A BIOTIN FUNCTION IN SUCCINIC ACID DECARBOXYLATION BY PROPIONIBACTERIUM PENTOSACEUM

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Through the demonstration of the importance of biotin to oleic acid synthesis, to oxalacetate decarboxylation and carbon dioxide fixation, and to certain amino acid deamination reactions, as reviewed by Stokstad and Jukes (1949), and to citrulline synthesis (MacLeod *et al.*, 1949), it is becoming increasingly apparent that the coenzyme form of this vitamin may be concerned in many different types of biological transformations.

Recent studies in our laboratory indicate that biotin is closely concerned with the decarboxylation of succinic acid by a strain of *Propionibacterium pentosaceum*. It is likely that the stimulation of the succinic decarboxylase system observed in repeated experiments is a reflection of coenzyme function occurring upon the addition of biotin to biotin-deficient cells.

EXPERIMENTAL METHODS

Strain E214 of *Propionibacterium pentosaceum* of the C. B. van Niel collection was shown in a previous study to require biotin for growth (Delwiche, 1949). A detailed investigation of the quantitative needs of the organism showed that the minimum amount of biotin necessary for maximum growth on synthetic medium was in the order of 10 mμg per ml, with growth stimulation occurring in the range of 0.001 mμg per ml up to the minimum required for maximum growth. Cells grown in a biotin concentration of 4 mμg per ml were consistently capable of marked stimulation by the addition of biotin to decarboxylating cells in a Warburg vessel. The basal culture medium was of the following composition:

Glucose	10 g	$MnSO_4 \cdot 4H_2O$	3.6 mg
Casein hydrolyzate	5 g	Adenine	10 mg
$NaC_2H_3O_2 \cdot 3H_2O$	8 g	Guanine	10 mg
Sodium thioglycolate	0.2 g	Uracil	10 mg
L-Cystine	0.05 g	Xanthine	10 mg
L-Tryptophan	0.05 g	Calcium pantothenate	1.0 mg
$K_2HPO_4 \cdot 3H_2O$	4 g	*p*-Aminobenzoic acid	1.0 mg
NaCl	4 g	Thiamine HCl	1.0 mg
$MgSO_4 \cdot 7H_2O$	0.25 g	H_2O to make one liter	
$FeSO_4 \cdot 7H_2O$	0.012 g	pH adjusted to 6.6 to 6.8	

A medium satisfactory for the production of biotin-deficient cells was obtained by the addition of a concentrated biotin stock solution in such amount as to give a final concentration of 4 mμg per ml of medium. This was accomplished by the addition of 1 ml of a biotin solution of a concentration of 1 μg per ml to 250 ml of basal synthetic medium.

The usual inoculation procedure consisted of the inoculation of 250 ml of medium with four-times-washed cells from 25 ml of a 48-hour culture from a complex medium composed of 0.5 per cent each of glucose, yeast extract, and pepticase. Cells were harvested by centrifugation after 40 to 42 hours of incubation at 30 C. They were washed once in distilled water and resuspended to the desired concentration.

The progress of the decarboxylation reaction was followed by the usual Warburg procedures for measuring CO_2. The general techniques used are described in a previous paper from this laboratory (Delwiche, 1948) dealing with the mechanism of propionate formation.

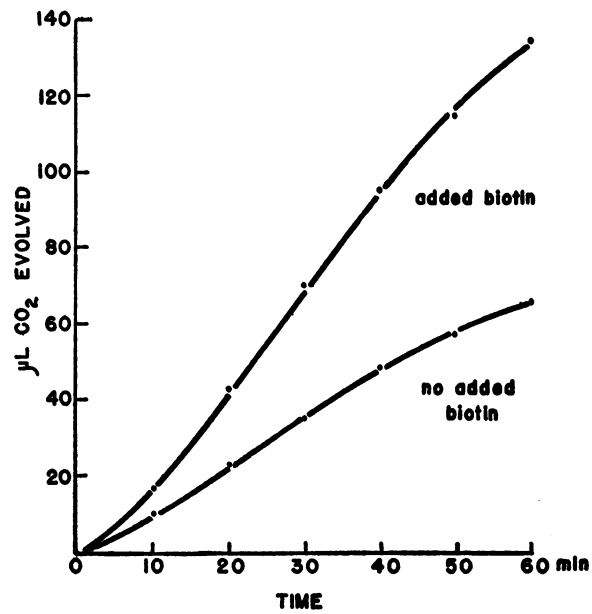

Figure 1. Effect of added biotin upon the activity of the succinic decarboxylase system of *Propionibacterium pentosaceum.*

