear that a knowledge of subunit structure was a prerequisite for elucidation of the relationship between structure and function of these glycolytic enzymes. Because glycolysis plays such a central role in metabolism and because so little was known about the subunit structure of the glycolytic enzymes, this laboratory embarked on a program designed to ultimately provide an analysis of the subunit structure and control of all the glycolytic enzymes from a single source. Since enzymes are known to often differ from species to species and from tissue to tissue, such a restriction seemed desirable in order to be able to correlate all the results into a complete picture of glycolysis. We chose to focus mainly on the enzymes from rabbit skeletal muscle.

Our attention was drawn to pyruvate kinase because of its large size [mol wt 237,000 (Warner, 1958)] and because the enzyme had been reported to bind 2 moles of PEP[1]/mole of the native enzyme (Reynard *et al.*, 1961). Recently, it has also been shown that 2 Mn^{2+} ions are bound/mole of enzyme (Mildvan and Cohn,

* From the Department of Biochemistry, Michigan State University, East Lansing, Michigan. *Received December 3, 1965.* Supported by grants from the Division of General Medical Sciences, National Institutes of Health, U. S. Public Health Service (GM-11170), and the U. S. Department of Agriculture through the Michigan Agricultural Experiment Station (932). Papers I and II in this series are, respectively, Deal *et al.* (1963a and 1963b).

† Predoctoral Fellow (5-F1-GM-22, 851-02) of the Division of General Medical Sciences, National Institutes of Health.

[1] Abbreviations: for $D^0_{20,w}$, $s^0_{20,w}$, M^0_w, M^0_z, and $M^0_w(s/D)$, the superscript, ^{0}, indicates that the quantity has been extrapolated to zero protein concentration; $M_w(s/D)$, weight-average molecular weight determined from the Svedberg equation; M_w, weight-average molecular weight; M_z, z-average molecular weight; PEP, phosphoenolpyruvate; C_0, initial protein concentration; $(C_m + C_b)/2$, average of the protein concentration at the meniscus, and bottom of the solution; rtic, rotor temperature indicator and control; ATP, adenosine triphosphate.



FIGURES 1–3: Extrapolation of the apparent weight-average (M_w^{app}) and apparent z-average (M_z^{app}) molecular weights to 0 pyruvate kinase concentration. Concentrations were evaluated as $(C_m + C_b)/2$ for M_w^{app} and $(C_m + C_b)$ for M_z^{app}. In addition to the dissociating agents listed, the solvent systems also contained 0.04 M Tris (HCl), 0.15 M NaCl, 0.001 M EDTA, and the pH was 8.0 (at 25°). The temperature for all studies was about 7°. Equilibrium centrifugation of the enzyme in 7.4 M urea solutions was carried out at 13,410 rpm while that in 6.8 M guanidine–HCl required a speed of 20,410 rpm.

1965). Pyruvate kinase has been well characterized and its properties are discussed in an excellent review by Boyer (1962). Morawiecki (1960) reported that pyruvate kinase dissociated into subunits of mol wt 150,000 in 6 M urea and concluded that the enzyme consisted of at least two polypeptide chains. However, it is part of our working hypothesis that most polypeptide chains will have mol wt <65,000. Thus, it seemed to us that the pyruvate kinase complex might consist of subunits smaller than 150,000 mol wt, which might simply require more stringent conditions to produce dissociation into ultimate subunits. Smaller subunits have now indeed been found to exist. This paper presents the results of a detailed study on the conditions for dissociation as well as a characterization of the properties of the subunits.

Results

The sedimentation equilibrium technique was used to provide the most precise determination of molecular weight values. The apparent molecular weights were extrapolated to 0 protein concentration to eliminate the effects of protein interactions upon the calculated molecular weights. All phases of the experiments were conducted at about 5°.

