the second molecule of glutamine used in hypoxanthine synthesis.

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ACETOACETYL COENZYME A AS INTERMEDIATE IN THE ENZYMATIC BREAKDOWN AND SYNTHESIS OF ACETOACETATE[1]

Sir:

In a recent note[2] evidence was presented that enzyme preparations from pig heart catalyze the following reversible reactions[3]

(1) Succinyl-S-CoA + acetoacetate $\rightleftharpoons$ succinate + acetoacetyl-S-CoA

(2) Acetoacetyl-S-CoA + CoA-SH $\rightleftharpoons$ 2 acetyl-S-CoA

(3) 2 Acetyl-S-CoA + 2 oxalacetate $\rightleftharpoons$ 2 citrate + 2 CoA-SH

The CoA transferase catalyzing Reaction 1 is analogous to the acetyl-propionyl CoA transphorase discovered by Stadtman[4] in *C. kluyveri*. Reaction 3 is catalyzed by the citrate condensing enzyme.[5] By various methods, including low temperature ethanol fractionation in the presence of zinc ions, the enzymes catalyzing Reactions 1 and 2 have now been separated and the intermediate acetoacetyl CoA has been isolated.

When succinyl CoA[6] and acetoacetate are incubated with the heart CoA transferase, an acid-stable intermediate accumulates which can be precipitated as a crude, alcohol insoluble barium salt. The intermediate yields no citrate (*i.e.*, no acetyl CoA) in the presence of CoA-SH, oxalacetate and citrate condensing enzyme, unless the acetoacetate condensing enzyme (Reaction 2) from either heart or liver is present. As shown in Table I one molecule of CoA-SH (determined as sulfhydryl) is released for each two molecules of citrate formed (*cf.* Reactions 2 and 3).

Acetoacetyl CoA can be further purified by paper chromatography. In ethanol–acetate[7] its R_F is 0.52 at 24° (R_F of acetoacetate, 0.75). Like acetyl CoA[8] it gives a nitroprusside reaction only after treatment with alkali. The absorption spectrum of acetoacetyl CoA eluates is similar to that of Lynen's analog S-acetoacetyl-N-acetyl thioethanolamine.[9] At *p*H 8.1 the compound shows a strong absorption band in the range 290–320 mμ with a peak at 305 mμ. The absorption is markedly increased by magnesium ions. The formation and disappearance of acetoacetyl CoA, through progress of Reaction 1 or 2 in either direction, can thus be readily followed spectrophotometrically (Fig. 1). By means of the optical method it has been possible to obtain an approximate estimate of the equilibrium constant (*p*H 8.1) of Reaction 2 (K^2_{eq}: (Acetyl-S-CoA)^2/(Acetoacetyl-S-CoA) (CoA-SH) $\simeq 5 \times 10^4$) which greatly favors acetoacetyl CoA cleavage. The equilibrium constant of Reaction 1 (K^1_{eq}: (Succinate) (Acetoacetyl-S-CoA)/(Succinyl-S-CoA) (Acetoacetate)) is about 10^{-2}.

