Acetoacetate Decarboxylase. The Molecular Weight of the Enzyme and Subunits*

Waichiro Tagaki† and F. H. Westheimer

ABSTRACT: The enzyme acetoacetate decarboxylase dissociates reversibly into dimers of the ultimate subunit, and irreversibly into the subunits themselves. The molecular weights of the enzyme, the subunit dimer, and the subunit determined ultracentrifugally by the method of Yphantis are 340,000 ± 10,000, 62,000 ± 2000, and 29,000 ± 1000, respectively.

The enzyme, acetoacetate decarboxylase, that catalyzes the decarboxylation of acetoacetate to acetone and CO_2 has previously been isolated from *Clostridium acetobutylicum*, purified, and crystallized (Hamilton and Westheimer, 1959a,b; Zerner *et al.*, 1966; Westheimer, 1968). In investigations of mechanism, it has been shown that acetoacetate reacts with the enzyme at a specific lysine residue to form a Schiff base, which is an essential intermediate in the decarboxylation process (Hamilton and Westheimer, 1959a,b; Fridovich and Westheimer, 1962; Warren *et al.*, 1966; Laursen and Westheimer, 1966; Westheimer, 1963). Prior investigation had also revealed that the enzyme consists of subunits, and that the native enzyme is a large molecule. Chemical investigation had suggested that the subunits have a molecular weight of about 30,000, whereas the molecular weight of the enzyme itself was crudely estimated at around 260,000 from ultracentrifugal measurements by the Archibald method (Lederer *et al.*, 1966). We have now carefully reinvestigated the molecular weights of the subunit, of a dimer of the subunit, and of the enzyme, and have found that the prior estimate for the weight of the subunit (although subject to a small correction) was approximately correct, whereas the earlier estimate for the molecular weight of the enzyme itself was substantially too low. Based on our present molecular weights of about 29,000 for the subunit and about 340,000 for the enzyme itself, it appears that the enzyme is composed of 12 subunits.

Experimental Section

Materials. Dithiothreitol was obtained from Calbiochem., Inc. Urea was purchased from Merck, and was tested to be sure it was free from cyanate (Werner, 1923; Stark *et al.*, 1960). Guanidinium chloride was prepared from the carbonate (Nozaki and Tanford, 1967). Other chemicals were reagent grade.

The preparation of crystalline acetoacetate decarboxylase was carried out according to Zerner *et al.* (1965), with slight modifications that have been introduced more recently (Westheimer, 1968). The enzyme was twice recrystallized from ammonium sulfate solution in pH 5.96 phosphate buffer, dialyzed against 0.05 M phosphate buffer (pH 5.96), and stored in this buffer. Bovine serum albumin was a gift of Professor G. Guidotti, Department of Biology, Harvard University. α-Chymotrypsin was purchased from Worthington Biochemical Corp. Catalase from beef liver was purchased from Sigma Chemical Co. It was stock no. C-100, twice recrystallized, and suspended in 0.1% thymol solution.

Preparation of Enzymes for Ultracentrifugation. The catalase suspension was put on a column (2.8 × 28 cm) of Sephadex G-25 and eluted with 300 ml of phosphate buffer (0.033 M KH_2PO_4–0.056 M Na_2HPO_4, μ 0.2, pH 7.04). The ultraviolet spectrum of the main fraction corresponds to that of Tauber and Petit (1952). Bovine serum albumin was made up as a 1% solution in acetate buffer (0.15 M NaCl, 0.02 M sodium acetate, and 0.03 M acetic acid, pH 4.42). The solution was dialyzed against this same buffer for 24 hr at 4°, with two changes of the external buffer. The ultraviolet spectrum of the final solution was normal. Crystalline chymotrypsin was dissolved in sodium phosphate buffer (μ 0.2, pH 6.18). The solution was passed through a column (2.8 × 28 cm) of Sephadex G-25 in a cold room. The center fractions from the eluate containing the enzyme were combined and dialyzed against the same buffer, with two changes of the external solution. Native acetoacetate decarboxylase (specific activity 42.4; OD_{320}/OD_{280} 0.108) was diluted from a stock solution (8.24 mg/ml) with 0.1 (pH 5.86) or 0.05 M (pH 5.96) potassium phosphate buffer to give about a 0.1–0.15% solution, and dialyzed at 4° against this same buffer, with three changes of the external buffer.

