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Acetoacetate Decarboxylase. Subunits and Properties*

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ABSTRACT: Acetoacetate decarboxylase from *Clostridium acetobutylicum* has a molecular weight of about 260,000. On treatment with acid, base, urea, guanidinium chloride, or dodecyl sulfate the enzyme dissociates into subunits, but the subunits have not as yet been reassembled into active enzyme. Amino acid analysis of the enzyme is presented. The decarboxylase shows N-terminal methionine and C-terminal lysine. The evidence from these analyses, and from analyses for tryptophan and cysteine, and from peptide mapping suggest that the enzyme consists of eight subunits with molecular weight probably around 30,000–35,000. The enzyme shows an isoelectric point around 4.9, inconsistent with that calculated from its amino acid content; it shows an unexplained weak absorption band at 320 mμ. These and other problems are discussed.

The previous papers of this series (Zerner *et al.*, 1966; Warren *et al.*, 1966) presented the procedure for the preparation of acetoacetate decarboxylase from *Clostridium acetobutylicum* and the detailed evidence that a particular lysine residue in the enzyme forms a Schiff base with the substrate; the experiments show that this Schiff base is an intermediate in the decarboxylation process. The evidence in those papers concerning mechanism supplements that previously presented (Hamilton and Westheimer, 1959; Fridovich and Westheimer, 1962; Westheimer, 1963).

In the present paper, considerable chemical and physical data describing the enzyme are assembled and discussed. Some of these experiments have been mentioned in the abstract: the amino acid composition of acetoacetate decarboxylase, its N-terminal and C-terminal residues, and the "fingerprint" for the tryptic peptides are presented. The large sedimentation coefficient for the enzyme had been interpreted (Hamilton and Westheimer, 1959) to indicate that its molecular weight is several hundred thousand; the molecular weight has now been accurately measured. Further investigations have shown that the decarboxylase, like other high molecular weight proteins, consists of subunits; the conditions for dissociating the enzyme into these subunits, and for inactivating it, together with further chemical and physical data concerning its properties are outlined. Several unresolved problems concerning the structure of the decarboxylase are discussed.

Experimental Section

Materials. The crystalline decarboxylase was prepared according to Zerner *et al.* (1966). Urea (Merck), White Label 2,4-dinitrofluorobenzene, guanidine hydrochloride and mercaptoethanol (Eastman), and potassium cyanate (Baker Analytical Reagent) were used without further purification. The anionic detergents were donated by Professor John Law as samples he received from Dr. L. W. Beck, Proctor and Gamble. Acetopyruvate had been prepared by Colman (1962).

Eastman's White Label *p*-dimethylaminobenzaldehyde was dissolved in Du Pont Reagent Grade sulfuric acid for tryptophan analyses (Ehrlich's reagent). 5,5′-Dithiobis-(2-nitrobenzoic acid) was synthesized by the method of Ellman (1959) from Aldrich 2-nitro-5-chlorobenzoic acid; mp 238–240° dec; lit. 237–238°.

Bovine serum albumin (Nutritional Biochemicals),

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N-acetyl-L-cysteine (Nutritional Biochemicals), and glutathione (Mann Laboratories) were used as standards in analyses. α-Chymotrypsin (3× crystallized) and trypsin (2× crystallized) were purchased from Worthington Biochemical Corp. Carboxypeptidases A and B (Worthington) had been treated with diisopropylfluorophosphate by the supplier. Dinitrophenyl derivatives of all the natural amino acids, plus those of cysteic acid and methionine sulfone, were purchased from Nutritional Biochemical Corporation. DL-Tryptophan was obtained from Nutritional Biochemicals Corporation and Eastman Kodak Co. (White Label).

Sephadex G-25, bead form, was manufactured by Pharmacia, Uppsula, Sweden. Dowex-50 (Bio-Rad AG-50-W-X2, 200–400 mesh) was purchased from Calbiochem and washed with acid and water before use. Dowex 1-X 8 (200–400 mesh) was purchased from Baker Chemical Co. and washed with alcohol and hydrochloric acid (6 and 0.2 M). Adsorbsil-1 (10% $CaSO_4$ binder) was purchased from the Applied Science Laboratories, Pennsylvania State University. Valine hydantoin was synthesized from DL-valine and potassium cyanate; it melted at 146–146.5°, as compared with Stark and Smyth's (1963) value of 146–148°, and with Boyd's (1933) value of 145° for the L-isomer.

Anal. Calcd for $C_6H_{10}N_2O_2$: C, 50.69; H, 7.09; N, 19.70. Found: C, 50.72; H, 7.11; N, 19.53. Methionine hydantoin was prepared from DL-methionine and potassium cyanate, and, after recrystallization from water melted at 103–104°, as compared to the value of 103–105° reported by Pierson *et al.* (1948). Other chemicals were reagent grade, used without further purification, except as noted in the descriptions of methods that follow.

Methods

Ultraviolet spectra were determined with a Cary 14 recording spectrophotometer, or a Zeiss PMQII spectrophotometer. Assay for the enzyme was conducted as previously described (Fridovich and Westheimer 1962; Zerner *et al.*, 1966). Spectrophotometric determination of enzyme concentration was carried out by measuring the optical density at 280 mμ. A weight standard was established by measuring the ultraviolet absorption (in buffer solution) of an aliquot that had been dialyzed against distilled water; the remainder of the sample was lyophilized and weighed on a Cahn Electrobalance. By coincidence, the optical density at 280 mμ is unity at a concentration of enzyme of 1 mg/ml.

Experiments on Denaturation of the Enzyme. The assay of enzymic solutions was generally carried out by mixing 2.6 ml of 0.10 M (pH 5.92) phosphate buffer, 0.3 ml of acetoacetate solution, and 0.1 ml of the solution to be tested, and measuring the change in optical density at 270 mμ on the Zeiss PMQII spectrophotometer in a thermostated cell compartment at 30°. However, this method is inadequate for studies of denaturing agents, since it dilutes the test solution 30-fold. If, therefore, the enzyme were inactivated reversibly by the reagent, the dilution of that reagent during assay could cause an instantaneous reversion to active enzyme. Therefore, assays with urea as denaturing agent were carried out by preparing the buffer in a solution of urea 10% more concentrated than the test solution; when the three assay solutions (buffer, enzyme, and acetoacetate solutions) were mixed in the spectrophotometer cell, the concentration of urea was the same as that of the test (enzyme) solution. At the same time, in an additional assay, the test solution was diluted with ordinary buffer, so as to reduce the urea concentration 30-fold. After 15-min incubation of this diluted solution, it was likewise assayed. These procedures gave two sets of results—one with and one without dilution of the urea; *i.e.*, one without and one with an opportunity for the enzyme to recover from the effects of the denaturing agent. Similar experiments were conducted with sodium dodecyl sulfate. However, in the experiments with guanidinium chloride and in the experiments on the effect of pH on the enzyme all assays were conducted after dilution with pH 5.92 buffer, so that the enzyme may partially have recovered from denaturation prior to measurement of its activity.

