Decarboxylation of 2-keto fatty acids by brain

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ABSTRACT An enzyme system consisting of washed acetone powder of pig brain, adenosine triphosphate, nicotinamide adenine dinucleotide, magnesium, fumaric acid, and ascorbic acid was found to catalyze the oxidative decarboxylation of 2-ketostearic acid. The products were carbon dioxide and heptadecanoic acid. Washing the enzyme powder with ethylenediaminetetraacetate and other chelating agents destroyed the activity. Further properties of the enzyme system are described. It is believed that this enzyme system accounts in part for the 1-carbon degradation route for brain fatty acids.

KEY WORDS 2-ketostearic acid · decarboxylation · brain · pig · ascorbic acid · fumaric acid · iron · fatty acid catabolism

On the basis of studies with labeled acetate in rats, it was concluded (1–3) that the odd-numbered fatty acids in brain are made by two routes: elongation of propionate with C_2 residues, and 1-carbon degradation of even-numbered fatty acids. The second route involves hydroxylation of the nonhydroxy acids at the 2-position (4), followed by decarboxylation to form the shorter acid. The nature of the decarboxylation has been studied by Levis and Mead in brain microsomes (5). They found that the microsomes needed ATP, NAD^+, and the supernatant fraction of brain homogenate for activity. 2-Ketostearate and heptadecanoate were found to be formed from hydroxystearate, and the keto acid underwent decarboxylation somewhat more rapidly than the hydroxy acid. This paper reports a study of the enzyme system that decarboxylates the keto acid.

METHODS AND MATERIALS

Substrate Preparation

Stearic acid-1-^{14}C was converted to 2-hydroxystearic acid (6), which was esterified with methanol and purified on a column of Florisil (Floridin Company, Tallahassee, Fla.) (7). The ester was oxidized to the 2-keto ester with CrO_3–HOAc (8) and purified on a column of silica gel (Unisil, Clarkson Chemical Company, Williamsport, Pa.), with hexane–ether 97:3 as eluent. The ester was saponified by standing for 1 hr at room temperature in N methanolic KOH (8) and the free acid was recovered by acidification and extraction. Final purification was by chromatography on silica gel, with hexane–ether 95:5. Evaluation of the purity of the ketostearate by TLC on Silica Gel G (chloroform–HOAc 96:4; bromothymol blue spray) showed only one spot, which contained all the ^{14}C. Although the keto acid might be expected to be less polar than the hydroxy acid, it had a slightly *lower* R_f. Spraying the plate with 0.5% 2,4-dinitrophenylhydrazine in 2 N HCl yielded a yellow spot with the keto acid and a white spot (on a pale yellow background) with the hydroxy acid.

The keto acid was emulsified for incubation by evaporating a solution of 1 mg of the acid (8.6×10^5 cpm), 6.5 mg of G-2159 (polyoxyethylene stearate),[1] and 12.5 mg of Tween 20 (polyoxyethylene sorbitan monolaurate)[1] in benzene to dryness with nitrogen. The residue was sonicated together with 0.15 ml of M Tris buffer (pH 7.5), 5.85 ml of water was added, and the mixture was sonicated further to yield a clear emulsion. The substrate was stored in a freezer.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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[1] Atlas Chemical Industries, Wilmington, Del.

Preparation of Enzyme System

Fresh pig brain was homogenized for 2 min in a Waring blendor with 10 volumes of acetone in a cold room. The liquid was removed by filtration; the powder was dried between filter papers and by pumping for several hours under suction from a water aspirator. The acetone powder was stable for three months at −20°C, but lost nearly all its activity during the following month.

Before use, a 100 mg portion of powder was suspended in 10 ml of water by means of an all glass homogenizer, then centrifuged at 105,000 × *g* for 60 min. The pellet was suspended in 5 ml of water and portions of the fine suspension were added to the incubation mixture.

Enzyme Assay Method

The activity of the enzyme was determined by measuring the release of $^{14}CO_2$, which was trapped in KOH suspended above the reaction mixture on a pleated piece of glass filter paper. The reaction vessel was a 75 × 18 mm O.D. test tube, with a side arm and flared mouth. The side arm held 0.2 ml of 5 N sulfuric acid. The (rubber) stopper for the tube had a single hole which was filled with a short glass tube, closed at the upper end. The pleated paper was suspended by inserting one end of it into this glass tube; the exposed end was wetted with 8 μl of 20% KOH.