A solution considered to consist of the subunit dimer (see Discussion) was prepared from stock

* From the James Bryant Conant Laboratory of the Department of Chemistry of Harvard University. This work was supported by Grant GM-04712 from the Institute of General Medical Sciences of the National Institutes of Health.

† Present address: Department of Applied Chemistry, Faculty of Engineering, Osaka City University, Osaka, Japan.

TABLE I: Proteins and Conditions for Ultracentrifuge Experiments.[a]

Proteins	Temp (°C)	Rpm	$\bar{V}$	Solvent (M)	pH	ρ 4°[b]
AAD	20	11,272	0.740	KP_i (0.1)	5.86	1.0081
AAD	20	17,250	0.740	KP_i (0.05)	5.96	1.0033
AAD	10	29,500	0.740	Urea (2.5)	8.11	1.0469
			0.730			
AAD	10	39,460	0.740	Glycine–HCl (0.1)	2.15	1.0050
			0.730			
AAD	20	39,460	0.740	Gu^+Cl^- (6)	5.80	1.1411
AAD	20	39,460	0.740	Urea (4)	8.20	1.0665
Catalase	20	17,250	0.730	KP_i–NaP_i (μ 0.2)	7.04	1.0060
BSA	20	24,630	0.734	NaCl–AcONa–AcOH	4.42	1.0060
α-Chymotrypsin	20	39,460	0.736	NaP_i (μ 0.2)	6.18	1.0060

[a] Abbreviations: AAD, acetoacetate decarboxylase; BSA, bovine serum albumin; KP_i, potassium phosphate; NaP_i, sodium phosphate; Gu^+Cl^-, guanidinium chloride. [b] Density of the solvent.

solution of enzyme (10.6 mg/ml; specific activity 44.1; OD_{320}/OD_{280} 0.075). This stock solution (0.2 ml) was diluted at 0° with 1.8 ml of 2.5 M urea solution in 0.1 M phosphate buffer (pH 8.11), containing 2×10^{-3} M dithiothreitol to give about a 0.1% solution. The mixture was then dialyzed at 4° for 23 hr against the same urea solutions, with three changes of external solution. This solution was divided into two parts, one for ultracentrifugal measurements and one set aside for a measurement of the amount of enzyme activity that could be recovered. The latter was kept, during the ultracentrifugation, at 10° (the same temperature as that of the ultracentrifugation). It was then dialyzed against 0.05 M potassium phosphate buffer (pH 5.96), according to the method previously published (Tagaki and Westheimer, 1968). A small amount of precipitate was removed by centrifugation. Measurement of the specific activity showed that 70% had been recovered.

Solutions considered to contain the monomeric subunit from acetoacetate decarboxylase (see Discussion) were prepared in three ways. (a) Glycine–HCl buffer pH 2.15: Stock solution of enzyme (10.6 mg/ml; specific activity 44.1; OD_{320}/OD_{280} 0.075) was diluted with nine times its volume of buffer (pH 2.15) containing 0.1 M HCl and 0.143 M glycine. The mixture (2 ml) was dialyzed for 36 hr at 4° against the same buffer, with three changes of the external buffer. (b) 6 M Guanidinium chloride–0.1 M 2-mercaptoethanol: Stock solution of enzyme (9.8 mg/ml; specific activity 37.4; OD_{320}/OD_{280} 0.119) was diluted with nine times its volume of a solution of 6 M guanidinium chloride, 0.1 M mercaptoethanol, and 0.05 M phosphate buffer (pH 5.80). The mixture (2 ml) was dialyzed for 24 hr at 4° against the same guanidinium chloride–mercaptoethanol–buffer solution with three changes of the external solution. (c) 4 M urea: Stock solution (12.7 mg/ml; specific activity 45.7; OD_{320}/OD_{280} 0.085) was mixed with four times its volume of 5 M urea in sodium phosphate buffer (pH 8.2). To prepare the more dilute solutions for ultracentrifugal measurements, the resulting solution was subsequently diluted fivefold with 4 M urea in 0.1 M sodium phosphate buffer, and then once again fivefold in this urea–buffer solution.

