Acetoacetate Decarboxylase. Identification of Lysine at the Active Site*

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ABSTRACT: When an aqueous solution of crystalline acetoacetate decarboxylase and lithium acetoacetate is reduced with sodium borohydride, the enzyme is largely inactivated. If the reaction is carried out with acetoacetate labeled in the β-position with ^{14}C, radioactive protein is obtained. Hydrolysis of this protein has produced ε-*N*-isopropyllysine as the only labeled amino acid. Control experiments show that, under proper experimental conditions, the reduction in the presence of acetoacetate is much more efficient than that with acetone. These experiments indicate that one of the lysine residues of the enzyme forms a Schiff base with the substrate as an essential step in the mechanism of decarboxylation.

The decomposition of acetoacetate to acetone and CO_2 in the presence of the crystalline decarboxylase from *Clostridium acetobutylicum* presumably occurs through the formation of a Schiff base between enzyme and substrate. This mechanism for the biochemical process was first proposed (Hamilton and Westheimer, 1959b) on the basis of the finding that acetoacetate labeled in the carbonyl group with ^{18}O undergoes enzymic decarboxylation to form isotopically normal acetone; this observation is consistent with and suggested the formation of a ketimine between enzyme and substrate. Later work (Fridovich and Westheimer, 1962) showed that the enzyme is largely inactivated by treatment with sodium borohydride in the presence of substrate, and that, when the acetoacetate is labeled in the β-position with ^{14}C, borohydride reduction introduces radiocarbon into the protein. Presumably borohydride reduces a Schiff base (or more precisely, a Schiff base salt) formed between enzyme and substrate. The general scheme is shown below (Scheme I) where the ketimines that are postulated as intermediates in the decarboxylation are designated as A and B, and where the enzyme is shown as ENH_2.

The general method of borohydride reduction for Schiff bases in enzyme chemistry was introduced in 1958 (Fischer *et al.*) and various applications of the method have recently been reviewed (Fischer, 1965). A general discussion of the mechanism of enzymic and nonenzymic decarboxylation of β-keto acids (including earlier evidence on acetoacetate decarboxylase) has been presented elsewhere (Westheimer, 1963). This present paper presents the pertinent data concerning the formation of a Schiff base between one of the lysine residues of the enzyme and acetoacetate.

Experimental Section

Materials. The preparation of crystalline acetoacetate decarboxylase is presented in an accompanying paper (Zerner *et al.*, 1966). Lithium acetoacetate was prepared by the method of Hall (1963). Ethyl acetoacetate labeled in the β-position with ^{14}C was obtained from New England Nuclear Corp. Radioactive lithium acetoacetate was prepared from this ester by the same method (Hall, 1963) as that for the synthesis of the nonradioactive salt. Sodium borohydride (98+% pure) was obtained from Metal Hydrides, Inc., Beverly, Mass. Other reagents were commercial preparations, usually of analytical grade.

Methods. Radioactivity was measured with a Nuclear Chicago scintillation counter, series 720. Scintillators were either toluene–ethanol–liquifluor (Pilot Chemicals, Inc.) (1832:2000:168) or a polyether solvent consisting of 3000 ml of dioxane, 500 ml of purified anisole, and 500 ml of diglycol dimethyl ether, plus 16 g of 2,5-diphenyloxazole and 0.8 g of *p*-bis-2-(5-phenyloxazolyl)benzene (Pilot Chemicals, Inc.). Infrared spectra were taken with a Perkin-Elmer Infracord, and nuclear magnetic resonance spectra with a Varian A-60 spectrometer.