Ultracentrifugal Measurements. Measurements of sedimentation velocity were obtained with a Beckman-Spinco analytical ultracentrifuge, Model E, which was made available to us through the courtesy of Professor Paul Doty. The ultracentrifuge was equipped with a phase plate for schlieren optics, using 12 mm, 4° sector centerpieces. The position of the boundaries were read from photographic slides with a two-coordinate Gaertner microcomparator. Densities of the buffers were measured by pycnometry. Measurements by the Archibald method were made with the same equipment.

Amino acid analyses were conducted on a Beckman-Spinco amino acid analyzer, Model 120 B, equipped for accelerated analysis. Its recorder was equipped with a resistor that allowed the scale to be expanded so that full pen deflection occurred over the region of 0.0–0.1 in optical density. Calibrations were carried out with standard mixtures containing 0.05 μmole/ml of each amino acid. Analytical samples applied to the column were obtained by the hydrolysis of 0.1–0.15 mg of protein. DL-Norleucine and α-amino-δ-guanidopropionic acid were used as internal standards. The protein was oxidized with performic acid prior to hydrolysis by the method of Hirs (1956) or of Moore (1963).

Starch gel electrophoresis was conducted with a Buchler Instruments, Inc., apparatus, made available to us by Professor John Law. The gel was prepared from 65 g of Connaught Medical Laboratories' Starch-Hydrolyzed and 500 ml of the appropriate buffer. The starch was stained with Buffalo Black (400 mg in 1 l. of a 5:5:1 mixture of methanol–water–glacial acetic acid). Starch gel electrophoresis was run vertically with 80 μg of protein for about 14 hr at 125 v (about 4 v/cm); the temperature of the gel was held below 28°. Phosphate buffers (pH 8.00, 7.15, and 5.92) and acetate buffers (pH 5.07, 4.77, 4.54, 4.30, and 4.17) were employed, at a concentration of 0.025 M for the gel and 0.05 M in the

electrode chambers. Experiments near the isoelectric point were also conducted with the poles reversed; the results were not significantly different.

Dinitrophenylation of the enzyme or of the oxidized enzyme was carried out by Sanger's procedure, with modifications (Fraenkel-Conrat *et al.*, 1956) and in particular using the thin layer chromatography of Brenner *et al.* (1961, 1965). When the protein was oxidized, the method of Hirs (1955) was used, with the data of Toennies and Homiller (1942), to calculate the amount of performic acid needed. The dinitrophenylated proteins were hydrolyzed according to Moore and Stein (1963). Extraction of the hydrolysates and paper chromatography was carried out by the method of Fraenkel-Conrat *et al.* (1956).

Action of Carboxypeptidases. Acetoacetate decarboxylase was treated with both carboxypeptidase A and B. Reaction with carboxypeptidase A was attempted in pH 8.3 Tris buffer, as described by Hirs *et al.* (1960) and in 6 M urea, pH 8, according to the method of Halsey and Neurath (1955). Neither of these procedures resulted in the hydrolysis of the decarboxylase. Digestion of the enzyme with carboxypeptidase B also followed the procedure of Hirs *et al.* (1960) except that a ninhydrin negative buffer (pH 8 sodium borate) was used. Worthington carboxypeptidases A and B had been treated with diisopropylfluorophosphate.

Peptide Mapping. A solution of acetoacetate decarboxylase in ammonium bicarbonate buffer was lyophilized, and the resulting powder dissolved in sufficient 0.1 M ammonium hydroxide solution to give a concentration of 3–10 mg/ml. The solution was heated for 3 min on the steam bath to denature the protein, and then cooled to room temperature. A drop of phenol red indicator was added, and sufficient Dry Ice to turn the indicator orange; at this point the protein precipitated. The solution was adjusted to pH 7–8 (pink color) with ammonium carbonate, and trypsin (1% in 0.001 M HCl) was added to the extent of 2% of the weight of the decarboxylase. The solution was incubated at 37° for 8–16 hr.

The mapping procedure was essentially that of Katz *et al.* (1959), *i.e.*, chromatography on Whatman 3MM paper in 1-butanol–acetic acid–water (4:1:5) in one direction, followed by electrophoresis in pyridine–acetic acid–water (1:10:289) at pH 3.5 at right angles. The electrophoresis was carried out with a Savant Instruments, Inc., water-cooled high-voltage paper electrophoresis apparatus. After the peptide maps were dried, they were developed with 0.2% ninhydrin in acetone–pyridine (100:1) according to Fondy *et al.* (1964). The peptides were made visible with ninhydrin; tryptophan was detected with Ehrlich's reagent, according to the method of Rodnight (1956). The reagent for paper chromatography consisted of 1.66 g of *p*-dimethylaminobenzaldehyde in 180 ml of acetone and 20 ml of concentrated HCl; the peptide map was dipped in this solution.

Carbamylation of the Enzyme. Lyophilized enzyme in 8 M urea solution in a pH 8 *N*-ethylmorpholine–acetate buffer was allowed to react with potassium cyanate according to Stark and Smyth (1963), and the resulting carbamylated protein was desalted over a Sephadex column. The hydantoin corresponding to the terminal amino acid was set free by reaction in HCl–acetic acid solution at 100° for an hour, and the hydantoin separated from hydrolysis products of the enzyme by chromatography over Dowex 50. The pure hydantoin may then be hydrolyzed with 0.2 M NaOH, and the resulting amino acid identified with the Beckman-Spinco amino acid analyzer. Valine hydantoin was added as an internal standard before alkaline hydrolysis in quantitative experiments to estimate the amount of N-terminal amino acid.

