JOURNAL OF BACTERIOLOGY, Oct. 1971, p. 175-178

Vol. 108, No. 1
Printed in U.S.A.

Succinate as a Growth Factor for *Bacteroides melaninogenicus*

M. LEV, K. C. KEUDELL, AND A. F. MILFORD

Department of Microbiology and Immunology and the Department of Surgery, Albert Einstein College of Medicine, Bronx, New York 10461

Received for publication 9 June 1971

Rumen strains of the obligate anaerobe *Bacteroides melaninogenicus* normally require medium supplemented with both heme and vitamin K. Sodium succinate was found to be an additional growth factor in that this compound can replace the requirement for heme in the presence of vitamin K, allowing good growth of the organism, and succinate can also partially replace the requirement for vitamin K in the presence of heme. The addition of succinate to a medium supplemented with both vitamin K and heme increases the growth rate of the culture. This ability to stimulate growth was specific for succinate, and cells grown without heme but with vitamin K and succinate were insensitive to cyanide. These experiments demonstrate a central role for succinate in the metabolism of *B. melaninogenicus*.

Rumen and other strains of *Bacteroides melaninogenicus* (*Fusiformis nigrescens*) are fastidious anaerobes requiring a complex of growth factors. These organisms have an obligate requirement for protoheme (2, 7) and for peptides, and many strains also have a requirement for vitamin K (5-7). In investigating some aspects of the nutrition of this organism in relation to a study of the mode of action of vitamin K, we recently found that succinate exhibited growth-factor properties for this microorganism. Of special interest were the observations that succinate could partially replace the vitamin K requirement of the organism when grown in the presence of blood and that succinate could also replace the requirement for protoheme when grown in the presence of vitamin K. The growth-factor property of succinate was found to be highly specific in that related compounds were inactive, suggesting a special role for succinate in the metabolism of this organism. These studies of the growth factor role of succinate are the subject of this report.

MATERIALS AND METHODS

The organism was a vitamin K-requiring rumen strain of *B. melaninogenicus* used in previous studies on vitamin K metabolism (5-7). The basal medium consisted of: Trypticase (BBL), 3%; yeast extract (Difco), 0.3%; and NaCl, 0.5%; adjusted to *p*H 7.4. Tubed basal medium (10 ml) was either freshly autoclaved or deaerated by heating in boiling water for 15 min and then, for routine use, supplemented by addition of 0.05 ml of a 1/20 dilution of packed horse red blood cells lysed in distilled water and an aqueous emulsion of vitamin K_1 (Nutritional Biochemicals Corp., Cleveland, Ohio) to give a final concentration of 0.1 μg/ml; little or no growth occurred in the medium supplemented only with blood or only with vitamin K. In some experiments, hemin (General Biochemicals Corp., Chagrin Falls, Ohio; recrystallized; 10 μg/ml) was used in place of the laked blood.

Routine subcultures were made weekly with an inoculum of 0.05 ml of a 1/20 dilution of a 2-day culture per 10 ml of medium. Cultures that had been incubated in an anaerobic jar for 2 days at 37 C in an atmosphere of 95% H_2 and 5% CO_2 were used as inocula in some experiments; in others, inocula were bacteria grown in medium supplemented with either succinate plus blood, or succinate plus vitamin K.

Growth curves were obtained with a modified anaerobic jar containing an Erlenmeyer flask sealed into the glass wall of the jar with a side tube projecting to the exterior of the anaerobic jar. A rubber tube connected the side tube to a Klett tube. With this apparatus, periodic nephelometer readings can be taken without disturbing the E_h of the culture and with little effect on the temperature (8). The growth-rate constant (k_G) of the cultures was calculated assuming that the absorbancy is proportional to cell number.

Purified, crystalline succinate (disodium salt, hexahydrate; Fisher Chemical Co., Pittsburgh, Pa.) was made up as a 25% solution and sterilized by autoclaving. Solutions of other compounds were sterilized by autoclaving or by membrane filtration and were added to medium at a final concentration of 10^{-2} M.

In view of the partial replacement of vitamin K by succinate, sodium succinate in some experiments was extracted three times with ether to remove possible contamination.

Uptake of acetate was examined by the addition of 40 μCi of acetate-*l*-^{14}C (Schwarz BioResearch, Inc., Orangeburg, N.Y.; 57 mCi/mmole) to 200 ml of me-

dium supplemented with vitamin K and hemin with the use of the modified anaerobic jar (8). Samples of 5 ml were removed, the turbidity was measured, and 0.5 ml was added to 0.5 ml of cold 10% trichloroacetic acid. The precipitate was washed three times with cold 5% trichloroacetic acid on a membrane filter and counted in a dioxane-based scintillation fluid. The uptake of succinate and fumarate were examined in a similar fashion, by the addition of 20 μCi of succinate-*2,3*-^{14}C (New England Nuclear Corp., Boston, Mass.; 5.84 mCi/mmole) or 10 μCi of fumarate-*2,3*-^{14}C (International Chemical and Nuclear Corp., Irvine, Calif.; 2.3 mCi/mmole) to 200 ml of medium.

RESULTS

During studies on the uptake of various ^{14}C-labeled substrates by *B. melaninogenicus* grown in the presence of vitamin K and under conditions of vitamin K depletion, it was found that the addition of unlabeled succinate, in amounts designed to chase the label, stimulated the growth of both vitamin K-grown and depleted bacteria.