Various methods were used for reduction. In a typical experiment, a solution of 0.6 mg of enzyme in 2.8 ml of 0.05 M potassium phosphate buffer (pH 5.9) was stirred magnetically in an Erlenmeyer flask at 0°. Then 0.1-ml samples of ice-cold solutions of acetoacetate (0.06 M in potassium phosphate buffer) and sodium borohydride (0.03 M in 0.001 M sodium hydroxide solution) were added simultaneously and as rapidly as possible from syringes; the time of addition

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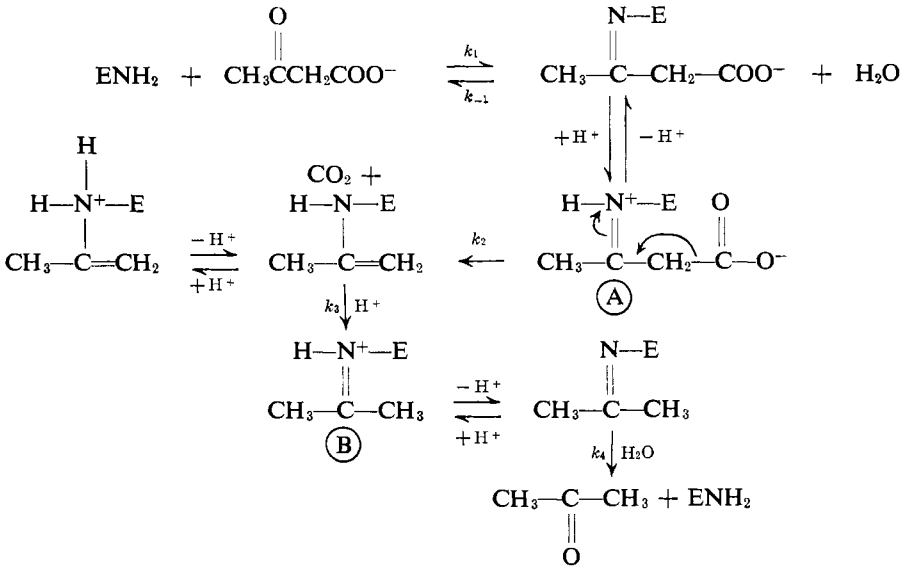
SCHEME I



ENH_2 = enzyme

did not exceed a second. The following considerations dictated the choice of experimental conditions. The solutions of borohydride were made up in sodium hydroxide solution in order to avoid acid-catalyzed decomposition (Stockmayer *et al.*, 1961; Davis and Gottbrath, 1962). The rates of reduction of acetone and acetoacetate by borohydride (Brown and Ichikawa, 1961; Freund and Nuenke,[1] 1962) are slow compared to the reduction of the Schiff base. The half-life of acetoacetate in the presence of 100 mg/l. of enzyme is about 20–30 sec at 30°, or probably about 4 min at 0°. The decomposition of borohydride is general acid catalyzed (Davis and Gottbrath, 1962) and the half-life of a solution in the buffers used at pH 5.9 and 0° is only about 10 sec. Therefore, the mixing during the reduction process must be prompt, and the reductions take place immediately or not at all. After the reductions were complete, the solutions were exhaustively dialyzed against 0.05 M phosphate buffer at pH 5.9 before assaying for enzymic activity or measuring the radioactivity of the product. The assay was conducted by observing the disappearance of the enol absorption band of acetoacetate at 270 mμ in 0.1 M pH 5.9 phosphate buffer at 30°, by a slight modification of the method previously developed (Fridovich and Westheimer, 1962; Fridovich, 1963).