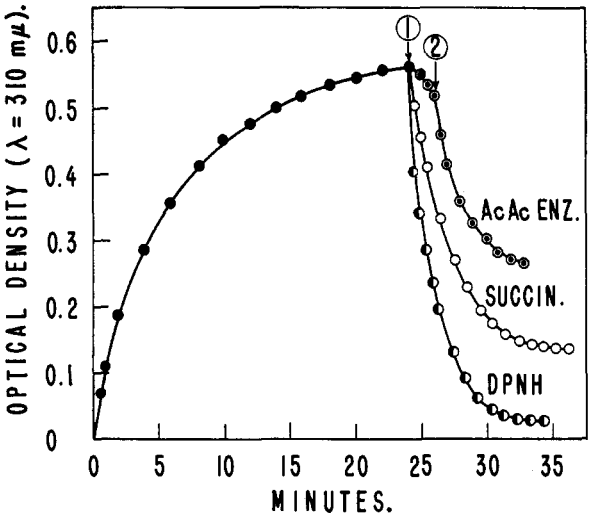

Fig. 1.—Ascending curve represents formation of acetoacetyl CoA on mixing, at *p*H 8.1, 0.4 μM. succinyl CoA and 100 μM. acetoacetate with 0.15 mg. CoA transferase protein (Reaction 1). The descending curves represent three separate experiments: (a) addition of 20 μM. succinate (arrow 1) shifts equilibrium of Reaction 1 to the left. (b) Addition of heart acetoacetate condensing enzyme fraction (0.35 mg. and 1.0 mg. protein at arrows 1 and 2) and CoA–SH causes cleavage of acetoacetyl CoA (Reaction 2). (c) Addition of 0.2 μM. DPNH (arrow 1) causes decrease in extinction through reduction of acetoacetyl CoA to β-hydroxybutyryl CoA.

TABLE I

50 μM. potassium phosphate buffer, *p*H 7.4, 4 μM. $MgCl_2$, 16 μM. potassium oxalacetate, 0.15 μM. CoA–SH (Pabst), potassium salt of intermediate (equivalent to 5.5 mg. of Ba salt), 0.03 mg. crystalline citrate condensing enzyme and, where indicated, ox liver acetoacetate condensing enzyme fraction (free of acetoacetyl CoA deacylase[2]); volume 1.2 ml.; incubation, 30 minutes at 38°.

Acetoacetate enzyme, mg. protein	0	4.3
SH released, μM.	...	0.12[a]
Citrate formed, μM.	0	0.26
SH/Citrate	...	0.46

[a] Corrected for CoA–SH liberated by spontaneous hydrolysis of succinyl CoA present in the acetoacetyl CoA preparation.

The CoA transferase, which has been purified over 100-fold from pig heart extracts, contains as a contaminant the β-hydroxybutyryl CoA dehydrogenase recently described by Lynen, *et al.*[9] DPNH is rapidly oxidized by acetoacetyl CoA (but not by

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(2) J. R. Stern, M. J. Coon and A. Del Campillo, *Nature*, **171**, 28 (1953).

(3) Abbreviations: Coenzyme A (reduced), CoA or CoA-SH; acyl coenzyme A derivatives, acyl CoA or acyl-S-CoA; reduced diphosphopyridine nucleotide, DPNH; μM., micromoles.

(4) E. R. Stadtman, *Federation Proc.*, **11**, 291 (1952).

(5) S. Ochoa, J. R. Stern and M. C. Schneider, *J. Biol. Chem.*, **193**, 691 (1951).

(6) Prepared synthetically from succinic anhydride and CoA-SH by an unpublished method of E. Simon and D. Shemin.

(7) E. R. Stadtman, *J. Biol. Chem.*, **196**, 535 (1952).

(8) F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1951).

(9) F. Lynen, L. Wessely, O. Wieland and L. Rueff, *Angew. Chem.*, **64**, 687 (1952).

acetoacetate) in the presence of transferase preparations; the reaction can be followed by the decrease in optical density at 310 (Fig. 1) or 340 mμ. The product, β-hydroxybutyryl CoA, has been isolated by chromatography as above (R_F, 0.64) and characterized enzymatically.

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COFACTOR REQUIREMENTS FOR THE DECARBOXYLATION OF SUCCINATE

Sir:

The decarboxylation of succinate, considered to be the main pathway of propionate formation in the propionibacteria and certain micrococci, was discovered in experiments with suspensions of whole cells[1,2,3] and consequently the cofactor requirements have not been determined. This communication presents evidence, obtained from experiments with cell-free preparations, that several cofactors are involved in the decarboxylation of succinate.