The incubation mixture had a total volume of 1 ml and consisted, in the complete system, of 100 μmoles of sodium phosphate (pH 7.5), 1 μmole of ATP, 2 μmoles of NAD^+, 0.05 μmole of ascorbic acid, 0.1 μmole of fumaric acid, 6 μmoles of $MgCl_2$, 0.1 ml of enzyme suspension (2 mg), and 56 mμmoles (15,000 cpm) of 2-ketostearate. After incubation at 37°C for 45 min in a Dubnoff shaker, the acid was tipped in, and the tube was shaken for an additional 45 min at 37°C to permit diffusion of the CO_2 to the KOH. The filter paper was then counted in 15 ml of toluene–absolute ethanol 85:15 containing the usual oxazolyl scintillators and having an over-all efficiency of 38%.

The efficiency of counting by the paper–KOH method was 68% of the efficiency of counting ketostearate in the same scintillation solution. This was shown by counting known samples of $^{14}CO_2$ (from sodium carbonate-^{14}C) and using an internal standard of hexadecane-^{14}C (Nuclear-Chicago Corporation, Des Plaines, Ill.).

RESULTS

Cofactor Requirements

The early work in this study was carried out with brain microsomes and the 100,000 × *g* supernatant fraction. As reported by Levis and Mead (5), the supernatant fraction stimulates the microsomal ketostearate oxidase and the active material is dialyzable. The active material was passed through a Dowex 50-K^+ column, which yielded an effluent containing only about one-half the activity. This, together with severe inhibition by EDTA in the whole system, suggested that a metal is involved. When this effluent was passed through a Dowex 1-OAc^- column, the remaining activity was removed, which suggests that an acid is responsible for part of the activity.

Further work was carried out with whole brain, since a preliminary study showed that mitochondria are also quite active toward ketostearate. The mitochondrial fraction was prepared by centrifuging a 9:1 homogenate in 0.25 M sucrose at 800 × *g* for 10 min (to remove debris), then centrifuging at 12,000 × *g* for 30 min. The mitochondria from 29 mg of rat brain yielded 6314 cpm in the standard assay system (with complete cofactors) while the microsomes from a similar amount of brain yielded somewhat less, 1606 cpm. For convenience in storing the enzyme, acetone powder of whole brain was used for further work.

Crude acetone powder with ATP and NAD^+ showed activity which was stimulated by Mg^{++}, but powder washed with water was almost inert in this medium. The aqueous extract resembled the cell supernatant fraction in that it activated the particulate enzyme and the activating material could be held back by an anion-exchanger. Elution of the ion-exchange column with 0.2 N HCl recovered the active material. The activator in the water extract was stable for 10 min at 60°C but not at 100°C. Exposure to 1.6 N HCl for 60 min at room temperature did not reduce its activity but 0.3 N NaOH destroyed the material.

From the above properties and the ineffectiveness of typical cofactors, it appeared likely that ascorbic acid was the cofactor. Tests showed it to be very effective at about 10^{-4} M, but inhibitory at higher concentrations. The effect of different concentrations is shown in Fig. 1. In an experiment with closer intervals, maximal activation was observed at 5 × 10^{-5} to 10^{-4} M ascorbate.

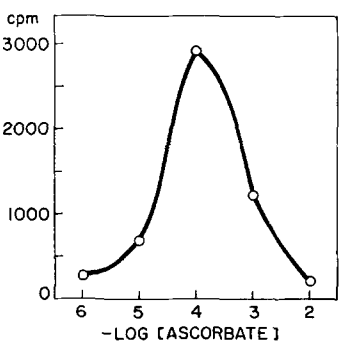

FIG. 1. Relation between decarboxylase activity and ascorbate concentration. The ordinate refers to the CO_2 activity. Standard assay as in text.

Ascorbic acid is a cofactor in the β-hydroxylation of dopamine by adrenal medulla, and fumaric acid greatly enhances the stimulatory effect of ascorbic acid (9). Fumarate stimulates the brain α-keto acid decarboxylase too, as shown in Table 1, but the effect is smaller. The

TABLE 1 COFACTOR DEPENDENCIES OF BRAIN KETOSTEARATE DECARBOXYLATION SYSTEM

Cofactor Omitted	Activity in CO_2
	cpm
None	5204
Ascorbate	294
Fumarate	4360
NAD^+	1526
ATP	4087
Mg^{++}	2147

Standard enzyme assay system used as described in text. Activities are corrected for value at zero time (60 cpm).

stimulatory effects of ATP, NAD^+, and Mg^{++} are also shown in the table.