ε-N-Isopropyllysine. ε-*N*-Isopropyl-L-lysine methyl ester dihydrobromide has been previously reported from our laboratory (Schellenberg, 1963). That publication presents the method of preparation of choice. A buffered aqueous solution of acetone and α-*N*-benzyloxycarbonyl-L-lysine methyl ester is reduced by borohydride, and the resulting α-*N*-benzyloxycarbonyl-ε-*N*-isopropyl-L-lysine methyl ester is hydrolyzed to the desired product. An additional, if less convenient preparation supplies confirmatory structural evidence. Two and eight-tenths grams of α-*N*-benzyloxycarbonyl-L-lysine (Bezas and Zervas, 1961) was converted to its methyl ester hydrochloride with thionyl chloride and methanol. After evaporation of the methanol, 30 ml of chloroform, 3 ml of triethylamine, and 1.1 ml of isopropyl iodide were added to the residue, and the solution refluxed for 13 hr. Fifty milliliters of chloroform was added, and anhydrous HBr bubbled through the solution, which was then set aside for 9 hr. Work-up gave a low yield of crystalline ε-*N*-isopropyllysine methyl ester dihydrobromide, mp 156.5–157.5. *Anal.* Calcd for $C_{10}H_{24}O_2N_2Br_2$: C, 32.98; H, 6.64; N, 7.69. Found: C, 33.22; H, 6.85; N, 8.07. The bromine analysis of a different sample has been reported by Schellenberg (1963). The samples gave the same infrared and nuclear magnetic resonance analyses, and showed no depression in a mixture melting point determination. The infrared spectrum shows the bands typical of ammonium salts at 4.09–4.10 μ, and those of an isopropyl group at 8.55 and 8.73 μ. The position of the isopropyl group was determined mass spectrometrically by Professor K. Biemann, unpublished data (1963),[2] who found a much more intense peak at mass 72 than at mass 30. He ascribed the peak at 72 to the ε-isopropyl isomer on the basis of the expected cleavage of the ε-isopropyl compound to yield $(CH_3)_2CH—NH=CH_2^+$; this fragment could only be obtained by a rearrangement process from the α-isomer, and would not be expected as an intense peak. This mass spectrometric

[1] Note that the rate constants in this communication are in error by 10^4 (too large).

[2] As per letter of 10/8/63 to F. H. Westheimer.

TABLE I: Chromatographic Comparison of ϵ-*N*-Isopropyllysine with the Radioactive Amino Acid from the Borohydride Reduction of Acetoacetate Decarboxylase and Acetoacetate-^{14}C.

Components of Solvent for Chromatography	Ratios	Colored Area		Equal Surrounding Area	
		(cpm)	% of Total Radioactivity	(cpm)	% of Total Radioactivity
1-Propanol–water–ammonia	55:30:10	153	89	14	8
1-Butanol–acetic acid–water	4:1:5	108	81	10	7
Pyridine–acetic acid–water	50:35:15	109	94	6	5

evidence and the second synthesis, despite its low yield, establish the structure of the isopropylated ester. ϵ-*N*-Isopropyllysine, itself, has not been obtained in crystalline form, but is prepared in solution from the methyl ester dihydrobromide by hydrolysis with 1 N HCl on the steam bath for 2 hr.

Identification of ϵ-N-Isopropyllysine. A sample of radioactive protein has previously been prepared by borohydride reduction at pH 6.99 of a solution of enzyme and of acetoacetate labeled in the β-position with ^{14}C (Fridovich and Westheimer, 1962). After hydrolysis of the protein, the amino acid mixture was subjected to two-dimensional paper chromatography in Redfield's system (1953), and to radioautography. The single radioactive spot was cut out and eluted, and the eluate mixed with a solution of synthetic ϵ-*N*-isopropyllysine. The mixture was then divided into three parts, and separately chromatographed in three solvent systems. In each system, the synthetic material was visualized with ninhydrin, and the colored area cut out, eluted, and counted. An equal area surrounding the colored spot was also eluted. The data from scintillation counting are shown in Table I.

In a second experiment, a reduction was carried out at pH 5.9 with 10^{-4} M borohydride solution, under experimental conditions where acetoacetate is more effective than acetone in reacting with the enzyme in the presence of borohydride. The resulting protein was 86% inhibited. After dialysis of this protein (0.2 mg) against distilled water, the contents of the dialysis sac were lyophilized, and the resulting white powder was hydrolyzed in 3 ml of 6 N HCl in an evacuated sealed tube for 48 hr at 110°. The solution was then evaporated to dryness on a rotatory evaporator, and the residue redissolved in 0.5 ml of water. One drop of a solution of authentic ϵ-*N*-isopropyllysine and 0.1 ml of the radioactive solution of amino acids were spotted together on Whatman No. 1 paper, and developed in three of the systems used by Cohen (1962). (They differ from those shown above only in that the second solvent is made from 1-butanol–glacial acetic acid–water in the ratios of 4:2:5. The mixture separates into two layers. The chromatography is conducted with the organic layer, while the aqueous layer is placed in the bottom of the chromatographic tank.) The synthetic material was visualized with ninhydrin, and the radioactive material detected with a Vanguard strip counter, used by the kind permission of Dr. F. Kahan. The data are shown in Table II.