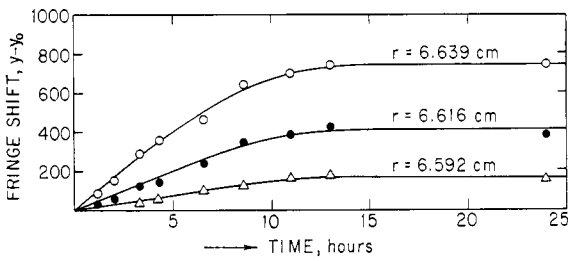

FIGURE 1: The vertical shift ($y - y_0$) of the schlieren fringes as a function of the time of ultracentrifugation, measured at three different distances from the axis of rotation for acetoacetate decarboxylase in solution with 6 M guanidinium chloride plus 0.1 M mercaptoethanol and 0.05 M phosphate buffer (pH 5.80).

Method. The ultracentrifugal experiments were carried out by the Yphantis method (Yphantis, 1964; Kawahara and Tanford, 1966) with a Beckman–Spinco Model E ultracentrifuge. Multichannel short-column equilibrium centerpieces for the Model E and Rayleigh interference optics were used through this work. Each channel was loaded with 0.01 ml of fluorocarbon from a Hamilton microsyringe, where the needle was covered with 3–4 cm of polyethylene tubing. The solution (0.11 ml) or solvent (0.12 ml) was introduced from a similar 0.5-ml syringe. The photographs were taken with Kodak spectroscopic plates (type II-G). The interference fringes on the plates were read with a Gaertner microcomparator according to the method outlined by van Holde (1967).

In order to carry out a determination of molecular

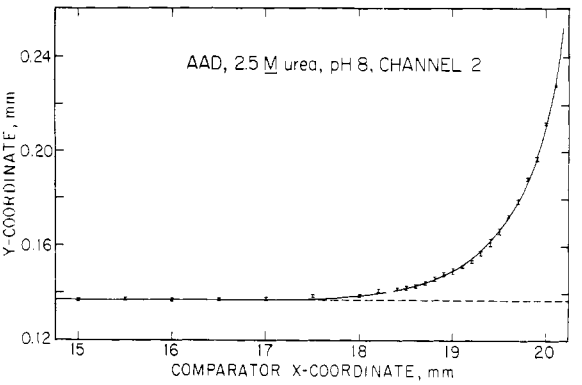

FIGURE 2: Vertical against horizontal comparator coordinates for acetoacetate decarboxylase in 2.5 M urea (pH 8) at 9° after equilibrium had been achieved. The base line (control) is satisfactorily flat.

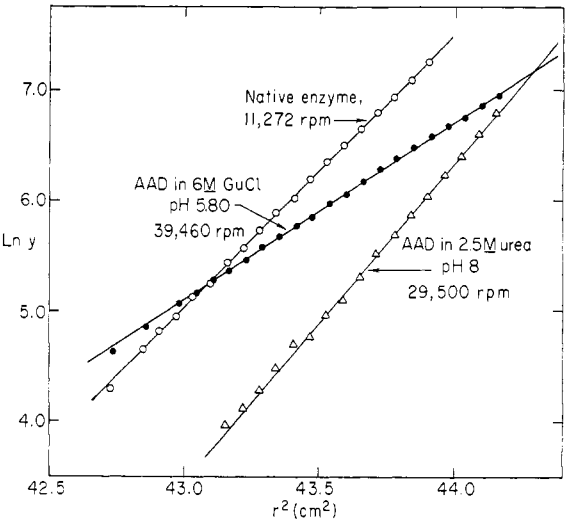

FIGURE 3: Examples of plots of the natural logarithm of the vertical comparator reading (y) against the square of the distance (r) from the center of the rotor for native enzyme in pH 5.92 buffer (○); for enzyme in 2.5 M urea plus 2×10^{-3} dithiothreitol in 0.05 M phosphate buffer (pH 8.11) (△); and for enzyme in 6 M guanidinium chloride plus 0.1 M mercaptoethanol and 0.05 M phosphate buffer (pH 5.80) (●).