Identification of Methionine. The position of the methionine sulfoxide peaks in the amino acid analyzer (Spackman *et al.*, 1958) was checked by heating methionine hydantoin with 0.2 M NaOH in a sealed and evacuated tube for 24 hr at 110°. The resulting solution was evaporated to dryness; the residue was dissolved in a minimum of water, and chromatographed on the amino acid analyzer both with and without aspartic acid as a marker. The chromatograms showed the presence of methionine sulfoxides as well as methionine (see Results). The methionine hydantoin was alternatively converted completely to the sulfone by oxidizing it with performic acid by the method of Moore (1963). The oxidized hydantoin was then hydrolyzed and the resulting compounds analyzed chromatographically on the amino acid analyzer. Test for cysteic acid as a possible end group was made by the method of Stark and Smyth (1963).

Hydrazinolysis. The enzyme was subjected to hydrazinolysis by the method of Spero *et al.* (1965). Samples of lyophilized acetoacetate decarboxylase were subjected to hydrazinolysis at 110°. Extraction of the reaction mixtures was carried out as described by Spero *et al.* (1965). An internal standard (0.05 μmole of arginine or of γ-guanidino-α-aminopropionic acid) was added for amino acid analysis.[1] The aqueous supernatants from the benzaldehyde treatment were evaporated to dryness on a rotary evaporator, and the residues, dissolved in pH 2.2 citrate buffer, were applied directly to a column of the amino acid analyzer.

Reactivation of Denatured Enzyme. A specific experiment where reactivation was attempted after the decarboxylase had been inactivated by urea is as follows: A sample with 0.36 mg/ml of enzyme was inactivated with 8 M urea for 1 hr at room temperature. It was then diluted 100 times with 0.1 phosphate buffer that contained 0.01 M mercaptoethanol and 0.1 mg/ml of bovine serum albumin (Epstein *et al.*, 1964). From time to time the diluted solution was assayed; after 24 hr it had recovered about 2% of its original activity.

Results

Molecular Weight of the Enzyme. The molecular weight of the enzyme was determined by the approach to

[1] If the standard is added before hydrazinolysis, the guanidino group will be destroyed (Akabori *et al.*, 1956).

TABLE I: Sedimentation Constant for Acetoacetate Decarboxylase.

Concn (mg/ml)	s_{20} (S)[a]
0.992	12.59
1.448	12.62
1.984	12.64
2.480	12.59

[a] Corrected for buffer density (1.0033).

equilibrium method of Archibald, as reported by Schachman (1957). The partial specific volume was estimated from the amino acid composition, as explained by Cohn and Edsall (1943), as 0.740 cc/g. The density of the phosphate buffer was 1.0033. Using a concentration of 9.7 mg/ml and an angular velocity of 2994 rpm at 19°, the molecular weight calculated from the top meniscus was 255,000 ± 7000 and that from the bottom meniscus 263,000 ± 7000. The sedimentation coefficient proved independent of concentration over the range from 1 to 2.5 mg/ml as shown in Table I.

Amino Acid Analyses. Seven separate analyses of the amino acid composition of the enzyme have been made. The results of these analyses are shown in Table II, where the figures represent micromoles of amino acid per milligram of protein. To obtain the final analyses, the values for valine and isoleucine were extrapolated to infinite time, to take account of the slow hydrolysis of peptides of these amino acids, whereas the values for serine and threonine were extrapolated to zero time to take account of the decomposition of these amino acids to ammonia and pyruvate or ketobutyrate, respectively. These extrapolation methods are discussed by Moore and Stein (1963).

The determinations of cysteine and of methionine were made with solutions of the decarboxylase that had been oxidized with performic acid. The ratios of the cysteic acid and methionine sulfone to aspartic acid, glutamic acid, glycine, and alanine were determined, and the amounts of cysteine and methionine estimated from these ratios and from the analyses of these stable nonsulfur-containing amino acids as recorded in Table II. The average of three separate sets of analyses for cysteic acid and methionine sulfone are included in the table. The amide nitrogen was determined in a separate experiment, where a control mixture containing all the components except the protein was subjected to the hydrolytic conditions and analyzed alongside the sample. The ratio of moles of ammonia to those of lysine is 0.844; the ratio to arginine is 1.317. These correspond

TABLE II: Amino Acid Analyses of Acetoacetic Acid Decarboxylase.[a]

	Experiment Number								
	1	2	3	4	5	6	7	8	9
Amino Acid	Time of Hydrolysis (hr)								Av Corrected for Moisture
	22	22	44	44	72	72	96	Av	
Lys	0.560	0.561	0.583	0.634	0.527	0.529	0.524	0.560	0.611
His	0.256	0.253	0.220	0.296	0.260	0.257	0.224	0.252	0.275
NH_3								0.465	0.507
Arg	0.357	0.349	0.361	0.365	0.311	0.317	0.368	0.347	0.380
Asp	0.774	0.774	0.821	0.769	0.823	0.799	0.839	0.800	0.879
Thr	0.399	0.417	0.395	0.390	0.397	0.375	0.372	0.424[b]	0.446[b]
Ser	0.340	0.357	0.298	0.322	0.282	0.274	0.260	0.395[b]	0.434[b]
Glu	0.507	0.540	0.559	0.534	0.550	0.549	0.581	0.546	0.600
Pro	0.547	0.507	0.562	0.499	0.556	0.554	0.578	0.543	0.597
Gly	0.400	0.417	0.419	0.409	0.436	0.418	0.427	0.418	0.459
Ala	0.558	0.576	0.601	0.558	0.575	0.580	0.578	0.575	0.632
0.5 Cys								0.0837[c]	0.0913[c]
Val	0.406	0.311	0.485	0.472	0.516	0.500	0.503	0.508[b]	0.558[b]
Met	(0.0733)	(0.0807)	(0.0553)	(0.0459)	(0.0380)	(0.0288)	(0.140)	0.230[c]	0.253[c]
Ileu	0.404	0.412	0.498	0.477	0.533	0.546	0.515	0.542[b]	0.596[b]
Leu	0.707	0.729	0.732	0.703	0.753	0.754	0.752	0.733	0.805
Tyr	0.377	0.393	0.335	0.335	0.290	0.319	0.422	0.435[b]	0.478[b]
Phe	0.260	0.223	0.217	0.234	0.217	0.232	0.230	0.230	0.253

[a] Values are given as millimoles of amino acid per gram of protein. [b] Extrapolated values. [c] Values derived from performic acid oxidized samples.

to 0.473 and 0.457 μmole of ammonia per mg of protein.

