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Requirement of Succinate for the Growth of *Vibrio succinogenes*

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Vibrio succinogenes required relatively small amounts of succinate for growth when formate plus nitrate was supplied as the energy source. The requirement for succinate was not apparent when formate plus fumarate was the energy source because fumarate is reduced to succinate. L-Asparagine, fumarate, and malate replaced succinate, and it appears likely that they do so by being converted to succinate. Formate plus L-aspartate or L-asparagine served as energy sources for growth. The stoichiometry of the reduction of aspartate with H_2 by resting cells suggests an aspartase reaction followed by reduction of fumarate to succinate. Oxalacetate or pyruvate plus bicarbonate did not substitute for succinate, nor did many other compounds that were tested. ^{14}C-succinate was mainly incorporated into the alcohol-soluble fraction of cells, although there was significant incorporation into the hot trichloroacetic acid-soluble and -insoluble fractions.

Vibrio succinogenes is a gram-negative, anaerobic vibrio that gains energy for growth by coupling the oxidation of hydrogen or formate to the reduction of fumarate or nitrate. The organism was isolated from bovine rumen fluid (7). Studies of the nutritional requirements of *V. succinogenes* showed that it did not require vitamins and could grow on a mineral salts medium containing L-glutamate, L-aspartate, L-alanine, and L-cysteine with formate plus fumarate as the energy source (7). None of the nutritional studies done tested nitrate as a substitute for fumarate. In early experiments with cultures grown in nitrate-containing media, the cultures used as the inoculum were cultivated in fumarate-containing media. It was subsequently found that serial transfers in formate-nitrate medium containing yeast extract were impossible. This difficulty in serial transfer was found to be due to a requirement of the organism for small amounts of succinate. Succinate was provided in ordinary transfers from fumarate medium because succinate is the product of fumarate reduction. This report is concerned with the demonstration of the succinate requirement.

MATERIALS AND METHODS

Growth of organism. *V. succinogenes* was routinely grown at 37 C in a medium (FF) which contained 0.1% $(NH_4)_2SO_4$, 0.5% K_2HPO_4, 0.3% fumaric acid, 0.3% sodium formate, 0.1% yeast extract (Difco), 0.02% $MgCl_2 \cdot 6H_2O$, and 0.001% $FeSO_4$. The *p*H was 7.0 to 7.2. Sterile, autoclaved sodium thioglycolate (Difco) was aseptically added before inoculation to a final concentration of 0.05%. For growth with nitrate or other electron acceptors, fumarate was omitted and 0.6% $NaNO_3$ or 0.3% L-malate or L-aspartate was added to the medium. Other additions to the medium are indicated in the text.

When a synthetic medium was used, sodium formate, $NaNO_3$, K_2HPO_4, sodium thioglycolate, $(NH_4)_2SO_4$, $MgCL_2 \cdot 6H_2O$, and $FeSO_4$ were added as in the above complex medium. Other additions were as follows: acid-hydrolyzed casein (Nutritional Biochemicals Corp.) 1.0%; L-cysteine, 0.01%; DL-tryptophan, 0.01%; $MnCl_2 \cdot 4H_2O$, 0.004%; adenine, guanine HCl, uracil, and xanthine, 10 μg of each per ml; thiamine HCl and pyridoxine HCl, 1 μg of each per ml; and biotin, cyanocabalamin, and folic acid, 1 ng of each per ml. The medium *p*H was 7.0.

The inoculum for all nutritional experiments was prepared by centrifuging a 16- to 24-hr culture grown on FF medium and resuspending the cells in 9 ml of sterile 0.1 M K_2HPO_4 (*p*H 7.0) in 0.9% NaCl plus 1.0 ml of 0.5% sodium thioglycolate which was sterilized separately. A drop inoculum was used, and cultures were incubated at 37 C. The optical density of cultures was measured at 660 nm with a Bausch & Lomb Spectronic 20 spectrophotometer with 18- by 160-mm cuvettes.

Radioactivity experiments. Analyses of growth medium and cells and cell fractions for radioactivity were as previously described (1). The cell fractionation procedure of Roberts et al. (6) was used to ob-

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tain cold and hot trichloroacetic acid-soluble and related fractions. Radioactivity in succinic acid was determined after isolation of the acid on a silicic acid column according to the procedure of Ramsey (5).

RESULTS

Requirement for succinate. The growth response of the vibrio to succinate shown in Fig. 1 was observed both in the medium which contained yeast extract and in a synthetic medium. No growth was obtained without either formate or nitrate in the media. The base level of growth observed in both types of media may be due, in part, to the lack of extensive washing of the inoculum, but it is more likely attributable to the presence of substances in yeast extract and in acid-hydrolyzed casein which will substitute for succinate. Compounds which have been found to substitute for succinate are malate, fumarate, aspartate, and asparagine. The fact that malate and fumarate substitute for the succinate requirement when nitrate is used as a growth substrate is not surprising in view of the fact that the organism can reduce both of these substances to succinate (7). *V. succinogenes* can use malate as a growth substrate substitute for fumarate or nitrate. When malate or fumarate was substituted for succinate in nitrate media, the levels required for a given growth response were about the same as those for succinate (Table 1), and were in insufficient quantity to serve as growth substrates. Approximately 25 μmoles of fumarate or malate per ml of media containing formate was necessary for maximal growth of *V. succinogenes*. About 3 μmoles of fumarate or malate per ml supported maximal growth when formate and nitrate were the growth substrate; these low levels of fumarate or malate did not support significant growth of the vibrio when formate alone was present.