weight by the Yphantis method, it is necessary to make sure that the ultracentrifugation is carried out for a period sufficiently long that equilibrium is attained; it is also necessary that the speed be sufficient that the protein concentration near the interface between air and solution be reduced to zero over a length sufficient to permit accurate reading of the base line. In each determination of molecular weight, the displacement of the fringes was measured at at least two distances, as a function of time, and conditions for molecular weight determinations were chosen appropriate to the protein under consideration. One graph (for enzyme in 6 M guanidinium chloride solution) is shown in Figure 1; equilibrium had been attained after about 15 hr at 39,460 rpm. Equilibrium had been attained for native enzyme in 10 hr at 11,272 rpm. All of the measurements of molecular weight were made after 24 hr, when the solutions were fully equilibrated. The base lines, measured for "blanks" (*i.e.*, for water or for the buffer used), were satisfactorily flat, with deviations that seldom exceeded the experimental error of the readings.

Partial Specific Volume. In determining the molecular weight of a protein, a value for the partial specific volume is required. For many proteins, the partial specific volume is in the neighborhood of 0.73–0.74. The value of 0.74 was calculated for acetoacetate decarboxylase from the amino acid composition (Lederer *et al.*, 1966) according to the method of McMeekin and Marshall (1952) (*cf.* Charlwood, 1957). The data of Taylor and Lowry (1956) and of Reithel and Sakura (1963) suggest that the partial specific volume of proteins diminishes at lower temperatures; we have therefore also used the value of 0.73 for $\bar{V}$ at 10°. The effect of guanidinium chloride and of urea on $\bar{V}$ is not certain, but probably small (Kielley and Harrington, 1960; Reithel, 1963). The density of the medium was measured or interpolated from the literature in all cases (see Table I).

Treatment of the Data. The vertical coordinates of the interference fringes were read three times for each horizontal setting. The weight-average molecular weights were calculated from the data using the equation (Yphantis, 1964)

$$\bar{M} = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{\mathrm{d} \ln C}{\mathrm{d}r^2} = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{\mathrm{d} \ln [y(r) - y_0]}{\mathrm{d}r^2} \quad (1)$$

where $\bar{M}$ is the weight-average molecular weight of the protein, $\bar{V}$ is the specific volume of the protein, ρ is the density of the solution, ω is the rotor speed in radians per second, and r is the distance in centimeters from the center of the rotor to the position where the interference fringe is read. C is the concentration of the protein, and $y(r)$ and y_0 are, respectively, the vertical displacements of the interference fringes at the distance r and at the position where ultracentrifugation has reduced the concentration of protein to zero. Since only the derivative of the logarithm of concentration with respect to r^2 is required, the derivative of the logarithm of the displacement may be substituted, as in eq 1; it is never necessary actually to determine protein concentrations.

Therefore $\ln [y(r) - y_0]$ was plotted against r^2. The slope of the resulting line was calculated from all the data points by least squares with an IBM 1620 computer. Each point was assigned a weight proportional to its displacement (y) on the assumption that the absolute error in reading the Gaertner comparator is constant.

Results

The molecular weights were determined by the Yphantis method, as outlined above. A typical graph

TABLE II: Acetoacetate Decarboxylase.