Almost all the amino acid analyses were carried out with protein that had been dialyzed against distilled water and subsequently lyophilized. The moisture content of the lyophilized protein was determined by drying a sample for 4 days at 110°. The slightly higher values for the amino acid contents indicate that the lyophilized protein contained 9% water. Recovery of amino acids from the dried protein amounted to 96% of theory. The molecular weight of the subunits of the protein has not yet been firmly established (see Discussion). However, the amino acid content of the protein, calculated for illustration on the assumption of a subunit weight of 33,000, is shown in Table III.

TABLE III: Amino Acid Composition of Acetoacetic Acid Decarboxylase.

Amino Acid	No. of Residues Calcd for 33,000g	
	Found	Integral No.
Lys	20.2	20
His	9.1	9
NH_3	16.7	17
Arg	12.5	12–13
Asp	29.0	29
Thr	15.4	15
Ser	14.3	14
Glu	19.8	20
Pro	19.7	20
Gly	15.1	15
Ala	20.8	21
0.5 Cys	3.0	3
Val	18.4	18
Met	8.3	8
Ileu	19.7	20
Leu	26.6	27
Tyr	15.8	16
Phe	8.3	8
Trp		1

Isoelectric Point. The experimentally obtained mobilities in starch gel electrophoresis for the enzyme are plotted in Figure 1 *vs.* pH. At pH 4.30, the enzyme was partially denatured, as shown by a long trailing streak on the electrophoretogram; at pH 4.17, it was completely denatured, and stayed at the origin. The relative stability of the enzyme in solution at this pH makes this result surprising, but other data suggest the possibility that it is an effect of insufficient ionic strength. Figure 1 shows that the isoelectric point is around pH 4.9. This value is consistent with the preliminary finding of Colman (1962).

Analysis for Metal Ions. Professor Bert Vallee (Peter Bent Brigham Hospital) was kind enough to arrange for analyses of acetoacetate decarboxylase for metal ions. Samples were prepared for analysis by prolonged dialysis of the protein against redistilled water, followed by lyophilization. The lyophilized protein was completely inactivated, and almost water insoluble. Several small samples were analyzed, but no metal was found in large concentration except calcium, and Professor Vallee wrote[2] that this is not an unusual contaminant even for proteins at an advanced stage of purification. One sample of decarboxylase contained about 0.3 atom of zinc for 33,000 mol wt units, but other samples were essentially zinc free. It is unlikely that essential metal was lost during dialysis against water, since dialysis of the enzyme against buffered solutions of EDTA (ethylenediaminetetraacetate) or 8-hydroxyquinoline-5-sulfonic acid does not affect enzymic activity.

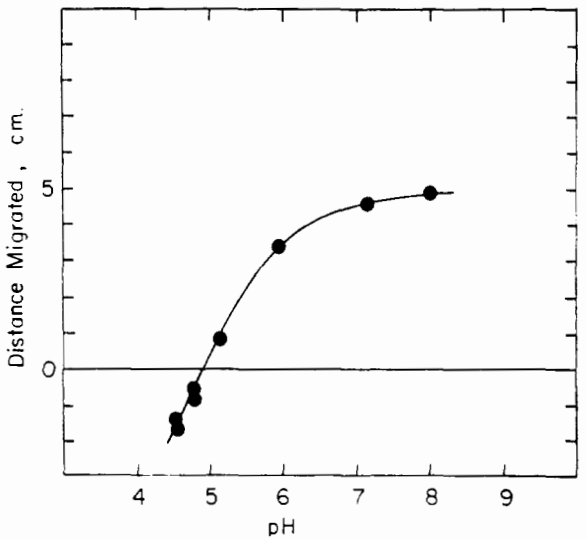

FIGURE 1: Mobility of the decarboxylase in starch gel electrophoresis as a function of pH, at 25–28° and 4 v/cm for 14 hr.

Analysis for Cysteine. Spectrophotometric analyses for cysteine, according to the method described by Ellman (1959), with 5,5′-dithiobis-(2-nitrobenzoic acid) were performed by measuring spectrophotometrically (Zeiss PMQII) the concentration of the 4-nitro-3-carboxyphenylthiolate anion formed by mercaptan–disulfide interchange between the enzyme and the reagent. The extinction coefficient of the anion at 412 mμ at pH 8 is 1.36×10^4. Control experiments with glutathione and with *N*-acetyl-L-cysteine showed exactly 1.0 sulfhydryl (SH)[3] group per molecule. Similar results were obtained with glutathione in buffer solution and in 4.0 M guanidinium chloride, pH 8.0.

When a solution of 1 mg of decarboxylase in 1 ml of

[2] Letter, B. Vallee to F. H. Westheimer, April 22, 1964.

[3] Abbreviation used in this work: SH, sulfhydryl or thiol group.

4 M guanidinium chloride, pH 8.0 was treated under nitrogen with Ellman's reagent the optical density measured immediately corresponded to about 2.2 sulfhydryl groups in 33,000 mol wt units. A second experiment confirmed the first. This number is less than that obtained by amino acid analysis of the oxidized protein, where 3 moles of cysteic acid was found per 33,000 g.

When the reaction was carried out in buffer, pH 8.0, but in the absence of guanidinium chloride, the reaction between the decarboxylase and the reagent was no longer instantaneous. After about an hour, the rate diminished sharply, but a slow and persistent increase in optical density continued; by contrast, no reaction at all was observed between Ellman's reagent and chymotrypsin which has no sulfhydryl groups (Tristram and Smith, 1963). When a correction was made for the slow reaction by extrapolating the final slope of the curve (OD *vs.* *t*) to zero time, the values of the optical density so found corresponded to 0.95 and 0.97 sulfhydryl group for each 33,000 mol wt units. Each subunit appears to contain one readily accessible sulfhydryl group, and a second that can be titrated only after the enzyme has been denatured. A third half-cystine residue has not yet been accounted for.

Analysis for Tryptophan. The analysis for tryptophan by the method of Spies and Chambers (1949) is essentially a colorimetric determination with Ehrlich's reagent. It was monitored with bovine serum albumin, which showed 2.06 and 2.07 molecules of tryptophan per molecule, in substantial agreement with Edsall and Wyman (1958) and Tristam and Smith (1963). With 0.402 mg of acetoacetate decarboxylase, the method showed 12.5 μg of tryptophan, corresponding to 1.03 moles of this amino acid per 33,000 g of enzyme; in a duplicate experiment, the ratio found was 0.96. In the peptide map of the tryptic peptides from the decarboxylase only one of the peptides gave a color with Ehrlich's reagent, and, therefore, only one peptide contains tryptophan.