Ascorbate could be replaced by dihydroxyfumarate (Nutritional Biochemicals Corp., Cleveland, Ohio) or by dehydroascorbate (methanol complex, Mann Research Labs. Inc., New York, N. Y.), but about ten times the concentration was needed to yield an equivalent amount of decarboxylation. Calcium ions were stimulatory but less so than Mg^{++}. Coenzyme A (1 μmole) and thiamine pyrophosphate (0.5 μmole) each increased the activity of the standard assay system by a small but distinct amount. Because of the expense and the smallness of the effect, these cofactors were omitted in the experiments described in this paper.

The Role of Iron

If the crude acetone powder is washed with 0.1 M EDTA (by centrifugation), then washed once with water to remove the EDTA and resuspended in water, the resultant enzyme preparation shows considerably less activity. The data from one experiment (lines 1 and 2, Table 2) show a drop of 45%. From this it would appear that Mg^{++} is not the only metal needed. In some experiments, as shown in Table 2, the addition of ferrous ions restored activity to the EDTA-washed enzyme suspension. Ferric ions were ineffective. Unfortunately, the stimulatory effect could not be demonstrated consistently. More specific chelators of iron, 0.01 M 2,2′-bipyridine and 1,10-phenanthroline, were completely inhibitory when added to the standard incubation mixture. They were more effective than EDTA in removing activity by washing the acetone powder, but reactivation with ferrous ions could not be accomplished. Addition of 0.33 mg of ferredoxin from *Clostridium pasteurianum*

TABLE 2 EFFECT OF METAL IONS ON KETOSTEARATE DECARBOXYLATION

Addition	Activity in CO_2	
	Enzyme Present	No Enzyme Present
	cpm	*cpm*
None*	2852	—
None	1574	—
None	1671	169
Fe^{++} 10^{-3} M	4021	130
Zn^{++} "	208	295
Mn^{++} "	128	830
Ca^{++} "	81	72

* This sample was run with standard enzyme preparation; all the remaining samples were run with EDTA-washed enzyme and complete set of cofactors.

(Worthington Biochemical Corp., Freehold, N. J.) did not restore activity to phenanthroline-washed enzyme.

The second part of Table 2 shows the effect of incubating EDTA-washed enzyme with additional metals. Zn^{++}, Mn^{++}, and Ca^{++} were inhibitory, although Zn^{++} and Mn^{++} showed some nonenzymatic decarboxylation activity (as did Fe^{+++}). It is interesting to note that the nonenzymatic activity of Mn^{++} and Zn^{++} was quenched by the addition of enzyme, possibly because of metal-binding by the enzyme or associated materials. It was shown in a separate experiment that $MnSO_4$ alone is a good decarboxylating agent and that its activity is reduced by the presence of ATP and NAD^+. Cupric ion was inhibitory, even at 10^{-6} M, as was Cu_2^{++}.

Properties of the Enzyme Preparation

The pH optimum was found to lie rather broadly between 5.8 and 7.5 (Fig. 2).

Although the acetone powder was rather stable, the aqueous suspension lost half its activity in 24 hr at 4°C.

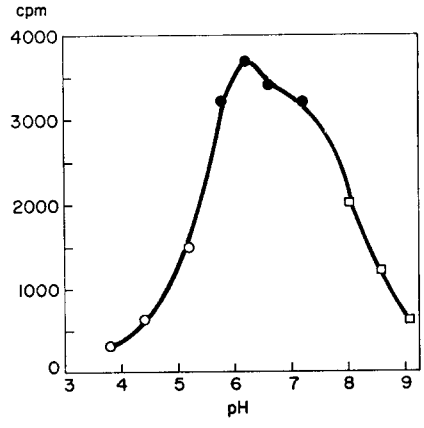

FIG. 2. Relation between pH and enzyme activity. Standard assay as in text. Buffers: ○ = acetate, ● = phosphate, □ = borate.

TABLE 3 EFFECT OF DESTRUCTIVE TREATMENTS ON BRAIN KETOSTEARATE DECARBOXYLASE

Treatment	Activity in CO_2*
	cpm
None	3731
Preincubation with protease	211
	186
Preincubation with denatured protease†	3468
	3391
Protease added without preincubation	1635
	1612
No protease, no incubation (zero time blank)	117
Preincubation with 0.025 N HCl 1 hr at 100°C	150
	135
Preincubation with 0.025 N NaOH 1 hr at 100°C	55
	90
Incubation with 0.025 N NaCl	1677
	1695
Normal incubation	1713
Enzyme preincubated 1 hr at 100°C	973
	978

Preincubation with protease (from *S. griseus*) was carried out with 1 mg of protease + 0.4 ml water + 0.1 ml pig brain enzyme suspension, 2 hr at 37°C. The assay was then carried out in the usual way. Preincubation with alkali and acid was followed by neutralization before assaying.