EXPERIMENTAL RESULTS AND DISCUSSION

The data represented in figure 1 were obtained by a simple manometric measurement of CO_2 evolution. The Warburg vessels contained 0.2 ml of M/5 succinate in the side arm, 0.2 ml of a solution of biotin of a concentration of 20 µg per ml when included, and 1 ml of a cell suspension of 20 mg per ml in distilled water in the main compartment of the flask. The volume was adjusted to 3.0 ml by the addition of M/10 phosphate buffer. The reaction mixture was initially at pH 5.2 and was so obtained by the addition of substrate and buffer of this pH. Incubation was at 30 C for 60 minutes. In all experiments control vessels were included for the correction of the final data for endogenous CO_2 production. The curves of figure 1 are so corrected for carbon dioxide production in the absence of substrate.

It is apparent that the addition of biotin more than doubled the decarboxylation rate, and a comparison of the rate ($Q_{CO_2} = 6.7$) of the vessel in which biotin was added to data obtained from cells grown in adequate amounts of biotin showed that complete activity was restored.

Biotin concentration in the particular experiment here presented was in excess of the total required in the Warburg vessel. Diminishing the concentration to one-tenth of the amount added still gave maximum stimulation. A decrease of a hundredfold gave lower stimulation, and a thousandth of the amount of biotin added in the experiment reported still gave detectable stimulation.

As reported in a previous publication from this laboratory (Delwiche, 1948), the activity upon succinate of cells grown on complex media was completely unaffected by the presence of M/100 semicarbazide. The inclusion of semicarbazide in the Warburg cup in a like concentration was also found to have no effect upon the stimulation by biotin of cells grown on synthetic medium under the conditions described. This latter fact is of considerable importance in that it is evidence against the possibility of the vitamin function here demonstrated being a result of oxalacetate decarboxylation. Although it is questionable if semicarbazide can react with oxalacetic acid in biological systems, it is well established that the semicarbazone of pyruvic acid is readily formed (Lichstein and Umbreit, 1947). To form oxalacetic acid from succinate, it is reasonable to assume that the intermediates, fumarate and malate, are necessarily formed. The oxidations would thereby require a hydrogen acceptor, which, if such did occur, would probably be pyruvate. In the form of the semicarbazone, however, pyruvate could not be reduced by a biological system and the process necessarily would come to a halt due to the saturation of hydrogen transport mechanisms. That the CO_2 evolution is unaffected by the presence of semicarbazide is adequate evidence, in our opinion, that oxalacetic acid decarboxylation is not occurring. Independent evidence is not lacking and is discussed in the previous publication in which the decarboxylation of succinic acid to propionic acid is offered as an important mechanism in the formation of propionic acid (Delwiche, 1948). In earlier experiments, in attempts to grow biotin-deficient cells it was observed that in some cases growth in lower concentrations of biotin (0.1 mμg per ml of medium) gave poor yields of cells, which were capable of biotin stimulation but also inhibitable by semicarbazide to the extent of 10 to 25 per cent of maximum activity with biotin present or absent. The cells grown in this low concentration of biotin were always low in activity, and, whenever biotin was increased in the culture medium, activity on succinate was greatly enhanced and semicarbazide sensitivity eliminated. This could constitute evidence for an alternate mechanism of succinate attack, and, indeed, such apparently occurs to a very limited extent as indicated by the slight production of acetic acid with succinate as a substrate (Delwiche, 1948). Such a hypothesis would also be in harmony with the observations of Anthony *et al.* (1949) in which isotope studies are reported as indicating different modes of propionate formation. Under the conditions of growth we employed, however, the elimination of semicarbazide inhibition was so complete as to preclude the possibility of an alternate mecha-

nism of CO_2 production from succinate involving an intermediate with a carbonyl group capable of reaction with semicarbazide.

SUMMARY

Data are presented that demonstrate that biotin is closely concerned with the decarboxylation of succinic acid by a strain of *Propionibacterium pentosaceum*.

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