Amino End Group. DNP (dinitrophenyl) Method. Acetoacetate decarboxylase was dinitrophenylated by the method of Fraenkel-Conrat *et al.* (1956). About 5 mg of dinitrophenylated protein was hydrolyzed for 16 hr in 1 ml of 6 N HCl by the method of Moore and Stein (1963). The hydrolysate was extracted to separate the neutral from the basic amino acids, and the paper chromatography was conducted as described in the section on Methods. Only one spot was obtained (in addition to those for dinitrophenol and dinitroaniline) among the ether soluble components. None except those anticipated (mono-DNP-lysine,-tyrosine, and-histidine) was found among the water soluble derivatives. A separate test for arginine with Sakaguchi's reagent (Fraenkel-Conrat *et al.*, 1956) was negative. The one new spot among the neutral derivatives corresponded to DNP-methionine. The spot was cut from the two-dimensional chromatogram and the colored material extracted and rechromatographed along with synthetic DNP-methionine on a thin layer plate of silica gel with chloroform–methanol–acetic acid (95:5:1) as solvent.

Similar experiments with acetoacetate decarboxylase that had been oxidized with performic acid gave a different spot (in addition to those of dinitrophenol and dinitroaniline), which was identified as dinitrophenyl-methionine sulfone by thin layer chromatography, along with an authentic sample, on Adsorbsil-I, using both the solvent system of Biserte and Osteux (1951) and chloroform–methanol–acetic acid (95:5:1). The dinitrophenylation procedures, however, did not yield quantitative results.

Amino End Group. Hydantoin method. Quantitative results were obtained by the method of Stark and Smyth (1963) for carbamylation of the enzyme. Preliminary qualitative analysis by the hydantoin method showed that methionine is the N-terminal amino acid, although several other amino acids were detected as minor contaminants. Cysteine was ruled out in a separate analysis. The original amino acid analyses showed considerable variations, and low results; in four separate experiments, the yields of methionine, obtained from the hydantoin, were 0.36, 0.50, 0.62, and 0.75 mole for 33,000 mol wt units. However, the variation of the yield of methionine was correlated with twin peaks, one of which is coincident with the aspartic acid position. The twin peaks were identified as the two stereoisomeric methionine sulfoxides (Levine, 1947) by analysis of a solution prepared by hydrolyzing a synthetic sample of methionine hydantoin with base. Apparently even with moderate precautions to remove air during alkaline hydrolysis, methionine is partially autoxidized to its sulfoxide. To obtain quantitative data two procedures were used: (1) the methionine was oxidized to the sulfone, which chromatographs on the amino acid analyzer as a separate peak between aspartic acid and threonine (Hirs, 1956), and (2) the mixture containing the sulfoxides was analyzed on a longer (150 cm) column which separates the sulfoxide peaks from that of aspartic acid.

The fraction corresponding to neutral and acidic hydantoins except those of tryptophan and homocitruline (Stark and Smyth, 1963) was oxidized with performic acid, and the excess performic acid was destroyed by the method of Moore (1963). After the mixture was hydrolyzed with 0.2 N NaOH, it was chromatographed on the amino acid analyzer; the expected peak for methionine sulfone was obtained in the fraction that appeared at 54 ml of effluent. The peak area corresponded to 0.81 residue of methionine sulfone for 33,000 mol wt units.

Two analyses of the unoxidized amino acid mixture were performed for us by Dr. William Knight on a Beckman-Spinco amino acid analyzer equipped with a 150-cm column. Here the three expected peaks were separated: that of methionine and the two peaks that have been identified as those of the sulfoxide. The peaks for the methionine sulfoxide were integrated by the absorbance method (Spackman *et al.*, 1958). The total of methionine plus the sulfoxide in two experiments corresponded to 1.0 and 1.1 residues per 33,000 mol wt units.

Carboxyl End Group. Acetoacetic acid decarboxylase

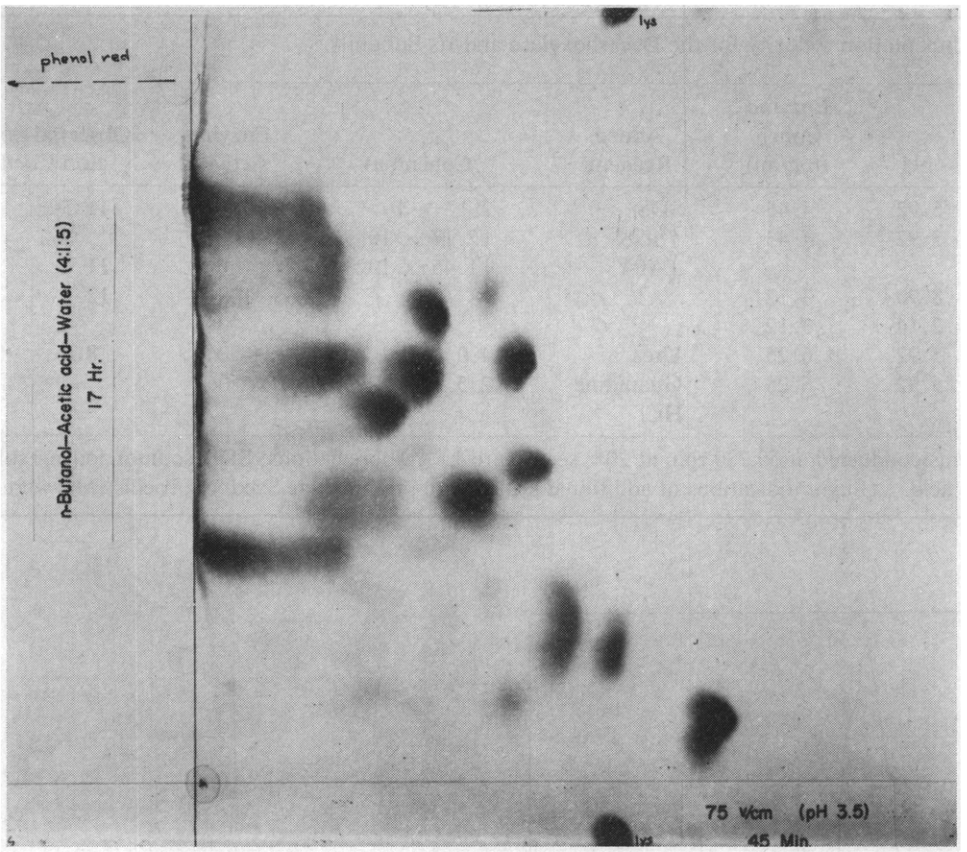

FIGURE 2: "Fingerprint" of the peptides obtained on tryptic hydrolysis of acetoacetate decarboxylase. Horizontal direction: paper chromatography with butanol–acetic acid–water (4:1:5); vertical direction, electrophoresis at 75 v/cm at pH 3.5. The lysine and phenol red spots are added markers.