Dissociation in Urea. Initial attempts to dissociate the enzyme into small subunits involved a series of short column sedimentation equilibrium experiments utilizing 7.4 M urea as the dissociation solvent. The results shown in Figure 1 extrapolated[2] to values of 60,600 and 75,700 for the weight-average (M_w^0) and z-average (M_z^0) molecular weights, respectively. This result proved that rabbit skeletal muscle pyruvate kinase consisted of subunits smaller than 150,000 mol wt, and probably contained 4 subunits of about 60,600 mol wt. However, the slight discrepancy in weight- and z-average molecular weights needed clarification since this could have been caused by either of two possibilities; namely, (1) aggregation, or (2) subunits of different molecular weights. Furthermore, the possibility of even smaller subunits, covalently linked by disulfide bonds, needed to be tested.

Dissociation in Urea and β-Mercaptoethanol. The dual need for a dissociation solvent which would prevent aggregation and also break disulfide bonds led us to study the effect of mercaptoethanol in the urea dissociation system. For this purpose, our next dissociation solvent possessed the disulfide bond-breaking ability of 0.12 M β-mercaptoethanol in addition to the noncovalent bond-breaking capabilities of 7.4 M urea. As seen in Figure 2, extrapolation[2] of the apparent molecular weights determined at the various enzyme concentrations yielded values of 54,300 for M_w^0 and 55,600 for M_z^0. This excellent agreement between M_w^0 and M_z^0 suggested that the subunits were *ca.* equal in size and that the discrepancy between the M_w and M_z^0 values in urea alone was due to aggregation. Furthermore, these subunit molecular weight results in 7.4 M urea and 0.12 M β-mercaptoethanol were *ca.* the same as those obtained in 7.4 M urea alone, indicating the absence of intersubunit disulfide bonds.

Dissociation in Guanidine and β-Mercaptoethanol. Examination of the molecular weight values obtained in the urea–mercaptoethanol solvent indicated that the

[2] For the actual extrapolation for M_w^0, a more expanded scale for molecular weight was used. This condensed scale allows presentation of the z-average and the weight-average molecular weight data together.

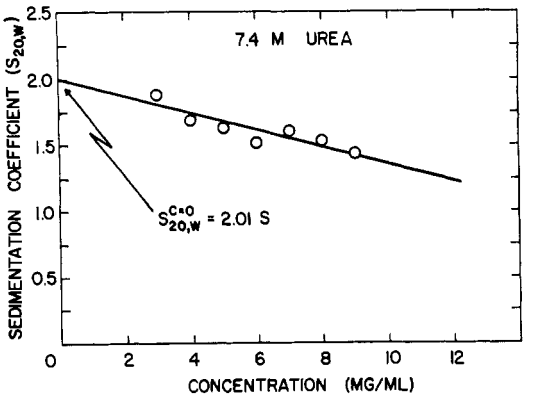


FIGURE 4: Extrapolation to 0 concentration of the sedimentation coefficients ($s_{20,w}$) of the subunits of pyruvate kinase in 7.4 M urea. For other experimental conditions, see text and Figures 1–3.

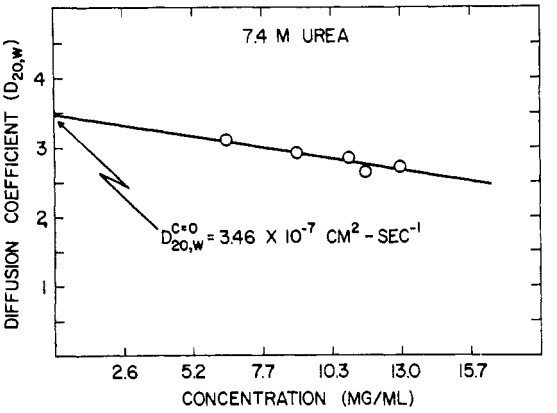


FIGURE 5: Extrapolation to 0 protein concentration of the diffusion coefficients ($D_{20,w}$) of the subunits of pyruvate kinase in 7.4 M urea. For other experimental conditions see text and Figures 1–3.

dissociated subunits did not exhibit pronounced aggregation. However, there did seem to be some inherent instability, as manifested by the slight scattering of the molecular weight values (Figure 2). In order to test a system in which the subunits might be more stable, and to further ensure that the native enzyme had been completely dissociated into its ultimate subunits, the enzyme was analyzed in a solution of 6.8 M guanidine–HCl and 0.12 M β-mercaptoethanol. As shown in Figure 3, the molecular weight values obtained under these conditions extrapolate[2] to a value of 57,100 for both M^0_w and M^0_z.