* Where two values are shown these are duplicates.

† Protease heated 20 min at 100°C before incubation with enzyme.

Only 8% of the activity was lost when the suspension was heated at 100°C for 10 min; 42% was lost after 60 min. Incubation with 1 mg of trypsin and 0.1 ml of enzyme suspension in a total volume of 0.5 ml at pH 7.5 at 37°C for 2 hr did not result in decreased activity, but a protease from *Streptomyces griseus* (Type V, Sigma Chemical Company, St. Louis, Mo.) brought the activity almost to the blank level. Comparison of lines 2 and 3 of Table 3 shows that the inhibitory effect of the protease is due to its proteolytic activity. The effect of other destructive treatments is shown in the table.

Fig. 3 shows the relationship between enzyme concentration and activity. The activity levels off well below the point of complete consumption of substrate, possibly because of increasing destruction of cofactors with increasing amounts of enzyme.

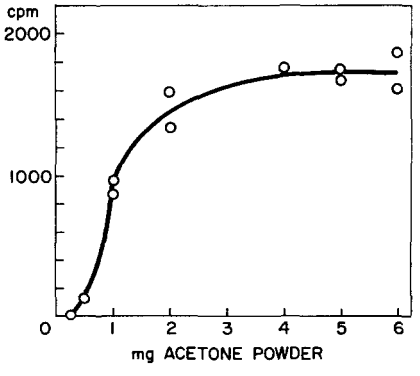

FIG. 3. Relation between amount of acetone powder and amount of radioactive CO_2 formed under standard assay conditions.

A time-course study showed that the decarboxylation was moderately linear for 90 min and that there was a short lag period (Fig. 4). Repetition of this experiment over a 30 min period confirmed the existence of the lag period and showed that preincubation of the enzyme with cofactors before the addition of substrate eliminated the lag.

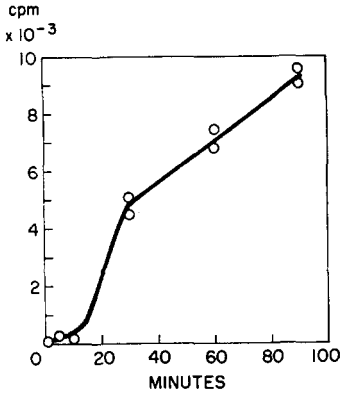

FIG. 4. Relation between time of incubation and amount of substrate destroyed. Conditions as in text but with double the amount of substrate.

Although pyruvate dehydrogenase utilizes somewhat different cofactors, the possibility existed that it is the enzyme that acts on the longer homologue, ketostearate. Table 4 shows the relative activities of rat brain homogenate and microsomes toward the two keto acids. As expected, the total homogenate was active toward both substrates but only the keto fatty acid is decarboxylated by the microsomes (particularly with added cell supernatant fraction). In comparing the activities toward the two substrates, one should note that the conditions for pyruvate decarboxylation were probably not optimal.

Table 4 also shows that liver resembles brain in possessing a microsomal fraction which attacks ketostearate and which is stimulated considerably by added cell supernatant fraction.

Characterization of the Reaction Products

Levis and Mead found the decarboxylation product of hydroxystearate to be the next lower acid, heptadecanoate (5). In the present study, incubating ketostearate under nitrogen resulted in a 90% decrease in activity, which would be expected if an oxidative decarboxylation to the same C_{17} acid is involved. Formation of this acid was shown directly by TLC and GLC with labeled ketostearate. Enzymic incubation was carried out, some of the

TABLE 4 DECARBOXYLASE ACTIVITY TOWARD PYRUVATE AND KETOSTEARATE

Enzyme Source*	Activity in CO_2	
	Pyruvate	Keto-stearate
	cpm	*cpm*
Brain homogenate	2806	5095
" microsomes	0	204
" " + brain supernatant fraction	66	2760
Liver homogenate	1702	5003
" microsomes	0	633
" supernatant	0	0
" microsomes + liver supernatant fraction	0	3169