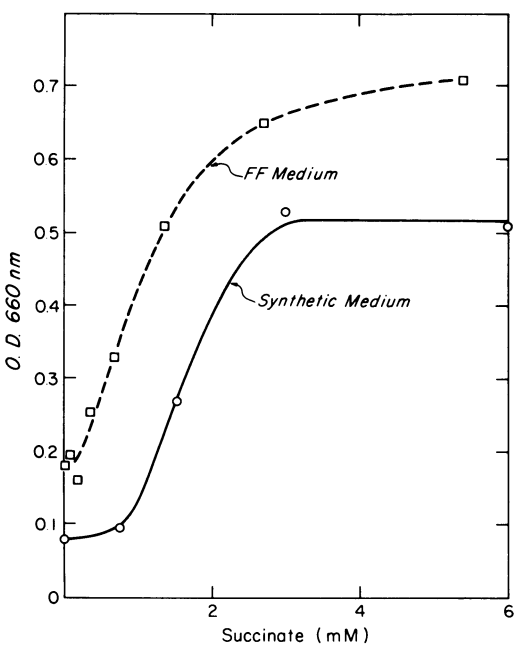

FIG. 1. *Growth response to succinate. Turbidity was measured after 24 hr. FF medium and the synthetic medium were used. The FF cultures were incubated in air, and the synthetic medium cultures were incubated in an N_2 atmosphere in a desiccator. Fumarate was omitted from the basal FF medium, and 0.6% $NaNO_3$ was substituted for the fumarate.*

Substition of aspartate for succinate. The substitution of L-aspartate for the succinate required for growth on nitrate initially suggested the possibility that the organism required the succinate or the other dicarboxylic acids for the synthesis of aspartate. Several lines of evidence, however, argue against this possibility and even suggest that aspartate can substitute for succinate because the amino acid can be converted to succinate which is actually the required compound. The amount of aspartate required as a substitute for succinate was approximately the same as the succinate requirement itself (Fig. 2). The amounts were considerably larger than would be expected for an amino acid requirement unless a serious permeability problem was involved. It was also found that L-aspartate and L-asparagine could substitute for fumarate, malate, or nitrate as a growth substrate in the presence of formate (Table 2). Approximately 10 times the

TABLE 1. *Dicarboxylic acids and growth on nitrate*[a]

Addition	Optical density	
	+ NO_3^-	− NO_3^-
None	.17	.04
Succinate		
0.5 mM	.27	.01
1.5 mM	.37	.02
3.0 mM	.51	.01
Fumarate		
0.5 mM	.21	.05
3.0 mM	.59	.10
Malate		
0.5 mM	.20	.02
1.5 mM	.45	.10

[a] Growth was in FF medium in air; turbidity was measured after 24 hr. Fumarate was omitted from the basal FF medium and added only where indicated. $NaNO_3$ was added, where indicated, to a final concentration of 0.6%.

amount of aspartate required for growth in nitrate media was required when aspartate was used as a growth substrate in place of nitrate. It was also found that resting cell suspensions catalyzed hydrogen uptake with L-aspartate as an electron acceptor. Approximately 1 mole of H_2 was taken up per mole of added aspartate. Although this reaction was not studied in detail, it is likely that an aspartase reaction is involved, in which aspartate is converted to fumarate, followed by reduction of the fumarate to succinate.

If the dicarboxylic acids required for growth on nitrate were subsequently converted to aspartate, it might be expected that oxalacetate or pyruvate plus bicarbonate might substitute for the succinate required. Oxalacetate (3 mM) or pyruvate (3 mM) plus $NaHCO_3$ (3 mM) did not substitute for the succinate required for growth on nitrate. Other compounds, tested at a level of 3 mM, which could not substitute for succinate were acetate, propionate, butyrate, L-glutamate, D-aspartate and Δ-aminolevulinic acid. The only compound, in addition to those mentioned, which substituted for succinate was L-asparagine.