To provide a direct test for stability, several selected concentrations were subjected to molecular weight analysis after dialysis and again after aging in the cold for 1 or 2 weeks. In experiments on five such samples, the reproducibility of the results was remarkable. In comparing the molecular weights from fresh and aged samples, three of the samples had molecular weight differences of <1% while the other two had differences of <2%.

Determination of $s^0_{20,w}$, $D^0_{20,w}$, and $M^0_w(s/D)$ in 7.4 M Urea. To further characterize the subunits, diffusion and sedimentation velocity experiments were performed in 7.4 M urea. The enzyme was prepared for study following the same procedure used in the sedimentation equilibrium experiments. Extrapolation of the $s^0_{20,w}$ values obtained at the various protein concentrations yielded a value for $s^0_{20,w}$ of 2.01 S (Figure 4). In a similar fashion, the plot (Figure 5) of the $D_{20,w}$ values as a function of protein concentration yielded a value for $D^0_{20,w}$ of 3.46 × 10^{-7} cm^2/sec. Calculation of a molecular weight using these values gave a value for $M^0_w(s/D)$ of 56,300. This value is in excellent agreement with those obtained by the sedimentation equilibrium techniques and allows greater confidence to be placed in the previous results.

The extrapolated values for the subunit sedimentation and diffusion coefficients are much lower than would be expected for a globular protein with a mol wt 57,200, suggesting that the subunits are extensively unfolded and/or asymmetric in shape. Specifically, the frictional ratio calculated for the subunits is 2.4, whereas that of the native enzyme is 1.3; this indicates a drastic change in structure, although a small portion of the change is probably due to differences in solvation of the native enzyme and the subunits.

Dissociation as a Function of Urea Concentration. A detailed analysis of the structural transitions of the enzyme as a function of urea concentration was also performed. For this study, a dialyzed stock native enzyme solution was diluted with concentrated urea solutions to various final urea concentrations ranging from 0.5 M to 6.0 M. All solutions contained 0.15 M KCl, 0.04 M Tris–HCl buffer, 0.001 M EDTA, and had a pH of 8.0 at 25°. The final enzyme concentration was 6 mg/ml for each sample. Except for the special cases mentioned in the legend for Figure 6, these were then dialyzed in the cold *vs.* the appropriate urea solvent for *ca.* 12 hr. Sedimentation velocity experiments on these samples at various urea concentrations were immediately conducted. Figure 6 shows that marked structural changes in the enzyme first occurred in 1.5–1.7 M urea. Here a single sedimenting peak was observed, with a sedimentation coefficient of $s^{0.6\%}_{20,w}$ = 7.3 S, in contrast to a value of 8.9 S in 1 M urea. Standing several hours in 1.6–1.7 M urea yielded partial conversion of the 7.3S species to a 3.6S product. Thus, in 1.6–1.7 M urea the equilibrium clearly allowed the 3.6S species, although the kinetics were so slow that only the 7.3S material was observed initially. This illustrates the general slowness of attainment of the equilibrium distribution of products which was observed in the 1.5 to 2.5 M range of urea concentration. In 3 M urea, a single peak (intermediate 2) was formed which possessed an $s^{0.6\%}_{20,w}$ of 3.6 S, representing the same product formed in increasing amounts in 1.5–2.5 M urea; this product in 3 M urea converted to the slower sedimenting species upon aging. In 4 M urea, the maximum structural change possible had occurred since