* Rat brain and liver homogenized with 9 volumes of 0.25 M sucrose, centrifuged at 12,000 × *g* for 30 min, the residue discarded, and the microsomes obtained by centrifuging 60 min at 104,000 × *g*. Microsomal pellet resuspended in 1.2 volumes sucrose (based on tissue weight). Used 0.2 ml of homogenate and supernatant solution, 0.1 ml of microsomes. Incubation carried out with 2.5 μmoles of ATP, 2.0 μmoles of NAD^+, 105 μmoles of Tris–HCl (pH 7.5) for 30 min at 37°C. Substrates: 0.1 μmole of Li pyruvate-1-^{14}C (30,000 cpm) or ketostearate (20,000 cpm); both in 0.2 ml water, with 0.25 mg of the detergent G-2159.

samples being used to measure the amount of CO_2 produced (by counting), others being acidified with two drops of 4 N HCl and extracted with 2 × 1.5 ml chloroform to extract the lipoid product. Before the extraction, 14.6 μg of arachidic acid and 10 μg of palmitaldehyde were added in 1 ml of chloroform as internal standards for GLC.

The aldehydes and acids were isolated by TLC on Silica Gel HR (Brinkmann Instruments, Westbury, N. Y.), with hexane–ether–HOAc 70:30:3 as developing solvent and bromothymol blue as detecting spray. The areas corresponding to the free fatty acids and aldehydes (identified by standards) were eluted with anhydrous ether (20 ml/g of silica gel). The ether was removed under nitrogen and the aldehyde fraction was examined by GLC, with diethyleneglycol succinate polyester in a flame ionization gas chromatograph (10). The chromatogram showed only the peak for palmitaldehyde (less than 0.5 μg of 17:0 aldehyde).

The fatty acid fraction was esterified with 0.1 ml of dimethoxypropane mixture (11) and the methyl esters were purified by TLC as above. The eluted esters were analyzed by GLC as with the aldehydes. Table 5 shows the resultant data for the incubated and control samples. A distinct peak for 17:0 was seen in the samples incubated with substrate. The yield of 17:0 acid, based on the radioactivity found in the samples used for collecting the $^{14}CO_2$, was 71%. The incomplete stoichiometric balance may be the result of adsorption losses.

An assortment of fatty acids was seen in both samples (with and without substrate), mainly 16:0, 18:0, and 18:1, but with a trace of 17:0 in the control sample as well. The "background" fatty acids, evidently derived from the enzyme preparation itself, were similar in amount in both samples. Analysis of the fatty acids from the sample acidified and incubated with sulfuric acid showed somewhat more of all the acids, apparently because of acid-catalyzed breakdown of a lipid in the acetone powder.

Effects of Inhibitors

Iodoacetamide and, to a lesser extent, Tris buffer were inhibitory, but sodium arsenite (10^{-3} M), aminopterin (10^{-4} M), and catalase (100 units) were not. *p*-Hydroxymercuribenzoate (10^{-4} M) inhibited 34%. In a cruder system (acetone powder plus the ultrafiltrate obtained from a homogenate supernatant fraction) 2 × 10^{-3} M mercaptoethanol inhibited by 27%.

DISCUSSION

Role of the Ketostearate Decarboxylating System

Although β-degradation of fatty acids is one of the most securely established degradative routes, there is surprisingly little evidence from work in vivo that this is actually the *major* route in operation. Some evidence has shown that ω-degradation takes place, and recently it has been demonstrated in vivo that the brain carries out α-degradation, at least with the very long fatty acids. This type of degradation, which appears to involve the sequence:

fatty acid → 2-hydroxy fatty acid → 2-keto fatty acid → CO_2 + fatty acid

may well take place throughout the body. 2-Hydroxy fatty acids, both even- and odd-numbered, occur in many organs (12), which suggests that at least the first step in the above sequence is widespread. The data in

TABLE 5 PRODUCTION OF HEPTADECANOIC ACID AND CARBON DIOXIDE FROM KETOSTEARIC ACID BY PIG BRAIN ENZYME

Enzyme Incubation Protocol*	17:0 Acid Formed	CO_2 Formed	
	mμmoles	*mμmoles*	*cpm*†
Standard incubation system, followed by HCl acidification	31 32		
Same, but without substrate	0.7 1.9		
Standard incubation system acidified with sulfuric acid and shaken 45 min at 37°C	—	44 45	6671 6843
Same, but acidified at time zero	—	0.8	147

* As in assay procedure, but with double the amounts and incubated an additional 15 min. Observed activity in substrate was 26,150 cpm per tube.