Exptl Condn	Temp (°C)	Time (hr)	Rotor Speed (rpm)	Channel	Protein Concn (%)	Mol Wt
pH 5.92 buffer	20	24	11,272	1	0.104	337,600 ± 2,500
		24		2	0.035	338,900 ± 2,200
		24		3	0.010	345,300 ± 3,600
	20	24	17,250	1	0.148	339,700 ± 5,200
		24		2	0.049	331,800 ± 6,300
		24		3	0.010	355,400 ± 5,500
2.5 M urea, pH 8	10	24	29,500	1	0.08	62,900 ± 600
		24		2	0.04	63,500 ± 600
		24		3	0.02	62,500 ± 600
Glycine–glycine–HCl buffer, pH 2.15	10	21	39,460	1	0.086	29,600 ± 400
		26		1		30,400 ± 200
		21		2	0.043	30,500 ± 500
		21		3	0.011	29,600 ± 400
Guanidium chloride, 6 M	20	24	39,460	1	0.08	29,300 ± 300
		24		2	0.04	29,300 ± 200
		24		3	0.02	29,400 ± 300
Urea, 4 M	20	47	39,460	3	0.012	28,500 ± 200

TABLE III: Proteins for Standardization.

Protein	Temp (°C)	Time (hr)	Rotor Speed (rpm)	Channel	Protein Concn (%)	Mol Wt	
						Exptl	Lit.
Catalase	20	24	17,250	2	0.027	244,300 ± 2,100	248,000[a]
		24		3	0.015	256,000 ± 8,400	
Serum albumin	20	24	24,630	1	0.10	66,400 ± 300	67,000[b]
		24		2	0.05	65,600 ± 300	
		24		3	0.02	67,700 ± 400	
α-Chymotrypsin	20	24	39,460	2	0.046	27,000 ± 100	25,000[c]
		24		3	0.010	26,800 ± 100	

[a] Sumner and Gralen (1938). [b] Edelstein and Schachman (1967). [c] Hartley (1964).

of the displacement against distance is given in Figure 2, and several examples of the plot of ln $\bar{y}$ against r^2 are shown in Figure 3. In all cases, the points lie on excellent straight lines, with only slight uncertainty. The conditions for the ultracentrifugal experiments are given in Table I.

The details of the experiments with acetoacetate decarboxylase are given in Table II, and those for the proteins used for standardization are given in Table III. Some comments on the individual determinations are given below. The determination of the molecular weight for native acetoacetate decarboxylase was better at the lower rotor speed, where the slope of the fringe deflections was ideal for measurement.

The most difficult determination was that for the subunit dimer. Urea (4 M) at 20° irreversibly dissociates the enzyme into monomeric subunits (see below). When an experiment was conducted in 4 M urea at 10°, it was doubtful whether a flat region (zero concentration of protein) was established near the air meniscus, and only 30–40% of enzymic activity could be recovered after the experiment. The molecular weights calculated crudely from the data ranged from 45,000 to 55,000. Thus all the evidence suggests that in this solution the enzyme was undergoing further incomplete dissociation to the monomer. When, however, the experiment was repeated at 10° with 2.5 M urea in the presence of dithiothreitol, the numbers shown in Table II were obtained; representative data are also shown in Figure 3. The ultraviolet spectrum of recovered protein was similar to that of native enzyme, and about 70% of the enzymic activity was recovered. These data therefore appear much better than those with higher urea concentrations.

TABLE IV: Summary of Molecular Weights.

Protein	$\bar{M}_w^0$ Obsd	Lit.
AAD, native	340,000[a]	
AAD in 2.5 M urea, pH 8	63,000,[a] 60,200[b]	
AAD in pH 2.15 buffer	30,000,[a] 28,900[b]	
6 M Gu^+Cl^-, pH 5.80	29,300[a]	
4 M urea, pH 8.2	28,500[a]	
Beef liver catalase	250,000	248,000[c]
Bovine serum albumin	67,000	67,000[c]
α-Chymotrypsin	27,000	25,000[c]

[a] $\bar{V} = 0.740$. [b] $\bar{V} = 0.730$. [c] See Table III.

Previous determinations of sedimentation velocity (Lederer *et al.*, 1966; Tagaki and Westheimer, 1968) had suggested three experimental conditions where the enzyme is dissociated to subunits: pH 2, 4 M urea at 25°, and 6 M guanidinium chloride solutions at pH 6 and low temperature. The results with the subunit are uniform, regardless of the method of its preparation. The results from channels 1 and 2 for the protein (presumably monomer) prepared with 4 M urea could not be calculated since the region near the air interface was not flat, presumably because of mismatching with the solvent blank.