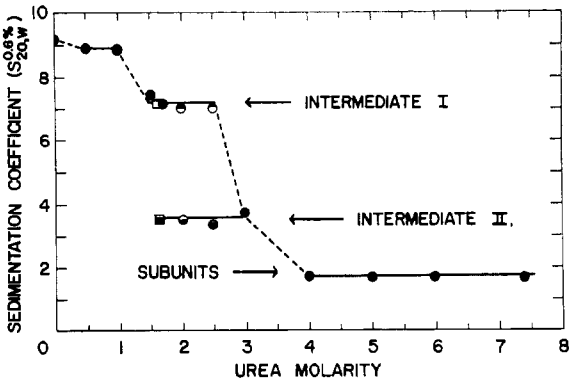


FIGURE 6: Effect of urea concentration on pyruvate kinase structure. The protein concentration was 6 mg/ml in each case. The enzyme was not dialyzed, in order to evaluate the kinetics of the reaction, for those experiments at 1.5, 1.6 and 1.7 M urea. The samples at 1.5 and 1.7 M urea were run immediately after dilution into urea, while that at 1.6 M urea (□) was run after 72 hr. All others were dialyzed for 12 hr and conducted as described in the text. The degree of shading of the circles and square in the 1.5–2.5 M urea region is proportional to the fractional amount of protein present as that particular species.

the sedimentation coefficient ($s^{0.6\%}_{20,w}$ = 1.7 S) observed at this concentration closely approximated that observed at all higher concentrations of urea (Figure 6), including 7.4 M urea (Figure 4).

Since the molecular weight data indicated dissociation into ultimate polypeptide chains in 7.4 M urea, this agreement between the sedimentation coefficients of the enzyme in 4 M and 7.4 M urea suggested that 4 M urea also produced dissociation into ultimate subunits. However, recalling that the subunits undergo random aggregation to some extent in 7.4 M urea, it seems likely that they are even less stable in 4 M urea.

The 7.3S value of the sedimentation coefficient for intermediate 1 suggested a splitting of pyruvate kinase into two halves, and since the native enzyme has two binding sites for PEP (Reynard *et al.*, 1961) and two for Mn^{2+} (Mildvan and Cohn, 1965), this raised the possibility that this 7.3S product might be a catalytically active particle of 120,000 mol wt. Preliminary studies are consistent with such a model but unequivocal proof must await the results of further work presently in progress. The enzyme is active in an assay solution with a urea concentration of 1.7 M.

The value of $s^{0.6\%}_{20,w}$ = 3.6 S found for intermediate 2 would be consistent with a slightly unfolded subunit of mol wt 57,200; it thus may well represent the folded form of the subunits. Since we cannot exclude, on the basis of present evidence, the satisfactory alternative possibilities of (1) a very unfolded dimer (protomer) or, (2) a rapid equilibrium between slightly unfolded dimers and monomers, definitive conclusions must await the results of work in progress.

Discussion

Considering (1) the value of 237,000 (Warner, 1958) for the molecular weight of the native enzyme, (2) the value of 57,200 for the molecular weight of the subunits, and (3) that the subunits are of nearly equal size, we conclude that the native rabbit skeletal muscle pyruvate kinase is composed of four subunits. The excellent agreement between the extrapolated values (Table I) of M^0_w,[1] M^0_z,[1] and $M^0_w(s/D)$[1] obtained in the various dissociating media allows considerable confidence to be placed on this subunit mol wt 57,200. These results are based on extrapolations to 0 protein concentration conducted in four separate series of experiments, performed in three separate dissociating solvents including (1) 7.4 M urea, (2) 7.4 M urea–0.12 M mercaptoethanol, and (3) 6.8 M guanidine–0.12 M mercaptoethanol. The extrapolated weight-average (M^0_w) and *z*-average (M^0_z) molecular weights obtained in the latter two dissociating solvents, where the subunits were more stable, were essentially the same. This indicated that the subunits had *ca.* equal molecular weights. As previously mentioned, Morawiecki (1960) reported the dissociation of pyruvate kinase into subunits of mol wt 150,000 in 6 M urea. A detailed comparison of our molecular weight results with those of Morawiecki (1960) does not appear to be justified because of the limited nature of his experiments.

TABLE I: Physical Properties of the Pyruvate Kinase Subunits.[a]

Solvent	Variable				
	M^0_w	M^0_z	(s/D) M^0_w	$s^0_{20,w}$	$D^0_{20,w}$
7.4 M urea	60,600	75,700[b]	56,300	2.01	3.46[c]
7.4 M urea + 0.12 M β-mercaptoethanol	55,600	54,300			
6.8 M guanidine + 0.12 M β-mercaptoethanol	57,100	57,100			

[a] See text for experimental details. [b] Probably high because of aggregation. [c] Units are 10^{-7} cm^2 sec^{-1}.

