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THE MECHANISM OF PROPIONIC ACID FORMATION BY SUCCINATE DECARBOXYLATION. I. THE ACTIVATION OF SUCCINATE

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The studies of Johns[1–8] and Delwiche[4] with suspensions of anaerobic micrococci (*Micrococcus lactilyticus* or *Veillonella gazogenes*) and *Propionibacterium* spp. showed that these bacteria produced propionate by the decarboxylation of succinate. This decarboxylation can also be accomplished by cell-free extracts of *M. lactilyticus* and the requirement for specific cofactors in this reaction has been briefly described.[5] The present paper contains detailed evidence for the participation of these cofactors in the activation of succinate and for the nature of the active product.

Methods.—M. lactilyticus (strain 221) was grown in tap water with 2–3% sodium lactate, 2% peptone, 1% yeast extract, salts, and 0.0015% thiamin. The latter was added routinely to all media, except when otherwise noted, because some batches of yeast extract, apparently deficient in thiamine, yielded small cell crops and extracts of such cells had low decar-

boxylase activity on a unit protein basis. When the deficient media were supplemented with thiamin, greater cell crops resulted, and extracts prepared from these cells had a high activity.

Cultures were incubated at 37°C. for 16–24 hours; the cells were then harvested, washed once in tap water, ground with alumina,[6] the cell paste extracted with distilled water, or with a 0.01% solution of a reducing agent, and centrifuged at approximately 12,000 × g for 20 minutes. Extracts so prepared, with cysteine as the reducing agent, could be stored for several days in the frozen state without great loss in activity.

Details of manometric procedure and of methods for the determination of volatile acids have been published elsewhere.[7] The protein content of the extracts was estimated by the method of Stadtman, *et al.*[8]

Total hydroxamic acid was determined according to Lipmann and Tuttle;[9] identification and quantitative estimation of the component acids was accomplished by paper chromatography.[10] For the separation of succinhydroxamic acid from other hydroxamic acids a 10:2 mixture of phenol-water was used.

TABLE 1

THE EFFECT OF pH ON THE DECARBOXYLATION OF SUCCINATE

pH	μL. CO_2/10 MG. EXTRACT PROTEIN/HOUR
5.0	490
5.3	543
5.5	560
5.7	520
6.0	512
6.5	359
7.0	199

Pabst Co. preparations of adenosine-tri-phosphate (ATP) and coenzyme A(CoA, approximately 200 units/mg.) were used. Concentrations of CoA are expressed in terms of units as defined by Kaplan and Lipmann.[11]

CoA and ATP were adsorbed from the extracts by treatment with Dowex-1 and Norit A;[8] 98% of the CoA, and 90–95% of the ATP content were thus removed. The former value was established by CoA assay with pigeon-liver enzyme,[11] the latter by measurement of absorption at 260 mμ.

Dialysis was performed at 5°C. against a 0.001% cysteine solution at pH 5.5; if continued too long, the extracts are permanently inactivated. Consequently the optimal duration of dialysis was established by determining the activation of the extracts at frequent intervals.

Results.—Conditions for testing the decarboxylation of succinate: All experiments were conducted under anaerobic conditions inasmuch as in air succinate is not decarboxylated but oxidized to acetate. The optimum

substrate concentration for carbon dioxide production is approximately 20 μM/ml. with 20 mg. of extract protein. The highest rate of decarboxylation occurred at pH 5.5 (table 1), and all experiments in which carbon dioxide production was measured were performed at this pH. With freshly prepared extracts the rate of decarboxylation was approximately the same in various buffer solutions (acetate, propionate, benzoate, man-