is inert to the action of carboxypeptidase A (see Experimental Section). Carboxypeptidase B attacks the enzyme, and lysine appears early in the hydrolysate; it was identified with the amino acid analyzer. The known specificity of these enzymes (Neurath, 1960) reinforces the finding of lysine as the C-terminal amino acid. Attempts to quantitate the reaction with carboxypeptidase B, however, failed. The amount of lysine liberated increases linearly with time and there is no apparent leveling-off, or break in the curve for amount of lysine against time for 24 hr. Furthermore, by this time all the other amino acids were also present, many of them in quantity. Additional study will be required to obtain quantitative results with this system.

The qualitative result was confirmed, and the quantitative answer obtained with the method of hydrazinolysis (Spero *et al.*, 1965; Akabori *et al.*, 1956). After hydrazinolysis and extraction with benzaldehyde only one amino acid could be detected chromatographically; this was lysine. Two quantitative experiments were performed, both with added arginine as a standard. The yield of lysine in these experiments was 0.92 and 0.99 mole/33,000 mol wt units. In addition, several experiments were carried out with α-amino-δ-guanidinoproprionic acid as a standard; although the standard peak chromatographed too close to the ammonia peak for accurate integration, the experiments showed that no arginine was released during the hydrazinolysis.

Peptide Mapping. The peptide map of the tryptic peptides is shown in Figure 2.

Ultraviolet Spectrum. The ultraviolet spectrum of the crystalline enzyme is presented in Figure 3. In addition to the usual protein absorption at 280 mμ, the enzyme shows a low but distinct band at 320 mμ. This band has been present in all samples of the enzyme—those from *Clostridium madisonii* (Lee, 1965) as well as those from *Cl. acetobutylicum*. The band is undiminished on chromatography of any particular sample of the enzyme on diethylaminoethylcellulose or Sephadex, or on recrystallization, or on dialysis against buffer or EDTA. However, the earlier samples of enzyme, with lower specific activity (Zerner *et al.*, 1966), showed a somewhat larger 320 mμ band than that shown in Figure 3. The cause of this absorption is unknown. It is not removed on borohydride reduction (Warren *et al.*, 1966) but loses definition on denaturation of the enzyme with urea, with acid, or with detergent.

Subunits. Like other large proteins, acetoacetate decarboxylase consists of subunits. The existence of subunits has been identified in ultracentrifugal experiments, but their molecular weight has so far been obtained only by chemical analysis. Examples of sedi-

TABLE IV: Sedimentation Velocity for the Decarboxylase and Its Subunits.[a]

Expt	pH	Enzyme Concn (mg/ml)	Added Reagent[b]	Concn (M)	Enzyme Activity	Observed Sedimentation Coefficients	
A	5.92	4.45	SDS	2.17×10^{-2}	0	11.5[c]	2.4
B	5.92	4.45	SDS APY	2.17×10^{-2} 1.45×10^{-2}	0	11.4	2.4
C	8.90	3.12	...	...	100%	12.5	
D	2.14	3.12	...	...	0		1.2
E	5.92	6.25	Urea	4.0	~20%	8.0[c]	1.2
F	5.92	6.25	Guanidine HCl	2.5	0		1.2

[a] Experiments conducted at 59,780 rpm at 20°; see Figure 4. [b] Abbreviations: SDS, sodium dodecyl sulfate; APY, acetylpyruvic acid. [c] Slight indications of additional peaks, with intermediate Svedberg coefficients, were observed.

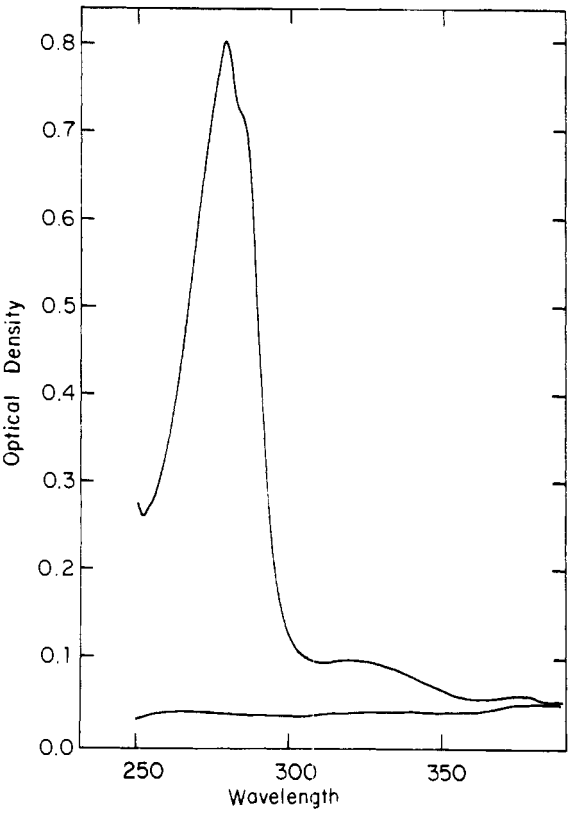

FIGURE 3: Ultraviolet spectrum of acetoacetate decarboxylase.

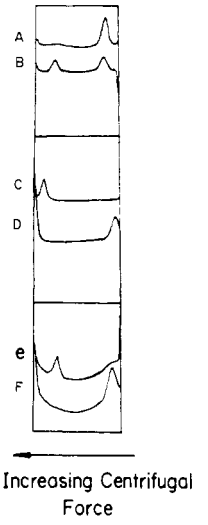

FIGURE 4: Sedimentation velocity at 59,780 rpm at 20°. The experiments are those described in Table IV. (A) Sodium dodecyl sulfate. (Photograph taken 68 min after rotor reached full speed.) (B) Same as (A), except that mixture was preincubated with acetopyruvic acid. (C) pH 8.9. (Photograph taken 90 min after rotor reached full speed.) (D) pH 2.14. (E) 4 M urea. (Photograph taken 88 min after rotor reached full speed.) (F) 2.5 M guanidinium chloride.

mentation velocity experiments are shown in Table IV and Figure 4, where both the intact enzyme and the subunits are visible.

