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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amount of ATP and is independent of CoA, thus suggesting that succinyl phosphate may be formed, (d) in experiments with sulfanilamide,[10] a CoA- and ATP-dependent decrease in free amide is observed; this decrease is greater if carbon dioxide production is inhibited, suggesting that succinyl-CoA, as well as succinyl phosphate, may be formed. A detailed treatment of the experimental results and of the postulated mechanism of succinate activation will be presented elsewhere.

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(10) D. R. Sanadi and J. W. Littlefield, *J. Biol. Chem.*, **193**, 683 (1951).

(11) Part of this work was done at the Hopkins Marine Station, Pacific Grove, California, on an Atomic Energy Commission Postdoctoral Fellowship.

SULFATED NITROGENOUS POLYSACCHARIDES AND THEIR ANTICOAGULANT ACTIVITY[1]

Sir:

N-Deacetylated chitin, previously swollen with pyridine, was heterogeneously sulfated at 100° for 1 hour with chlorosulfonic acid and pyridine to yield a product, isolated (inorganic salts were removed by dialysis) as the amorphous, water-soluble sodium salt, containing essentially two N-sulfate and one O-sulfate groups per anhydrodisaccharide unit; $[\alpha]^{25}D$ −23° (*c* 1.5, water). *Anal.* Calcd. for $C_{12}H_{19}O_7(NSO_3Na)_2(OSO_3Na)$: C, 22.93; H, 3.05; N, 4.46; S, 15.31; Na, 10.98. Found: C, 22.68; H, 3.08; N, 4.02; S, 15.6; Na, 11.2; $-NH_2$ (by ninhydrin), absent; NAc, absent. This preparation exhibited the behavior in the Van Slyke amino acid assay characteristic of the acid-labile N-sulfate group present in heparin.[2,3] Its anticoagulant activity was 56 International Units (I. U.)/mg. The animal (mouse intravenous) toxicity was approximately double that of heparin, a finding believed to be due to the unsuitably high molecular size of the substance.

(1) Supported by the Bristol Laboratories, Inc., Syracuse, N. Y., (R. F. Project 432).

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Chondroitinsulfuric acid (from cartilage) was essentially homogeneously N-deacetylated with 45% NaOH (25, 48 hr.) under nitrogen and in the presence of antioxidants (benzyl alcohol and sodium sulfite) and was sulfated as described above (but at 80–90°). The product was isolated as the amorphous sodium salt and is under further analytical characterization; $-NH_2$ (by ninhydrin), absent. The anticoagulant activity was 48 I. U./mg. The same sample of sodium chondroitin sulfate was subjected to the above sulfation procedure without preliminary N-deacetylation and the product, isolated in the same manner, showed an anticoagulant activity of *ca.* 10 I. U./mg. Sodium heparinate was re-sulfated under these conditions with a reduction in its activity from 110 to 55 I. U./mg. and an increase in the sulfur content from 12 (initial) to 14.4%.

Methyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride[4] was sulfated as above to produce the amorphous, water-soluble barium salt of the N-sulfate, tri-O-sulfate; $[\alpha]^{25}D$ +4° (*c* 3.4, H_2O). *Anal.* Calcd. for $C_7H_{11}NO_{17}S_4Ba_2 \cdot 2H_2O$: S, 15.63; Ba, 33.48. Found: S, 15.64; Ba, 32.98. A 3×10^{-4} *M* solution of this substance in 0.004 *N* HCl at 95° lost 1.0 mole of sulfate in ≦20 min. with the concomitant release of the free amino group (ninhydrin). The O-sulfate was removed relatively more slowly and only completely so after 12 hr. Previously reported results[2] on the inactivation of heparin by mild acidity were considered to involve a negligible sulfate loss. On the basis of our present knowledge of the heparin molecule,[5] this sulfate loss is about equivalent to the amino group released so that a sulfate group shift[5] is not a required postulation.

The above results show that the sulfamic acid group is a potent contributor to anticoagulant activity. Experiments are now underway to determine the optimum molecular size for these chemically modified polysaccharides.

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