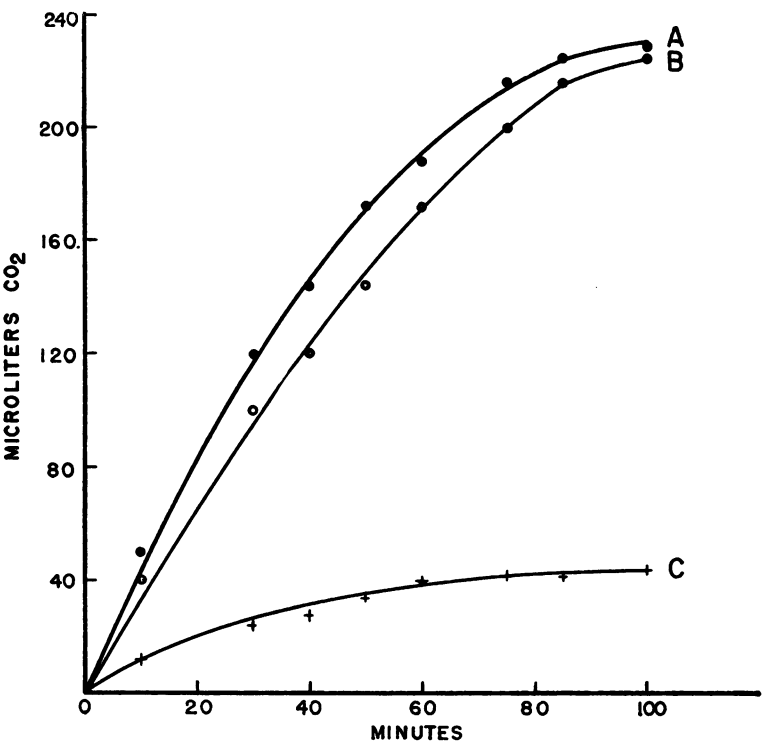

FIGURE 1

Decarboxylation of succinate by extracts of *M. lactilyticus*. A: Original extract, 10 μM succinate. B: Supernatant fluid after centrifugation at 60,000 × g, 10 μM succinate. C: Sediment after centrifugation at 60,000 × g, 10 μM succinate; also endogenous, original extract.

delate, and phthalate); citrate buffer was strongly inhibitory, and phosphate caused a slight decrease in the rate of decarboxylation. Since $MgCl_2$ partially relieved the inhibition by citrate, but not by phosphate, the action of the former was attributed to the binding of metal ions. The addition of $MgCl_2$ and reducing agents increased the rate of succinate decarboxylation, the latter probably by maintaining the cofactors in the reduced (active) state. Cysteine, glutathione, thioglycollate, and 1,3-

dimercaptopropanol at a final concentration of approximately 0.01 *M* were equally effective; Na_2S was toxic. NaF was added to inhibit an ATP-ase present in the crude extracts. Thus, carbon dioxide production from succinate by freshly prepared bacterial extracts was measured at 30°C., in N_2, with the following reaction mixture: 1 ml. extract containing 10–20 mg. protein, 10 μM reducing agent, 10 μM $MgCl_2$, 100 μM NaF, 50 μM succinate, and 50μM buffer, the choice of buffer depending on the nature of the experiment; total volume 2.2 ml.

Figure 1 shows the production of carbon dioxide from succinate by an extract of *M. lactilyticus*. If an extract prepared as described is further centrifuged at approximately 60,000 × g for 40 minutes, a small amount of sediment is found. This sediment is not capable of decarboxylating succinate, but the supernatant fluid is, and at a rate comparable to that determined for the original extract. This shows that the enzymes involved in the decarboxylation of succinate are soluble.

Suspensions of intact cells decompose succinate to equimolar amounts of carbon dioxide and propionate;[1, 2] this is true also for extracts. The decomposition of succinate is therefore a straight decarboxylation.†

Cofactor requirements: Freshly prepared extracts, tested under the above conditions, decarboxylate succinate rapidly without the addition of any cofactors (Fig. 1). As described previously,[5] an increase in the rate of carbon dioxide production occasionally results from the addition of cocarboxylase. This effect is restricted to extracts prepared from cells grown in a thiamine-deficient medium, or to extracts subjected to prolonged dialysis.

Experiments with cell suspensions of biotin-deficient *Propionibacterium pentosaceum*[12] showed that biotin increased the rate of succinate decarboxylation. Since *M. lactilyticus* does not require this cofactor for growth,[13] biotin-deficient cells could not be obtained. However, the addition of biotin to untreated or "aged" cell suspensions,[14] or to freshly prepared, dialyzed, or depleted extracts did not increase the rate of succinate decarboxylation. Adenylic acid and a factor prepared by autoclaving glucose with H_2SO_4 have been reported to be involved in biotin-mediated reactions;[15, 16] their addition, singly or in combination with biotin, did not affect the decarboxylation of succinate by the above-described preparations of *M. lactilyticus*.