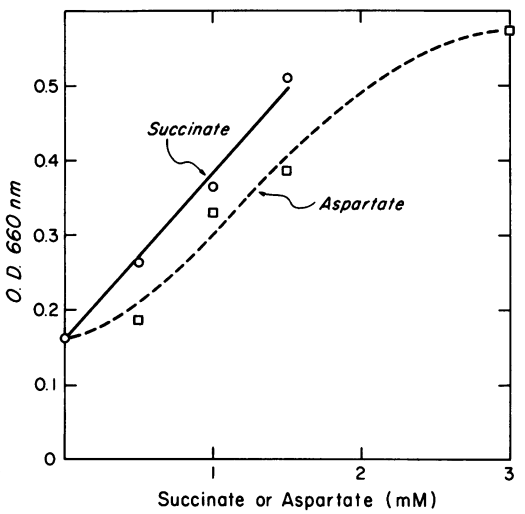

FIG. 2. *Growth response to L-aspartate. FF medium was used, and the cultures were incubated in air for 24 hr. Fumarate was omitted from the basal FF medium and 0.6% $NaNO_3$ was substituted for the fumarate.*

TABLE 2. *Aspartate and asparagine as growth substrates*[a]

Addition	Optical density	
	+ Formate (0.3%)	− Formate
L-Aspartate, 0.3%	.42	.01
L-Asparagine, 0.3%	.52	.01
Fumarate, 0.3%	.49	.03
None	.06	.04

[a] Growth was in FF medium in air; turbidity was measured after 24 hr. Fumarate was omitted from the basal FF medium and added only where indicated.

Growth on labeled succinate. It was thought that an examination of the fate of labeled succinate might give some indication of the reason that *V. succinogenes* requires succinate for growth on nitrate. *V. succinogenes* was grown on a synthetic medium containing 3 mmoles of unlabeled succinate; 1.5 μg of succinate-2-^{14}C (approximately 0.5 μCi) and 1.7 μg of succinate-*1,4*-^{14}C (approximately 0.5 μCi) were added to the medium. The cultures were incubated for 12 hr at 37 C in an N_2 atmosphere and then analyzed for radioactivity in CO_2, cells, and culture filtrate. The distribution of radioactivity in the various fractions is shown in Table 3. The great bulk of the radioactivity remained in the culture filtrate. Column chromatography of the culture filtrate recovered 93% of the radioactivity as succinic acid.

The distribution of the cell radioactivity in various cell fractions is shown in Table 4. Of the original cell radioactivity, 38.5% was recovered in the alcohol-soluble fraction, and the remaining radioactivity was about equally distributed between the hot trichloroacetic acid-soluble and -insoluble fractions. The counts in the protein fraction were not made directly on

TABLE 3. *Distribution of radioactivity in a culture grown on ^{14}C-succinate*

Fraction	Radioactivity[a]
Acidified cell-free medium	88.4
Cells	7.6
$^{14}CO_2$	2.8
Total recovered	98.8

[a] Percentage of the total radioactivity added to the medium.

TABLE 4. *Distribution of radioactivity in fractions from cells grown on ^{14}C-succinate*

Fraction	Radioactivity[a]
Cold trichloroacetic acid-soluble	0.0
Alcohol-soluble	38.5
Alcohol-ether-soluble	0.0
Hot trichloroacetic acid-soluble	18.4
Alcohol-ether wash	0.4
Protein hydrolysate	14.0

[a] Percentage of total radioactivity in cell fractions.

the hot trichloroacetic acid-insoluble precipitate after cell fractionation but were determined after hydrolysis of the hot trichloroacetic acid-insoluble material in 6 N HCl for 16 hr. Some ^{14}C may have been lost during the hydrolysis, which may account for the low overall recovery of radioactivity in the cell fractions. Significant amounts of radioactivity were present in the hot trichloroacetic acid-soluble fraction.

DISCUSSION

It is difficult to deduce a specific explanation for the nutritional requirement for succinate on the basis of the nutritional and labeling experiments. The amount of succinate carbon taken up by growing cells (Table 3) was approximately 1 mg in a total yield of 21 mg (dry weight) of cells. Since the carbon content of bacterial cells is usually about 50% of the dry weight (4), succinate does not appear to be required as a major carbon source. The distribution of radioactivity in the various cell fractions, however, does indicate that succinate carbon can be incorporated into a variety of cell components. A careful analysis of the specific activities of particular compounds and comparison of these activities with that of the starting succinate is necessary before it can be decided whether succinate is uniquely used for the synthesis of any cell component.

The initial observation of the succinate requirement immediately suggested the possibility of a need for a dicarboxylic acid for aspartic acid and aspartic acid family amino acid synthesis. Although aspartate does replace the succinate required the fact that aspartate can replace fumarate as an electron acceptor for growth with formate, and that aspartate can be reduced by hydrogen, suggests that aspartate can be converted to succinate. We cannot rule out the possibility, however, that succinate is required for conversion to aspartate or dicarboxylic acids, which are then used in essential reactions.

V. succinogenes was isolated from bovine rumen contents (7), and succinate is an important intermediate in propionate formation in the rumen (2). Other rumen bacteria have been studied which require components of rumen fluid such as acetate, valerate and isovalerate, ammonia, and CO_2 for growth. The only existing report of a nutritional role for succinate in a rumen bacterium is the recent report of the obligatory requirement for succinate of a rumen strain of *Bacteroides melaninogenicus* in the absence of vitamin K and in the presence of heme, and also in the absence of heme but in the presence of vitamin K (3).

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