TABLE II: Comparison of the R_F values of ϵ-*N*-Isopropyllysine with those of the Radioactive Amino Acid from the Borohydride Reduction of Acetoacetate Decarboxylase and Acetoacetate-^{14}C.

Solvent	Radioactive Peak (R_F)	Synthetic ϵ-*N*-Isopropyllysine (R_F)
I	0.76	0.78
II	0.21	0.21
III	0.54	0.52

More recent experiments, conducted by R. Laursen, show that the radioactive amino acid (ϵ-*N*-isopropyl-L-lysine) can be separated from all the naturally occurring amino acids except histidine by the usual procedures of column chromatography on a Beckman automatic amino acid analyzer. ϵ-*N*-Isopropyl-L-lysine is easily separated from histidine by paper chromatography, electrophoresis, or by column chromatography at pH 4.25 on the short column of the automatic amino acid analyzer.

Results

The Reductions. Our prior communication (Fridovich

TABLE III: Comparison of Inhibition with Acetone and Acetoacetate by Borohydride Reductions at High Concentrations of Substrate.

Buffer (M)	Enzyme (mg/l.)	Acetone (10^{-2} M)	Potassium Acetoacetate (10^{-2} M)	Sodium Borohydride (10^{-4} M)	% Inhibition
0.10	123				0.0
0.10	123	2.5		3.54	90
0.10	123	25		3.54	89
0.10	123		2.53	3.54	90

TABLE IV: Comparison of Inhibition with Acetone and Acetoacetate by Borohydride Reductions at Low Concentrations of Substrate.