Freshly prepared extracts respond to the addition of ATP and CoA, although a greater increase in decarboxylase activity upon the addition of these cofactors can be demonstrated with extracts that have been dialyzed or stored in the frozen state. The greatest effect is observed with preparations from which CoA and ATP have first been removed by treatment with adsorbents. Such extracts have low activity, but almost complete reactivation can be accomplished by adding both ATP and CoA, in the

presence of cocarboxylase,[5] provided the pretreatment of the extract has not resulted in significant inactivation of the enzymes concerned.

If traces of ATP remain in the extract after adsorption, the addition of CoA alone is sufficient to cause considerable carbon dioxide production.[5] However, a more complete removal yields extracts that do not respond to the addition of either cofactor alone; both ATP and CoA are required for succinate decarboxylation. The rate of carbon dioxide production by such extracts increases, though not proportionally, with increasing amounts of ATP. The experimental results, summarized in table 2, show that the addition of even small amounts of ATP causes the decomposition of large amounts of succinate in the presence of CoA.

TABLE 2

THE EFFECT OF ATP AND CoA ON THE DECARBOXYLATION OF SUCCINATE BY DEPLETED EXTRACTS

	NO CoA ADDED		18 UNITS CoA ADDED				
μM ATP added	0	10.0	0	1.0	2.5	5.0	10.0
μl. CO_2/10 mg. extract protein/hour	46	40	56	226	234	338	380

TABLE 3

THE EFFECT OF ATP ON THE FORMATION OF SUCCINHYDROXAMIC ACID BY A DEPLETED EXTRACT

μM ATP ADDED	μM HYDROXAMIC ACID/10 MG. EXTRACT PROTEIN/HOUR
0	0.2
5.0	1.7
10.0	3.5
15.0	4.0
20.0	4.2
10.0	3.1[a]
10.0	0.0[b]

Reaction mixture contained: 1000 μM hydroxylamine, 500 μM TRIS buffer pH 7.3, 50 μM succinate, 10 μM glutathione, 10 μM $MgCl_2$, 100 μM NaF; incubated at 30°C. under pure N_2.

[a] Hydroxylamine added at end of incubation period.

[b] Reaction mixture heated for 5 minutes at 100°C. (pH 5.0) at end of incubation period and hydroxylamine added.

Activation of succinate: The reactivation of depleted extracts by ATP and CoA indicates an initial conversion of succinate to succinyl-CoA or succinyl phosphate prior to decarboxylation. This inference is supported by the fact that a hydroxamic acid, identified as succinhydroxamic acid by chromatography, is formed in extracts to which hydroxylamine has been added. The following observations suggest that, under certain conditions, the substance reacting with hydroxylamine is succinyl phosphate rather than succinyl-CoA.

The addition of hydroxylamine at the start of the incubation period completely suppresses carbon dioxide evolution; the amount of succinhydroxamic acid formed under these conditions does not depend on the quantity of CoA present,[5] but is solely determined by the amount of ATP (table 3). The addition of hydroxylamine at the end yields only traces of hydroxamic acid if sufficient CoA is present; in this case, decarboxylation of succinate occurs. With extracts supplied with ATP, but unable to decarboxylate succinate owing to the virtually complete removal of CoA, nearly the same amount of succinhydroxamic acid is obtained when hydroxylamine is added at the end, as when it was present from the start. Since the quantity of activated succinate produced in the absence of hydroxylamine by depleted

TABLE 4

THE EFFECT OF VARIOUS SUBSTANCES ON CARBON DIOXIDE PRODUCTION AND SUCCINHYDROXAMIC ACID FORMATION

ADDITION[a]	IN % OF CONTROL	
	CO_2	HYDROXAMIC ACID
Potassium arsenate	5	1
Sodium chloride	32	63
Lithium chloride	57	70
Potassium phosphate	20	130

[a] Final concentration 0.01 *M*.

TABLE 5

THE EFFECT OF ARSENATE ON THE FORMATION OF SUCCINHYDROXAMIC ACID BY A DEPLETED EXTRACT

UNITS CoA ADDED	μM ARSENATE ADDED			
	0.0	20.0	50.0	100.0
0	1.63	1.56	1.43	1.33
9	..	0.28	1.20	0.00
18	..	0.10	0.00	..
36	1.50[a]	0.05	..	..