Attempts to determine the molecular weight of the subunits by the Archibald method have so far proved unsuccessful. The experiments have been restricted to enzyme denatured with acid (pH 2.05 glycine buffer). The data are internally inconsistent, with different values for the molecular weight calculated from the upper and lower menisci. Although chemical analysis suggests a probable size for the subunits, an accurate determination of molecular weight by physical means is obviously a first priority for further research with the decarboxylase.

Stability of the Enzyme and Its Inactivation. The stability of the enzyme to pH, urea, guanidinium chloride, and detergents is recorded in Figures 5–8. These

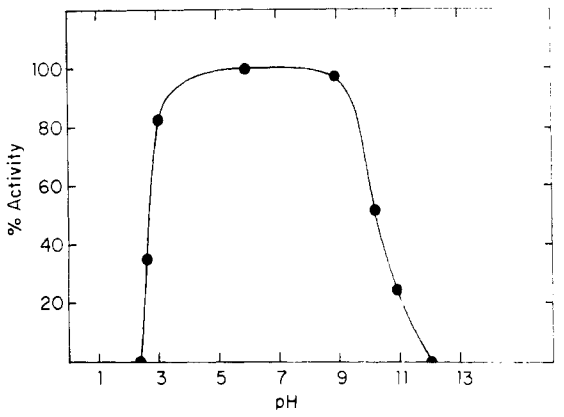

FIGURE 5: Stability of acetoacetate decarboxylase at 25° as a function of pH. These assays were performed with samples of the less active, more stable enzyme originally obtained; see text.

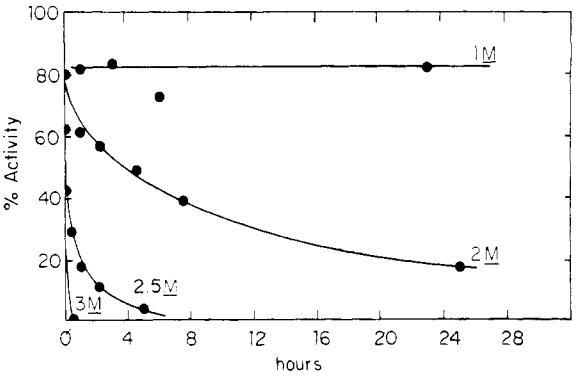

FIGURE 6: Effect of guanidinium chloride on the activity of acetoacetate decarboxylase at pH 5.9. These assays were performed after diluting the test solution 30-fold with buffer; see text.

data are approximate. As has been pointed out (Zerner *et al.*, 1966) the activity of recent preparations of the enzyme is higher than that found earlier, but its stability is less.[4]

The stability of the enzyme as a function of pH at 25° is shown in Figure 5. (These data were obtained with old enzyme.) The action of guanidinium chloride is shown in Figure 6. Guanidinium chloride at 1 M causes an instantaneous diminution of activity of about 20%; the activity however remains fairly constant with time. Fridovich (1963) has shown that the decarboxylase is about 50% inhibited by 0.05 M chloride ion. In the assay solutions, the 1 M guanidinium chloride has been diluted 30-fold, so that the concentration of chloride ion is about 0.03 M—a concentration that should have produced a somewhat greater inhibition than that observed. However, with concentrations of guanidinium chloride higher than 1 M, a time-dependent and eventually complete denaturation occurs. Experiments with urea are shown in Figure 7; its behavior differs from that of guanidinium chloride in that, since urea is electrically neutral, no instantaneous anion inhibition is observed. However, the slow and progressive denaturation is similar for both reagents. As noted earlier, the enzyme as prepared several years ago was less active but more stable than that now at hand, although the two materials appear chromatographically the same, and crystallize in the same manner. With the old enzyme, the rate of denaturation by urea is much less than with the more active enzyme recently prepared. Typical procedures for attempts to reactivate enzyme that had been allowed to stand in urea solution are described in the Experimental Section. The data obtained with 3 M urea are shown in Figure 8.

The effect of sodium dodecyl sulfate is shown in Figure 9. The ultracentrifugal results illustrated in Figure 4 were obtained with solutions about 0.02 M, *i.e.*, corresponding to the most concentrated solutions here shown, and therefore to complete denaturation and loss of activity. The effect of sodium dodecyl sulfate can be partially prevented by acetopyruvate (Figure 4 (B)). At concentrations of sodium dodecyl sulfate lower than 1.2×10^{-3} M, the inhibitory effect of the detergent was completely reversible, and resembled that of other anions (Fridovich, 1963); at concentrations slightly above this value, the denaturation is time dependent and irreversible (Figure 8).

Discussion

The data here presented show that acetoacetate decarboxylase has a molecular weight of about 260,000, and consists of subunits that can be observed in sedimentation velocity experiments. A few of the experiments are presented in Table IV, and in Figure 4. In expt A and B, the intact protein as well as subunits were observed; in (C), only the intact protein was present. The sedimentation constants in Table IV have not been corrected for the density or viscosity of the solutions. These corrections are minimal for the solutions in expt A–D, but large for expt E, conducted in 4 M urea. When the appropriate corrections are applied, the calculated sedimentation constant is about 11.7; quite probably the species observed in (E) is the same as that seen in (A)–(D). Since the diffusion coefficients for the subunits have not been measured, the molecular weights cannot be calculated from these sedimentation velocities, and the large difference between the sedimentation constant observed in dodecyl sulfate solution (expt A and B) and those observed in acid, urea, or guanidine (expt D–F) cannot yet be interpreted. Perhaps considerable detergent is adsorbed on the subunits, raising their molecular weights, but further experimentation is needed to clarify the situation.

Analytical chemistry, however, suggests that the

[4] The earlier preparations of acetoacetate decarboxylase are here referred to as old enzyme.

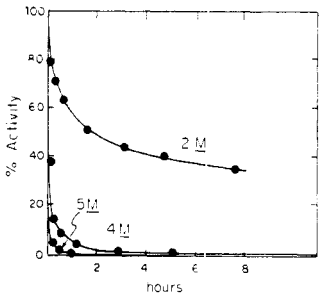

FIGURE 7: Effect of urea on the activity of acetoacetate decarboxylase at pH 5.9. The assay solutions contained the indicated concentrations of urea.