The data further suggest that the tetramer[3] of pyruvate kinase (mol wt 237,000) consists of two identical catalytic particles, or protomers[3] (mol wt 115,000 each),

[3] Tetramer is used here in its broadest meaning of four polypeptide chains; this does not infer that the chains are identical. Identical subparticles are designated protomers (Monod *et al.*, 1965).

with each protomer in turn consisting of two polypeptide chains (mol wt 57,200 each). Furthermore, since there is only one site for PEP and one site for Mn^{2+} per protomer, the polypeptide chains within the protomers probably are not identical, even though they are quite similar in mass. The conclusions above are based on the reports that pyruvate kinase possesses only two PEP[1] binding sites (Reynard *et al.*, 1961) and two Mn^{2+} sites (Mildvan and Cohn, 1965) per native enzyme complex of 237,000. From this it seems probable that two of the subunits bind PEP while two do not, and the same is true of Mn^{2+}; if this be true, the subunits must be different. Although not unique, the two-protomer interpretation seems plausible and is supported by other preliminary results. The 7.3S intermediate found in 1.5 M urea is probably a 120,000 mol wt protomer possessing catalytic activity.

If so, this would also provide an apparent size correlation between the rabbit skeletal muscle pyruvate kinase and human erythrocyte and leucocyte pyruvate kinases which have been shown to have sedimentation coefficients of about 7.1 S and to possibly consist of two different kinds of subunits on the basis of genetic evidence (Koler *et al.*, 1964). Various tissues of the rat show electrophoretically distinguishable enzymes (Von Fellenberg *et al.*, 1963; Tanaka *et al.*, 1965) which, in some cases, suggest the presence of nonidentical subunits.

The suggestion of nonidentical subunits also raises the possibility that some of the subunits of pyruvate kinase may be concerned with catalytic function while others may have regulatory functions, as has been shown recently to occur for aspartate transcarbamylase (Gerhart and Schachman, 1965). Skeletal muscle pyruvate kinase activity in the direction of glycolysis is already known to be inhibited by ATP at physiological levels (Reynard *et al.*, 1961). Kimberg and Yielding (1962) have reported slight changes in the viscosity and electrophoretic mobility of rabbit skeletal muscle pyruvate kinase in the presence of diethylstilbesterol and, to a lesser extent, certain steroid hormones. It is also possible that the dependence of the enzyme upon magnesium and potassium for catalytic activity (Boyer, 1962) may be further sources of control of its activity, although a simple mechanism for accomplishing this is presently lacking. Pyruvate kinase of the gluconeogenic organ, liver, has been shown to be under both dietary and hormonal control (Weber *et al.*, 1965; Krebs and Eggleston, 1965) and is thus considered to be a control point for glycolysis and glyconeogenesis. Pye and Eddy (1965) have also implicated the enzyme in the control of glycolysis in yeast.

Comparison of the dissociation properties of rabbit muscle glycolytic enzymes reveals several similarities. The 3.6S intermediate produced by pyruvate kinase in 2–3 M urea is strikingly similar to the 3.6S intermediate of aldolase found at pH 3.4 (Deal and Van Holde, 1962; Deal *et al.*, 1963b). The high degree of unfolding of the subunits of pyruvate kinase is also reminiscent of that of the aldolase subunits, whose molecular weight, sedimentation coefficient, and frictional ratio are only slightly lower than those of the pyruvate kinase subunits. A third similarity is that the requirement for a minimum dissociation condition of 4 M urea is the same as that for aldolase (Stellwagen and Schachman, 1962) and yeast and rabbit muscle glyceraldehyde 3-phosphate dehydrogenases (Deal, 1963; Deal and Holleman, 1964; W. C. Deal, 1965, in preparation).

The subunits are most stable in the guanidine–mercaptoethanol solvent. Although complete dissociation of the enzyme is achieved in the presence of urea, the subunits tend to aggregate upon standing in the urea, and to a lesser extent, in the urea–mercaptoethanol solvents. However, in the guanidine–mercaptoethanol system, not only is there practically no scatter of the molecular weights but in five experiments where samples were run again after 1 or 2 weeks, the reproducibility of the guanidine–mercaptoethanol data was remarkable.