Values given as μM hydroxamic acid/10 mg. extract protein/hour; 10 μM ATP in all reaction mixtures; experimental conditions listed under table 3; hydroxylamine added at end of incubation period.

[a] Hydroxylamine added at start of incubation period.

extracts depends on the ATP concentration, and far exceeds the trace amounts of CoA present, it appears likely that succinyl phosphate rather than succinyl-CoA is the compound giving rise to succinhydroxamic acid.

If the reaction mixture resulting from the incubation of a depleted extract, succinate, and ATP is heated for 5 minutes at 100°C. (pH 5.0), the addition of hydroxylamine no longer yields hydroxamic acid (table 3). Hence, the precursor of the latter is heat labile, a characteristic of acylphosphates, but not of acyl-CoA compounds,[17] although Sanadi and Littlefield[18] recently found that CoA esters may also be heat labile in crude preparations. However, in an aliquot of the *M. lactilyticus* extract used for testing the stability of the hydroxamic acid precursor, acetyl-CoA

was not decomposed by heat, thus supporting the proposal that the former is succinyl phosphate, rather than succinyl-CoA.

Supplementary evidence of a more indirect nature for the formation of a phosphate derivative comes from experiments with arsenate. The addition of arsenate completely inhibits carbon dioxide evolution and the production of succinhydroxamic acid (table 4), provided CoA is present. With increasing amounts of CoA, the arsenate inhibition of succinhydroxamic acid formation also increases (table 5). This effect can be accounted for by assuming a reversible, enzyme-mediated, reaction between succinyl phosphate and CoA with the formation of phosphate and succinyl-CoA. Substitution of arsenate for phosphate in the back reaction yields CoA and succinyl arsenate, followed by an instantaneous hydrolysis of the latter. This is analogous to the mechanism proposed for the disappearance of acetyl phosphate in solutions with phosphotransacetylase, arsenate, and CoA.[19]

Experiments on the effect of Na^+, Li^+, and $PO^{\equiv}_4$, on the activity of extracts also support the view that succinyl phosphate is produced (table 4). These ions differentially inhibit carbon dioxide production and the formation of succinhydroxamic acid. Na^+ and Li^+ inhibit both, but the addition of $PO^{\equiv}_4$ causes an increase in succinhydroxamic acid production and a marked decrease in carbon dioxide evolution. It is known that Na^+ and Li^+ have an inhibitory effect on phosphotransacetylase;[19] the action of $PO^{\equiv}_4$ is explicable by postulating the reversal of a reaction in which phosphate is an end-product, such as the above-mentioned reaction between succinyl phosphate and CoA.

The results of these experiments therefore show that succinate is initially activated in cell-free extracts of *M. lactilyticus*. The ATP-dependent accumulation of a substance capable of forming succinhydroxamic acid suggests that under certain conditions succinyl phosphate is the "active" form of succinate; experiments with inhibitors imply that succinyl-CoA may be formed. Evidence has recently been obtained for the formation of the both compounds by heart muscle preparations during the oxidation of alpha ketoglutarate.[20, 21]

Succinyl phosphate may be produced in the bacterial extract either from succinate and ATP, or by the initial formation of succinyl-CoA from succinate, ATP and CoA, followed by trans-succinylation.

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† The production of equivalent amounts of carbon dioxide and propionate from

succinate can be explained most simply as the result of succinate decarboxylation and this interpretation is used in the present papers. It is, however, recognized that experiments with purified enzymes may show that the process involves successive oxidations of four-carbon compounds and reductions of three-carbon compounds.

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THE MECHANISM OF PROPIONIC ACID FORMATION BY SUCCINATE DECARBOXYLATION. II. THE FORMATION AND DECARBOXYLATION OF SUCCINYL-CoA

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The effects of arsenate, phosphate, Na^+, and Li^+, described in the preceding paper,[1] suggest that succinyl-CoA is enzymatically produced from succinyl phosphate and CoA in extracts of *Micrococcus lactilyticus*. The CoA requirement for succinate decarboxylation thus indicated that succinyl-CoA might be the actual substrate for the decarboxylase. Evidence is presented here for the decarboxylation of succinyl-CoA and the mechanism of its regeneration in such extracts.

Methods.—Sulfanilamide acylation was tested by the method of Kaplan and Lipmann,[2] sulfanilamide being determined according to Bratton and Marshall.[3]