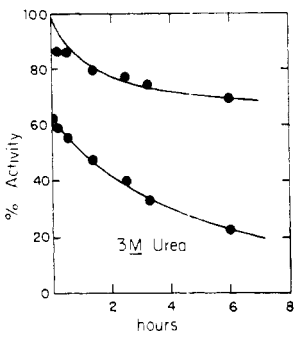

FIGURE 8: Recovery of enzymic activity after treatment of acetoacetate decarboxylase with 3 M urea. Lower curve: assay solutions contained 3 M urea. Upper curve: solutions diluted 30-fold 15 min prior to assay, pH 5.9.

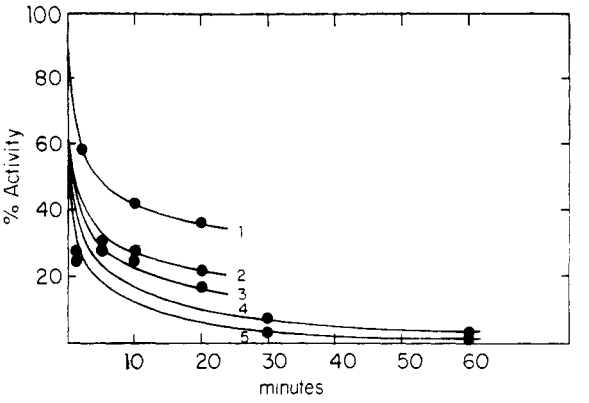

FIGURE 9: Effect of sodium dodecyl sulfate on the activity of acetoacetate decarboxylase. Molarity of sodium dodecyl sulfate for curves 1–5, respectively: 1.38×10^{-3} M, 1.84×10^{-3} M, 2.46×10^{-3} M, 1.65×10^{-2} M, and 3.30×10^{-2} M, pH 5.9.

enzyme consists of eight identical subunits with molecular weight somewhere between 30,000 and 35,000. The evidence is this: (1) Tryptic hydrolysis followed by peptide mapping shows that about 20–25 distinguishable peptides are produced on the tryptic digestion of the denatured protein. Inspection of Figure 2 shows that the exact number is uncertain; some of the peptides separate partially on chromatography, but remain near the origin in electrophoresis, so that counting them is difficult; furthermore, a number of weak spots are somewhat questionable. Despite the rather inexact nature of these data from tryptic peptides, they strongly suggest subunits. The enzyme contains 12 arginine and 20 lysine residues for 33,000 mol wt units, so that 32 separate tryptic peptides would have been expected for a molecular weight of 33,000. The peptide map (or "fingerprint") certainly suggests that the molecular weight of the subunit is moderate, and is consistent with a weight even less than 33,000. (2) The enzyme contains approximately one tryptophan residue for 33,000 mol wt units. Provided that there is only one kind of subunit, the molecular weight must be either around 33,000 or a higher multiple of this value; peptide mapping favors the first alternative both because of the number of peptides found and because mapping shows that only one peptide contains tryptophan. (3) Methionine appears to be the only N-terminal group; quantitative work shows 0.8–1.1 methionine end-groups per 33,000 mol wt units. (4) Lysine appears to be the only C-terminal group; quantitative determinations show ~0.95 lysine residue per 33,000 mol wt units. The analytical data for the N-terminal and C-terminal groups, the analytical data for tryptophan, together with the data for peptide mapping, are all consistent with a single chain of molecular weight around 30,000–35,000 for the subunits of acetoacetate decarboxylase. However, the precise molecular weight for the subunits has not yet been obtained.

Although the existence of subunits for the enzyme, and much of the analytical data, are now reasonably clear, several problems concerning the structure and composition of the decarboxylase still remain. The number of cysteine residues in the decarboxylase is not certain. Amino acid analysis shows three half-cysteine residues for each subunit, whereas analysis for free —SH shows less than three, and perhaps only two; the problem remains to be clarified. The isoelectric point also presents a challenge. The amino acid composition of the enzyme (Table III) shows the presence of 20 lysine residues, 12–13 arginine residues, and 9 histidine residues per 33,000 mol wt units. These are partially compensated by 29 aspartic acid residues and 20 glutamic acid residues, where 17 of these latter are bound to ammonia as amides. At pH 5, one would expect this protein to be heavily positively charged; in fact, the amino acid content would suggest that the isoelectric point should be close to the pK of histidine, *i.e.*, about 7. Even if the analysis for ammonia is somewhat too high (because of the partial decomposition during acid hydrolysis of threonine and serine to generate ammonia) the relationship between amino acid composition and isoelectric point is anomalous. A similar anomaly has been noted by Matsubara *et al.* (1965) for subtilisin BPN′.

The data so far obtained do not permit a decision as to whether the enzyme can be reconstituted from its subunits.[5] The partial recovery of activity (*e.g.*, Figure 8) may of course represent such reassembly; it may, however, merely represent a refolding of partially unfolded enzyme, where the subunit structure of the intact enzyme had been incompletely broken, or perhaps remained completely unbroken. Acetopyruvic acid is a powerful inhibitor for the enzyme (Warren *et al.*, 1966) but also protects it against attack by other reagents, such as sodium borohydride plus substrate. This inhibitor also partially prevents the dissociation of the enzyme into subunits, under the conditions of expt 4 (B).

The inhibition of the enzyme by sodium dodecyl sulfate is instantaneous and completely reversible up to a concentration of 1.2×10^{-3} M, but time dependent and irreversible (by present techniques) at higher concentrations. The sharp change from reversible to irreversible denaturation with increase in concentration of detergent resembles the transition to micelle formation at a critical concentration. The critical micelle concentration has not been determined in 0.05 M phosphate buffer; predictions from the data of Matsuura *et al.* (1962) suggest that micelles of sodium dodecyl sulfate might require a somewhat higher concentration (perhaps twice) of detergent than that needed for irreversible denaturation. Attempts to reactivate the protein by removing the detergent, either by dialysis or by filtration through Sephadex, have failed. On the other hand, the interpretation of the apparent partial success in regenerating activity from protein that had been incompletely denatured by urea is ambiguous, since it is not clear to what extent, if any, the active enzyme has been regenerated from dissociated protein, rather than from protein that had been otherwise inactivated by urea.

Acknowledgments

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[5] Dr. Waichiro Tagaki has now succeeded in reassembling active enzyme from dimers formed at pH 8 in 4 M urea.