Whether smaller chains of about 15,000–30,000 mol wt exist within the 57,200 mol wt subunits of pyruvate kinase is open to question. However, the strength of the guanidine–mercaptoethanol solvent as a dissociating medium is generally assumed to be adequate to achieve complete dissociation into ultimate polypeptide chains. Implicit in this hypothesis is the assumption that the only covalent link possible between polypeptide chains is the disulfide bond. Since the molecular weights obtained in urea in the absence of the disulfide bond-breaking reagent are essentially identical with those observed in the guanidine–mercaptoethanol system, we conclude that there are no disulfide bonds holding the subunits together. Thus, weak noncovalent attractive forces must maintain the native enzyme in its subunit complex. Therefore, it appears that if subunits smaller than 57,200 exist, the attractive forces or bonds maintaining the 57,200 subunit structure must be of such an unusual nature that they either have not been detected before, or are thought not to be significant in protein bonding.

Finally, our hypothesis that most ultimate polypeptide chains will have a molecular weight less than about 65,000 is supported by the pyruvate kinase data. Furthermore, an enzyme whose structure had been thought to contradict this hypothesis has now been found to support it. The subunit molecular weight of *Escherichia coli* β-galactosidase is now thought to be *ca.* 50,000 (Steers *et al.*, 1965). This general concept of maximum size of polypeptide chains cannot be classed as an inviolable rule because apparent exceptions to it still exist; however, it does appear to hold true for most proteins whose structures have been intensively investigated. One apparent major exception to this theory is the myosin molecule which is reported (Kielley and Harrington, 1960; Kielley and Barnett, 1961) to have three polypeptide chains of about 200,000 mol wt each.

Studies are currently in progress on the reversal of the dissociation process of pyruvate kinase, on the chemical characterization of the enzyme and its subunits, and on the control of pyruvate kinase activity.

Experimental Section

Enzyme Techniques and Reagents. Pyruvate kinase

was prepared from frozen rabbit muscle (Tietz and Ochoa, 1958). Following the second heat step, the protein was precipitated by $(NH_4)_2SO_4$ between 40 and 60% saturation, resuspended in 0.02 M imidazole buffer, pH 7.0, and stored under these conditions at a protein concentration of 50–80 mg/ml. Enzyme preparations were assayed for activity (Kayne and Suelter, 1965) utilizing a Radiometer TTT-1/SBR2-SBU1/TTA31 automatic recording titrator. They were found to have relatively high specific activities, with a range of 120–180 μmoles of substrate cleaved/min per mg. The reagents used in activity assays were: adenosine 5′-diphosphate, sodium salt (Pabst), and phosphenolpyruvic acid, tricyclohexylamine salt (Sigma). Urea (Baker's Analyzed Reagent) was recrystallized from 95% ethanol. The crystals were dried in a vacuum oven at 40° to remove residual ethanol. Guanidine–HCl was prepared from Eastman guanidine carbonate (Anson, 1941). Eastman β-mercaptoethanol was used. The dissociating solvents were always prepared just prior to use. All reagents were made 0.001 M in EDTA. The dialysis casing had been boiled and stored in 0.001 M EDTA.

Ultracentrifugal Analysis. With but one noted exception, the enzyme was prepared for ultracentrifugation as follows: stock enzyme solutions at high concentrations (50–80 mg/ml) were diluted with various dissociating and reducing agents containing 0.15 M NaCl or KCl, 0.04 M Tris–HCl buffer, and 0.001 M EDTA, pH 8.0 (measured at 25°). The resulting stock enzyme solution was dialyzed *vs.* these reagents for at least 3 days at 5°. For the experiments at different protein concentrations, samples were then diluted directly with the dialysate to yield enzyme concentrations of 1.5–19.0 mg/ml.