Expt No.	pH 5.9 Buffer (M)	Enzyme (mg/l.)	Aceto-pyruvate (10^{-5} M)	Acetone (10^{-5} M)	Lithium Acetoacetate (10^{-5} M)	$NaBH_4$ (10^{-5} M)	% Inhibition	Atom ^{14}C/ 33,000 (mol wt units) (cpm)	Atom ^{14}C/ 33,000 (mol wt units)
1	0.093	5.2				1.64	0.0		
2	0.090	5.2				3.27[a]	13.0		
3	0.083	5.2		3.3		3.27[a]	13.5		
4	0.083	5.2			6.7	3.27[a]	84.2		
5	0.085	5.2			5.0[b]	3.27[a]	82.1	732	0.45
6	0.083	5.2	6.7			3.27[a]	7.0		
7	0.076	5.2	6.7		6.7	3.27[a]	6.4		
8	0.078	5.2	6.7		5.0[b]	3.27[a]	9.9	112	0.06
9	0.187	5.2				0.00	4.8		
10	0.180	5.2				1.23	0.0		
11	0.173	5.2		6.7		1.23	7.5		
12	0.173	5.2			6.7	1.23	64.5		
13	0.173	5.2		5.0[b,c]		1.23	16.3	119	0.05
14	0.180	5.2			5.0	0.00	2.5	66	0.05
15	0.180	5.2			6.7	0.00	9.7		
16	0.173	5.2		6.7[c]		1.23	12.0		
17	0.187	1.6				0.00	3.6		
18	0.180	1.6				1.00	0.0		
19	0.167	1.6		6.7		1.00	10.0		
20	0.167	1.6			6.7	1.00	85.4		
21	0.147	1.6			16.7[b]	1.00	78.9	75	0.31
22	0.187	2.1				0.00	0.3		
23	0.180	2.1				1.07	0.0		
24	0.167	2.1		6.7		1.07	42.7		
25	0.167	2.1			6.7[b]	1.07	75.5		
26	0.167	2.1			6.7[b]	1.07	87.9	82	0.26
27	0.173	2.1			6.7[b]	0.00	1.9	27	0.08
28	0.173	21.0			20.0[b]	10.0	85.9	687	0.23
29	0.173	2.1		6.7[b,c]		1.07	16.2	27	0.08
30	0.187	13.2				0.00	0.0		
31	0.180	13.2				1.67	7.2		
32	0.167	13.2		6.7		1.67	25.4		
33	0.167	13.2			6.7	1.67	27.9		
34	0.167	13.2			6.7[b]	1.67	76.0	1826	0.31
35	0.154	13.2	6.7		6.7	1.67	32.6		
36	0.167	13.2	6.7			1.67	19.6		
37	0.153	13.2	6.7		6.7[b]	1.67	26.8	34	0.01
38	0.193	2.54				0.00	0.0		
39	0.180	2.54				0.86[a]	9.0		
40	0.167	2.54		3.3		0.86[a]	40.6		
41	0.167	2.54			6.7	0.86[a]	89.3		
42	0.167	2.54			6.7[b]	0.86[a]	86.8	503	0.45
43	0.167	2.54			6.7[b]	0.86[a]	85.3	439	0.39
44	0.167	2.54			6.7	0.86[a]	86.6		
45	0.173	2.54[d]			6.7[b]	0.43	[f]	40	
46	0.173	2.54[e]			6.7[b]	0.43	[f]	69	0.05
47	0.193	1.70				0.00	0.0		0.09
48	0.187	1.70				0.40	14.7		
49	0.173	1.70			6.7	0.40	78.7		
50	0.173	1.70			6.7[b]	0.40	47.5	363	0.35
51	0.160	1.70	6.7		6.7	0.40	3.3		
52	0.140	1.70	6.7		6.7[b]	0.40	11.5	253	0.25

[a] Added in two portions. [b] Labeled with ^{14}C in the β-position. [c] Acetone by decarboxylation of acetoacetate. [d] α-Chymotrypsin. [e] Bovine serum albumin. [f] No activity.

and Westheimer, 1962) reported the inactivation of acetoacetate decarboxylase on reaction with borohydride in the presence of acetoacetate. However, further control experiments showed that the enzyme is likewise inactivated by borohydride reduction in the presence of acetone, as shown in Table III.

Since the enzyme catalyzes the exchange of ^{18}O between the carbonyl group of acetone and water, the interaction of acetone and the enzyme could be anticipated, and is in no way inconsistent with the Schiff base mechanism for the decarboxylation. Nevertheless, on the basis of the data in Table III alone, it might have been inferred that decarboxylation precedes the condensation between the enzyme and the carbonyl compound, so that a Schiff base between acetone and lysine might have been formed after, rather than during the enzymic decarboxylation. However, at lower pH and at much lower concentrations, experimental conditions have been found where reduction in the presence of acetoacetate leads to inactivation of the enzyme, and (when radioactive acetoacetate is used) to labeling of the enzyme, whereas similar reduction in the presence of acetone leads to little, if any, inactivation of the enzyme or incorporation of radioactivity into the protein. The appropriate data are shown in Table IV, which is arranged so that experiments of any series, performed at the same time, are grouped together; a group is distinguished by identical enzyme concentrations.

For example, expt 12, with 6.7×10^{-5} M lithium acetoacetate, gave a product which was 64% inactivated; expt 11, with an identical concentration of acetone, showed only 7.5% inactivation. Similarly, expt 20, with acetoacetate, shows 85% inactivation, whereas expt 19, with an identical concentration of acetone, gave only 10% inactivation. Furthermore, expt 13 and 29, with radioactive acetone (prepared by the prior decarboxylation[3] of acetoacetate-β-^{14}C), showed an incorporation of radiocarbon on reduction far below the levels achieved with acetoacetate. It should be noted, however, that occasional experiments with acetone (*e.g.*, no. 24) gave rather larger percentages of inhibition than usual; the reason for the variability is not known.