Micrococcus lactilyticus, strain 221, an anaerobic micrococcus capable of fermenting organic acids[4] and purines,[5] was used. Cells were grown in a medium composed of inorganic salts,[5] 2–3% lactate, 1% Difco yeast extract, 2% peptone and 0.0015% thiamin. Extracts were prepared by grinding the harvested cells with alumina,[6] extracting the paste with 0.01% cysteine solution, and centrifuging at high speed.

The quantitative decomposition of succinate to carbon dioxide and propionate by such extracts is considerable without the addition of any cofactors, although the presence of reducing agents and magnesium chloride increases the rate of carbon dioxide production. It can be demonstrated, however, that coenzyme A (CoA), adenosine-tri-phosphate (ATP), and cocarboxylase take part in succinate decarboxylation. A great decrease in decarboxylase activity is produced by treatment of the extract with Dowex-1 and Norit.[7] It was found that this treatment reduces the CoA content by 98% (determined by the assay method of Kaplan and Lipmann[8]) and the ATP content by 90%. Since the method of treatment does not remove all of the ATP and CoA from the extract, some decarboxylation occurs without the addition of cofactors, and a rather large increase in CO_2 production can be observed with the addition of CoA alone (Table I). Since small amounts of ATP (data not presented here) are sufficient to initiate decarboxylation and are as effective as large amounts, it appears that ATP acts as a "sparker." Decarboxylase activity is almost completely regained by the addition of ATP, CoA and cocarboxylase, as shown in Table I.

TABLE I

EFFECT OF COFACTORS ON SUCCINATE DECARBOXYLATION

Warburg vessels contained: 50 μM. succinate, 50 μM. acetate buffer *p*H 5.5, 100 μM. NaF, 10 μM. glutathione, 10 μM. $MgCl_2$, 1.0 ml. extract containing 20 mg. protein, total volume 2.5 ml. Incubated under pure N_2, at 30° for 2 hours. Pabst ATP and CoA (approximately 200 units/mg.) used.

Extract	Additions	μl. CO_2[a]
Untreated	None	458
Treated	None	35
Treated	10 μM. ATP	48
Treated	10 units CoA	249
Treated	10 units CoA, 10 μM. ATP	390
Treated	10 units CoA, 10 μM. ATP, 25γ cocarboxylase	416

[a] Values corrected for bound CO_2.

The effect of cocarboxylase can be demonstrated more clearly by using extracts of cells grown in media with suboptimal concentrations of yeast extract and thiamin, as shown in Table II.

TABLE II

EFFECT OF COCARBOXYLASE ON SUCCINATE DECARBOXYLATION

Experimental conditions as given under Table I. Extract from cells grown under suboptimal conditions

Cocarboxylase added per vessel	μl. CO_2[a]
None	209
0.5γ	214
1.5γ	226
5.0γ	255
15.0γ	270
50.0γ	270

[a] Values corrected for bound CO_2.

An investigation of the possible role of these cofactors has shown that succinate is activated prior to decarboxylation. This conclusion is based on the following evidence: (a) in the presence of hydroxylamine, succinhydroxamic acid, identified by paper chromatography,[9] is formed (Table III), (b) with

TABLE III

THE EFFECT OF ATP AND CoA ON THE FORMATION OF SUCCINHYDROXAMIC ACID

Extract used and experimental conditions as listed under Table I except that 1000 μM. hydroxylamine present as a trapping agent and M TRIS buffer, *p*H 7.0, substituted for acetate buffer.

Extract	Additions	μM. succinhydroxamic acid[a]
Untreated	None	4.54
Treated	None	0.20
Treated	10 μM. ATP	3.02
Treated	10 units CoA	0.22
Treated	10 units CoA, 10 μM. ATP	3.06

[a] Values corrected for endogenous control.

treated extracts, in the presence of added ATP and the absence of added CoA, the same amount of succinhydroxamic acid is formed whether hydroxylamine is added initially as a trapping agent or at the end of the incubation period, (c) the amount of succinhydroxamic acid formed is dependent upon the

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