Spinco analytical Model E ultracentrifuges with phase-plate schlieren optics were used for these studies. The short column sedimentation equilibrium techniques (Van Holde and Baldwin, 1958) were complemented by new methods which are described in a forthcoming paper (W. C. Deal, in preparation). Techniques were devised to allow multicell (2 or 3 cells) operation using the schlieren optical system, and calculations were developed for extrapolation to 0 protein concentration using the data from a single experiment. The calculations include statistical analysis of data and were performed, using fully tested programs, on a Control Data Corporation 3600 digital computer. The value (Warner, 1958) of 0.74 (10°) for the partial specific volume of rabbit muscle pyruvate kinase was used. The solution column depth in the cells was 1.7 mm (0.06 ml of protein sample) and all sedimentation equilibrium experiments were carried out at *ca.* 7° for at least 36 hr. Due to the appearance of a low molecular weight species upon allowing the enzyme to remain in the presence of 0.12 M β-mercaptoethanol in urea or guanidine for more than 24 hr, all synthetic boundary experiments were performed immediately after dialysis. A detailed, careful study of the production and effect of this low molecular weight species showed[4] that the low molecular weight material was not derived from the enzyme nor did it influence the results (W. C. Deal, Jr., and M. A. Steinmetz, in preparation).

Sedimentation velocity experiments were run at 59,780 rpm near 7°; exact temperatures were obtained from the rtic unit. The diffusion coefficient experiments were performed in a double-sector synthetic boundary cell at 4059 rpm at a temperature of 7°. Diffusion coefficients were calculated (Schachman, 1957) using height-to-area analysis and converted, as were the sedimentation coefficients, to standard conditions of 20° and water. Densities of the solvents were measured with a hydrometer. Viscosities of urea solutions were interpolated from a graph based on data from the International Critical Tables (1929). Viscosity contributions of buffers and salts were estimated from other reference tables (Svedberg and Pederson, 1940).

References

Anson, M. L. (1941), *J. Gen. Physiol. 24*, 399.

Boyer, P. D. (1962), *Enzymes 6*, 95.

Deal, W. C., Jr. (1963), *Federation Proc. 22*, 290.

Deal, W. C., Jr., and Holleman, W. H. (1964), *Federation Proc. 23*, 264.

Deal, W. C., Jr., Rutter, W. J., Massey, V., and Van Holde, K. E. (1963a), *Biochem. Biophys. Res. Commun. 10*, 49.

Deal, W. C., Jr., Rutter, W. J., and Van Holde, K. E. (1963b), *Biochemistry 2*, 246.

Deal, W. C., Jr., and Van Holde, K. E. (1962), *Federation Proc. 21*, 254.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

International Critical Tables, (1929), *5*, 22.

Kayne, F. J., and Suelter, C. H. (1965), *J. Am. Chem. Soc. 87*, 897.

Kielley, W. W., and Barnett, L. M. (1961), *Biochim. Biophys. Acta 51*, 589.

Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.

Kimberg, D. V., and Yielding, K. L. (1962), *J. Biol. Chem. 237*, 3233.

Koler, R. D., Bigley, R. H., Jones, R. T., Rigas, D. A., Vanbellinghen, P., and Thompson, P. (1964), *Cold Spring Harbor Symp. Quant. Biol. 29*, 213.

Krebs, H. A., and Eggleston, L. V. (1965), *Biochem. J. 49*, 3C.

Mildvan, A. S., and Cohn, M. (1965), *J. Biol. Chem. 240*, 238.

Monod, J., Wyman, J., Changeux, J. (1965), *J. Mol.*

[4] The following considerations provide a partial basis for this conclusion: (1) The effect is associated with mercaptoethanol; dialyzed protein solutions containing urea or guanidine alone did not show the low-molecular weight species after aging for several days, whereas those with mercaptoethanol did; (2) the low molecular weight material is not derived from the protein; after several days aging of the mercaptoethanol-containing urea or guanidine solutions of protein described in (1), synthetic boundary analysis revealed that the area under the curve had increased significantly, with a concomitant appearance of the low molecular weight species.