Davies (1943) showed that acetopyruvate is an effective inhibitor for acetoacetate decarboxylase. It also prevents incorporation of radioactivity into the enzyme. Contrast, for example, expt 5 and 8, with identical concentrations of radioactive lithium acetoacetate, but where expt 8 was conducted in the presence of 6.7×10^{-5} M acetopyruvate. In the absence of acetopyruvate, the enzyme was reductively inhibited to the extent of 82%, and 0.45 atom of ^{14}C was incorporated for each 33,000 mol wt units. In the presence of acetopyruvate, the inhibition (after extensive dialysis to remove acetopyruvate) was less than 10%, and only 0.06 atom of ^{14}C was incorporated for each subunit. Thus, the inhibitor occupies the active site or otherwise prevents the normal enzymic reaction. On the other hand, the chemistry of the reaction of the enzyme with acetopyruvate differs from that with the substrate, since the enzyme is not irreversibly inactivated by treatment with acetopyruvate and borohydride; in expt 6, *e.g.*, only 7% of the enzymic activity was lost on treatment with borohydride and acetopyruvate, followed by extensive dialysis to remove residual acetopyruvate. Yet acetopyruvate is an extraordinarily effective inhibitor; it reacts slowly with the enzyme, but the inhibition constant is probably no greater than 2×10^{-7} M (Colman, 1962).

Control experiments (no. 45 and 46) shown in Table IV include attempts to carry out the borohydride reduction in the presence of acetoacetate and α-chymotrypsin and bovine serum albumin. Neither of these proteins catalyzes the decarboxylation at all, and incorporation of radioactivity into them on borohydride reduction in the presence of labeled acetoacetate is minimal. In fact, the radioactivity scarcely exceeds that which is obtained on mixing protein and acetoacetate, and then dialyzing (without addition of borohydride), and so presumably represents mechanically held material.

Maximum Inhibition and Incorporation of Radioactivity. The data of Tables III and IV show that reduction of the enzyme by borohydride in the presence of acetoacetate leads to inhibition which seldom exceeds 90%, and to an incorporation of radiocarbon which is not greater than 0.45 atom of ^{14}C for each subunit of mol wt 33,000. Recently, however, Laursen, unpublished data (1965), has been able to incorporate about 0.7 atom of ^{14}C into each 33,000 mol wt units, with accompanying inhibition of 90%.

An attempt was made to increase the percentage of inactivation by repeated reductions in the presence of acetoacetate and borohydride. The reductions were attempted one right after the other, and alternatively with dialysis of the reaction mixture between reductions. The latter was tried in order to remove borate or other negative ions which might inhibit the enzyme (Fridovich, 1963). A few of the data are shown in Table V; the scatter (as in the corresponding data from Table IV) was large.[4] In these experiments, successive increments of ordinary acetoacetate and borohydride were added to a buffered solution of the enzyme. Finally, a last reduction with borohydride was carried out in the presence of radioactive acetoacetate, and the

[3] Experiments with radioacetone purchased from New England Nuclear Corporation showed a large incorporation of radiocarbon into the enzyme with or without borohydride reduction; presumably the compound contained an unknown reactive radioactive impurity. Such difficulties were not observed with acetone prepared by the decarboxylation of radioactive acetoacetate.

[4] Although the results presented in Table V are representative of a large number of experiments that have been carried out with the same type of results, on two occasions reductions introduced large amounts of radioactivity into the enzyme. In these particular experiments, a first reduction with borohydride in the presence of radioactive acetoacetate gave normal results, while a second reduction introduced three or more atoms of ^{14}C per 33,000 mol wt units. The reasons for these two aberrant experiments are unknown.