Similarly, minor technical difficulties prevented use of the results from all the channels for the control proteins. Slight variations (±1°) in the base line were found to cause changes of as much as 10% in the calculated molecular weight for serum albumin; for this reason, great care was taken in aligning the comparator plates.

The molecular weights (Table IV) obtained for catalase, serum albumin, and chymotrypsin serve as standardization for the method for the determinations with the native enzyme, subunit dimer, and subunit. The agreement is quite good between the measurements here reported and those in the literature for catalase and serum albumin, but the molecular weight we determined for chymotrypsin is about 8% too high. The variation in the molecular weight from the two determinations for catalase is also somewhat greater than might be desired. Nevertheless, the values are sufficiently accurate to provide assurance that the molecular weights here reported are at least approximately correct.

Discussion

The molecular weight of the monomeric subunit from the enzyme is in the neighborhood of 29,000; this value was found regardless of whether the subunit was obtained by treatment with a buffer of low pH (2.15), or with 4 M urea, or with 6 M guanidinium chloride. The uniformity of the result is quite satisfactory in view of the different method of preparing the monomer and in view of the fact that the enzyme cannot be reconstituted from the subunits, which are presumably denatured. The molecular weight is in reasonable agreement with that determined by chemical methods. The latter value is about 31,500, and so differs from that determined by the Yphantis method by only 5%. We had previously reported (Lederer *et al.*, 1966) that the analysis of the enzyme for end groups and for tryptophan led to a molecular weight of about 33,000. That value, however, was based on standardization obtained by weighing the dried enzyme. Subsequently, the protein content of a solution was determined by the Kjeldahl method assuming that the acetoacetate decarboxylase contains 16.9% nitrogen, in accordance with its amino acid composition. The average result of the Kjeldahl analysis was to indicate that the dried enzyme had a water content of about 5%, so that the chemical analyses had overestimated the molecular weight by about 5%.

The molecular weight of the enzyme itself is about 340,000. Thus it is apparently a dodecamer, rather than an octamer as previously reported. Twelve times the molecular weight of the monomer is 348,000, or about 3% more than the molecular weight found experimentally.

The greatest uncertainties pertain to the interpretation of the molecular weight obtained in 2.5 M urea solution at 10°. The value was 63,000, which corresponds fairly well with that for two subunits; it is perhaps 8% too high. Since 70% of the original enzyme activity can be recovered from the solution, the dominant species in solution is presumably at least 70% pure, and it is reasonable to assume that this species is the subunit dimer. This conclusion is reinforced by inspection of the plot (Figure 3) of $\ln [y(r) - y_0]$ against r^2 for the 2.5 M urea solutions; the linear graph is that expected from a single, homogeneous material.

However, this evidence is not conclusive. If a mixture of two species (*e.g.*, dodecamer and monomer) were in rapid equilibrium, the mixture would behave to ultracentrifugation as if it were a single component. Since the enzyme dissociates rapidly into subunits, the possibility must be considered that the molecular weight around 60,000 is an artifact, produced by measuring a rapidly equilibrating mixture of materials of higher and lower molecular weights, which coincidentally appear as subunit dimer. However, in 4 M urea the Yphantis plots are curved, so that the presence of two different species is clearly evident. Further, although the possibility of different species in rapid equilibrium in 2.5 M urea solution cannot be completely ruled out, it may be considered only in combination with several additional and quite arbitrary assumptions, and is therefore unlikely. These additional *ad hoc* assumptions follow. The solution obtained in 2.5 M urea is enzymically inactive; therefore if one postulates a protein of higher molecular weight than the subunit dimer, this material cannot be native enzyme. On the other hand, 70% of enzymic activity can be recovered from the

solution in 2.5 M urea, whereas no method has yet been devised that will serve to recover activity from the subunits themselves. Therefore the material present in 2.5 M urea at 10° cannot contain ordinary subunits.