† Activities uncorrected for counting efficiency.

Table 4 show that liver can decarboxylate ketostearate. While it is true that liver does not contain detectable amounts of 2-hydroxy fatty acids (12), this may mean only that liver lacks a substrate or enzyme for utilization of these acids in the synthesis of complex lipids.

α-Degradation takes place in plants also, as shown by Martin and Stumpf (13) and Hitchcock and James (14). The former workers found an aldehyde as the degradation product, and no evidence for intermediate formation of a 2-hydroxy acid. However, the system studied by the latter workers seems similar to the brain system.

Cofactor Requirements and Hydroxylation

The finding of highly efficient activation by ascorbate gives this interesting cofactor a new function. It is likely that the function is physiologically operative, for the ascorbate level in brain is roughly 10^{-3} M (15, 16), a concentration which gives some (albeit not optimal) activation of ketostearate decarboxylation (Fig. 1).

Addition of ascorbate, fumarate, and ATP to an adrenal enzyme system stimulates the β-hydroxylation of dopamine (9) and the similarity to our system is great enough to warrant considering whether ketostearate decarboxylation also involves a hydroxylation. Ascorbate is also involved in the hydroxylation of proline (17), steroids (18), tryptophan (19), and phenols (20). Ascorbate, together with ferrous ion, may produce hydroxyl radicals and hydroxyl ions. The former could attack the 2-keto carbon atom to displace the carboxyl ion, as shown in the equations:

$$Fe^{++} + O_2 + \text{ascorbate} \rightarrow Fe^{+++} + OH\cdot + OH^- + \text{dehydroascorbate}$$

$$R{-}\underset{\underset{O}{\|}}{C}{-}COO^- + OH\cdot \rightarrow R{-}\underset{\underset{O\cdot}{|}}{\overset{\overset{OH}{|}}{C}}{-}COO^- \xrightarrow{+OH\cdot} R{-}COO^- + H_2O + CO_2$$

The failure of catalase to inhibit the ketostearate decarboxylation rules out a role for hydrogen peroxide in the mechanism.

Attempts to demonstrate the formation of dehydroascorbate during the incubation failed because of the very small amount involved.

The acidic activating substance in the brain supernatant fraction was not rigorously identified as ascorbic acid. Beside the evidence cited before on heat, acid, and alkali effects and elution from an anion-exchange resin, there is the observation that the unknown factor can pass through a dialysis membrane. Moreover, we found the factor to be removed by charcoal yet not recoverable with 0.07 N ammonia in 50% ethanol. Ascorbate is rapidly oxidized under these conditions. The inhibitory effect of high concentrations of ascorbate (Fig. 1) is paralleled by the inhibitory effect of high concentrations of brain supernatant fraction observed in experiments with microsomes.

Ascorbate has been shown to have small activating activity in a variety of oxidative enzyme reactions. Perhaps a more careful evaluation with somewhat lower ascorbate concentrations would reveal an inhibitory effect at the more usual "survey" concentrations.

In a note published after the submission of this manuscript, Levis (21) reported a similar stimulating effect of ascorbate on the decarboxylation of ketostearate by microsomes, and observed the inhibitory effect of higher ascorbate concentrations. He also found chelators to be strongly inhibitory.

The involvement of iron in the decarboxylation reaction is supported by the inhibitory effects of very low levels of *o*-phenanthroline and bipyridine ("dipyridyl"), both of which are particularly good chelators of iron. A number of enzymes containing nonheme iron are now known (22–24); these too show erratic or no restoration of activity on addition of iron. However, copper as the active metal cannot be ruled out, as the decarboxylase is strongly inhibited by Cu^{++}, an inhibitor of Cu-containing enzymes (25). Levis (21) found diethyldithiocarbamate, an effective copper chelator, to inhibit ketostearate decarboxylation. Dopamine β-hydroxylase, a Cu enzyme, is inhibited by phenanthroline (25), ordinarily considered an iron chelator but capable of combining with Cu^{++}.

The failure of aminopterin to inhibit the decarboxylation suggests that tetrahydropteridines, cofactors active in some hydroxylations (26, 27), are not involved in keto acid decarboxylase. The active material found to be present in the cell supernatant fraction was retained by Dowex 1 in the acetate form; pteridines would not behave in this way.

Further characterization of the keto acid decarboxylation system is underway.

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