TABLE V: Repeated Borohydride Reductions of Acetoacetate Decarboxylase and Acetoacetate, Followed by Reduction in the Presence of Acetoacetate-^{14}C.

No. of Cycles with Nonradioactive Acetoacetate	No. of Cycles with Radioactive Acetoacetate	% Inhibition[a]	Atoms ^{14}C/33,000 (mol wt units[a])
3	1	79	0.15
6	1	82	0.09
9	1	87	0.03

[a] Average of four determinations.

enzymic activity and radioactivity of the samples were assayed. Table V shows that even repeated reductions fail completely to eliminate the enzymic activity but that eventually the amount of reaction between acetoacetate and enzyme reaches a maximum. After a few successive reductions, the additional amount of radioactivity which is introduced does not appreciably exceed the amounts obtained in control experiments shown in Table IV, *e.g.*, it is less than the small amounts of incorporation which are found in chymotrypsin and bovine serum albumin under the same experimental conditions.

As shown in the following paper (Lederer *et al.*, 1966), each subunit of acetoacetate decarboxylase contains 20 lysine residues for 33,000 g. Therefore, the data of Table V show that the reduction is by no means random, but is restricted to a single reactive lysine. This conclusion is confirmed by the work of Laursen, unpublished data (1965), who has found that one of the peptides produced on tryptic digestion of labeled enzyme contains most of the radioactivity, and on hydrolysis yields ϵ-*N*-isopropyllysine.

Discussion

The data presented in this paper show the following: (1) The enzyme acetoacetate decarboxylase from *Clostridium acetobutylicum* is inhibited by reduction by borohydride in the presence of either acetone or acetoacetate, but the reduction with acetoacetate is much more efficient; at pH 5.9 and with low concentrations of reagents, the enzyme is substantially inhibited (70–85%) by reduction in the presence of acetoacetate, but almost unaffected by reduction in the presence of acetone. (2) Under conditions where the reduction is efficient only with acetoacetate, as well as under experimental conditions where reduction occurs either with acetone or acetoacetate, the modified protein obtained from the reduction with labeled substrate can be hydrolyzed to produce ϵ-*N*-isopropyllysine as the only radioactive product. The ϵ-*N*-isopropyllysine has been independently synthesized, and its structure proved. (3) A particular lysine is responsible for the formation of a Schiff base which can be reduced by borohydride.

These data strongly support the hypothesis of a Schiff base as an intermediate in the enzymic decarboxylation of acetoacetic acid, in accordance with the equations in the introductory section of this paper. Since isopropyllysine is formed, and not ϵ-*N*-1-carboxy-2-propyl-L-lysine, the reduction must occur with the ketimine, B, from acetone, and not with the ketimine, A, from acetoacetic acid itself. That the reductions occur with Schiff base salts, rather than with the Schiff bases themselves, has been demonstrated for model compounds (Schellenberg, 1963) and is electronically reasonable.

The findings here reported are strongly supported by previous experiments. Our earlier discovery of obligatory exchange of ^{18}O between the carbonyl group of acetoacetate and water during enzymic decarboxylation has been mentioned in the introduction. Furthermore, the details of the cyanide inhibition of the decarboxylation accord with the Schiff base mechanism (Westheimer, 1963).

Yet problems remain. These problems include the fact that repeated reductions fail completely to eliminate enzymic activity, and that the number of atoms of ^{14}C (*i.e.*, the number of isopropyl groups) introduced per molecule of protein is not clearly defined. Possibly the variation in the specific activity of the enzyme noted in the accompanying paper (Zerner *et al.*, 1966) and the scatter in the labeling results are related. Perhaps some inactive (or inhibited) protein is present, and can neither enter into enzymic reaction nor be labeled by the borohydride reduction. Alternatively, perhaps the key to these problems lies in the details of the mode of aggregation of subunits to form the enzyme. Nevertheless, the identification of the site of enzymic reaction as a specific lysine and the evidence for the formation of a Schiff base as an intermediate in the enzymic decarboxylation appear to be well established.

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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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