The existence of dimers of the subunits as discrete entities is further strengthened by the observations on sedimentation velocities and by the observations on the reconstitution of active enzyme from dissociated enzyme; both topics are discussed in the accompanying article (Tagaki and Westheimer, 1968). In the ultracentrifuge, a species has been observed with sedimentation constant about double that for the completely dissociated subunits; it is the major constituent of the solution under the experimental conditions here used for the determination of the molecular weight of the "dimer." The intermediate value of the sedimentation constant cannot easily be explained except by postulating a protein intermediate in size between native enzyme and the ultimate subunit. More significantly, when the enzyme is completely dissociated, all attempts at reconstitution of activity fail; when the enzyme is treated under the experimental conditions that have been shown to produce dimers of subunits, enzyme activity can be restored by dilution or dialysis (Tagaki and Westheimer, 1968). The formation of a species distinct from either native enzyme or the ultimate subunit therefore has been demonstrated.

Other chemical experiments are conveniently interpreted in terms of a subunit dimer. Acetopyruvate reacts with the enzyme to inactivate it, and in this inactivation a mole of acetopyruvate reacts with about 60,000 mol wt units (*i.e.*, with an amount equivalent to two subunits (Tagaki *et al.*, 1968). Similarly, acetic anhydride (O'Leary and Westheimer, 1968) reacts with the enzyme to inactivate it, with the introduction of one acetyl group for a weight equivalent to about two subunits. The chemical evidence is unfortunately not entirely firm, since the possibility exists that the purest enzyme so far obtained still contains inactive or inhibited subunits, and therefore the apparent stoichiometry may be really an artifact. Nevertheless, the physical evidence established the existence of dimers of the subunit as discrete particles.

Acknowledgments

The authors gratefully acknowledge the skilled technical assistance of Mr. Malcolm Smart, who operated the ultracentrifuge. We wish to thank Professor P. M. Doty for the use of the ultracentrifuge and Professor G. Guidotti for helpful discussions.

References

Charlwood, P. A. (1957), *J. Am. Chem. Soc. 79*, 776.
Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.
Fridovich, I., and Westheimer, F. H. (1962), *J. Am. Chem. Soc. 84*, 3208.
Hamilton, G., and Westheimer, F. H. (1959a), *J. Am. Chem. Soc. 81*, 2277.
Hamilton, G., and Westheimer, F. H. (1959b), *J. Am. Chem. Soc. 81*, 6332.
Hartley, B. S. (1964), *Nature 201*, 1284.
Kawahara, K., and Tanford, C. (1966), *Biochemistry 5*, 1578.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Laursen, R. A., and Westheimer, F. H. (1966), *J. Am. Chem. Soc. 88*, 3426.
Lederer, F., Coutts, S. M., Laursen, R. A., and Westheimer, F. H. (1966), *Biochemistry 5*, 823.
McMeekin, T. L., and Marshall, K. (1952), *Science 116*, 142.
Nozaki, Y., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 736.
O'Leary, M., and Westheimer, F. H. (1968), *Biochemistry 7*, 913 (this issue; paper 5).
Reithel, F. J., and Sakura, J. D. (1963), *J. Phys. Chem. 67*, 2497.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Sumner, J. B., and Gralen, N. (1938), *J. Biol. Chem. 125*, 33.
Tagaki, W., Guthrie, J. P., and Westheimer, F. H. (1968), *Biochemistry 7*, 905 (this issue; paper 4).
Tagaki, W., and Westheimer, F. H. (1968), *Biochemistry 7*, 891 (this issue; paper 1).
Tauber, H., and Petit, E. L. (1952), *J. Biol. Chem. 195*, 703.
Taylor, J. F., and Lowry, C. (1956), *Biochim. Biophys. Acta 20*, 109.
van Holde, K. E. (1967), Fractions, No. 1, Beckman Instruments, Inc.
Warren, S., Zerner, B., and Westheimer, F. H. (1966), *Biochemistry 5*, 817.
Werner, E. A. (1923), *J. Chem. Soc. 123*, 2577.
Westheimer, F. H. (1963), *Proc. Chem. Soc.*, 253.
Westheimer, F. H. (1968), *Methods Enzymol.* (in press).
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zerner, B., Coutts, S. M., Lederer, F., Waters, H. H., and Westheimer, F. H. (1966), *Biochemistry 5*, 813.