Biol. 12, 88.
Morawiecki, A. (1960), *Biochim. Biophys. Acta. 44*, 604.
Pye, E. K., and Eddy, A. A. (1965), *Federation Proc. 49*, 537.
Reynard, A. M., Hass, L. F., Jacobsen, D. D., and Boyer, P. D. (1961), *J. Biol. Chem. 236*, 2277.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 2.
Steers, E., Jr., Cranen, R., and Anfinsen, C. B. (1965), *J. Biol. Chem. 240*, 2478.
Stellwagen, E., and Schachman, H. K. (1962), *Biochemistry 1*, 1056.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, New York, N. Y., Oxford University Press, Appendix III.
Tanaka, T., Harano, Y., Morimura, H., and Mori, R. (1965), *Biochem. Biophys. Res. Commun. 21*, 55.
Tietz, A., and Ochoa, S. (1958), *Arch. Biochem. Biophys. 78*, 477.
Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.
Von Fellenberg, R., Richterich, R., and Aebi, H. (1963), *Enzymol. Biol. Clin. 3*, 240.
Warner, R. C. (1958), *Arch. Biochem. Biophys. 78*, 494.
Weber, G., Stamm, N. B., and Fisher, E. A. (1965), *Science 149*, 65.

Heme Proteins. VI. Crystalline Pineapple Peroxidase B*

Charles Beaudreau† and Kerry T. Yasunobu‡

ABSTRACT: The indoleacetic oxidase activity reported to be present in pineapple stem tissues has been shown to be due to the presence of peroxidases. Pineapple stem peroxidase B has been purified from both fresh pineapple stems and a commercial concentrate of pineapple stems and obtained in a crystalline form. From the enzymatic and physicochemical properties of the enzyme, it is shown to be an atypical peroxidase. The atypical properties include an acidic pH optimum, low specific activity, and anomalous Soret absorption. It is the first atypical peroxidase to be obtained in a crystalline form.

Plant peroxidases isolated thus far show great similarities in enzymatic and chemical properties and can be considered under the group called classical peroxidases (Theorell, 1951; Paul, 1963). Pineapple peroxidase B has been isolated in crystalline form and shown to be an atypical plant peroxidase. The atypical characteristics include low specific activity, a more acidic pH optimum, and anomalous Soret absorption. However, the other enzymatic and chemical properties of the enzyme are similar to the properties of the classical peroxidases.

Methods and Materials

Materials. The enzyme was isolated from both fresh pineapple stem tissue and Dolzyme T-20, a pineapple stem juice concentrate which was generously provided by Dr. Ralph Heinicke of the Dole Pineapple Co. Hydroxylapatite was prepared according to the procedure of Tiselius *et al.* (1956). Carboxymethylcellulose was purchased from Brown and Co. Sulfoethyl-Sephadex was purchased from Pharmacia Fine Chemicals, Incorp. Guaiacol and indoleacetic acid[1] were purchased from the Eastman Chemical Co. *p*-Coumaric acid was obtained from the Mann Research Laboratories, Inc.

Methods. The peroxidase activity was measured by a modified procedure of Devlin (Chance and Maehly, 1955) based on the rate of formation of tetraguaiacol by observing the absorbancy at 470 mμ. For this assay, 1.8 ml of 0.2 M sodium acetate buffer, pH 4.4, 1 ml of 0.02 M guaiacol, and 0.1 ml of diluted enzyme were placed in a 10 × 75 mm cuvet. The reaction was started by adding 0.1 ml of 1.63 M (5%) hydrogen peroxide. Absorbancy readings were taken every 15 sec, and the reaction was run at room temperature (29–30°). Activity (1 unit) was defined as the amount of enzyme which catalyzed a change of 0.001 absorbance unit/min using this assay system. Specific activity was defined as units of activity per milligram of protein.

The indoleacetic acid oxidase activity was measured spectrophotometrically as suggested by Ray (1956).

* From the Department of Biochemistry and Biophysics and the Department of Chemistry, University of Hawaii, Honolulu, Hawaii. *Received October 1, 1965; revised January 21, 1966.* Supported by Research Grant GB 1363 from the National Science Foundation.

† Present address: Department of Physiological Chemistry, Johns Hopkins University, Baltimore, Md.

‡ Address correspondence regarding this paper to the second author.

[1] Abbreviation used in this work: IAA, indole-3-acetic acid.