The growth rate of a culture supplemented in the routine way with vitamin K_1 and blood was compared with the growth rate of a similar culture to which 2.5 mg of succinate per ml had been added before inoculation (Fig. 1). The growth-rate constant of the former was found to be 0.39; with added succinate, the growth-rate constant was significantly increased to 0.53. Because of this effect of succinate on the fully (vitamin K plus blood) supplemented culture, the effect of this compound on the growth of vitamin K-depleted and heme-depleted cultures was individually investigated.

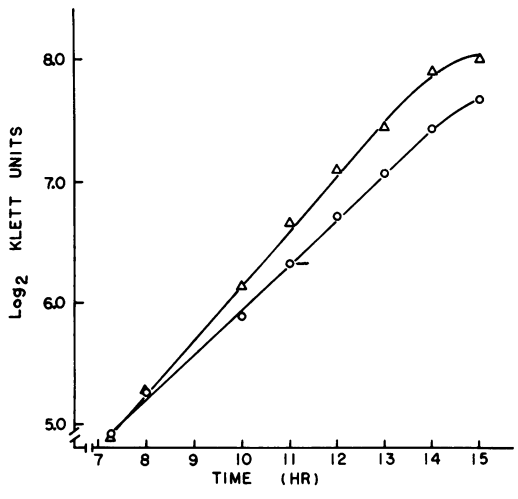

FIG. 1. *Growth curves of cultures of Bacteroides melaninogenicus supplemented with vitamin K and blood* (O) *and with vitamin K plus blood plus 2.5 mg of succinate per ml* (Δ). *After further incubation, both cultures reached the same final turbidity.*

Vitamin K-depleted cultures. Cell cultures without vitamin K but in the presence of blood and succinate grew without adaption at a slow rate (k_G = 0.11) to a limiting turbidity of 200 Klett units [0.75 mg (dry weight) of cells per ml] compared to routine cultures which reach a density of 300 to 320 units [1.1 mg (dry weight) of cells per ml]. Cultures inoculated into media containing blood but from which vitamin K had been omitted showed no growth. The succinate-supplemented vitamin K-depleted cells differ from deficient (D) cells in their ability to grow from small inocula in serial subculture in the absence of vitamin K. Succinate which had been ether extracted to remove possible contamination by traces of vitamin K was as effective as unextracted succinate. Cells in the basal medium supplemented with succinate and blood or succinate and heme in the absence of vitamin K grew in serial subculture. These cells, however, were still essentially vitamin K-requiring, since they responded with an increased rate of growth on subculture into fresh medium containing vitamin K. Moreover, the morphology of the cells on Gram staining resembles that of vitamin K-deficient cells (D cells) which become abnormally elongated during their growth with heme alone (6). Their lack of odor and of any mucoid nature is also in contrast to the characteristic pungent odor of cultures grown with vitamin K and heme.

The concentration of succinate required for maximum growth was determined in the presence of blood. Figure 2 shows that maximum growth was obtained at a level of 500 μg/ml and that of the final turbidity reached was significantly less than that obtained with cells grown with vitamin K.

Experiments were made to determine the specificity of succinate with respect to the replacement of vitamin K in the growth stimulation of *B. melaninogenicus*. Acetate, fumarate, oxaloacetate, malate, citrate, lactate, pyruvate, propionate, *n*-butyrate, α-ketoglutarate, δ-amino levulinate, methionine, aspartate, and threonine were added individually to the medium (10^{-2} M) in the presence of blood. None of these compounds was found to stimulate the growth of the microorganism.

Heme-depleted (vitamin K and succinate-supplemented) cultures. In view of the ability of succinate to replace vitamin K by allowing a limited amount of growth, its ability to replace heme—

which the bacterium likewise requires—was investigated. Cells inoculated into basal medium supplemented with vitamin K and 2.5 mg of succinate per ml grew rapidly (k_G = 0.35) after a short initial period of adaptation, reaching a final turbidity of approximately 300 Klett units [1.1 mg (dry weight) of cells per ml] which approaches that of a normal vitamin K and blood-supplemented control. These cells were able to be subcultured serially in the absence of heme, and, in contrast to the succinate and heme-supplemented (vitamin K depleted) cultures, the appearance of the microorganisms was similar to cells grown in the routine way in the presence of vitamin K and heme.

The growth response of a culture to increasing amounts (Fig. 2) shows that, in the presence of 0.1 μg of vitamin K per ml, a maximum response is obtained at 300 μg of succinate per ml, and cells grow to a greater degree per unit amount of succinate than do cells grown with heme in the absence of vitamin K. The specificity of succinate with respect to the replacement of heme as a growth factor was tested with the same series of compounds as above; none had the growth-promoting effect of succinate.

The growth-stimulating property of succinate for *B. melaninogenicus* under various cultural conditions shows that combinations of any two of the factors, vitamin K, heme, and succinate, are required for growth; growth does not occur in the presence of any one factor.

It is of interest that succinate was unable to replace the peptide requirements of *B. melaninogenicus*. Cells inoculated into a medium supplemented with vitamin K_1, blood, and succinate, in which the Trypticase was replaced by 3% Casamino Acids and 0.05% tryptophan, failed to grow.

The ability of *B. melaninogenicus* to grow in medium supplemented with succinate plus blood or, alternatively, with succinate plus vitamin K (but not with succinate alone), suggests that there are two pathways of succinate metabolism mediated by heme and vitamin K, respectively. In an attempt to differentiate these two pathways, cells were cultured in medium supplemented with (i) vitamin K plus blood, (ii) succinate plus blood, and (iii) succinate plus vitamin K. Potassium cyanide was added to these three cultures at levels of 10^{-2}, 10^{-3}, and 10^{-4} M. On examination at 2 days, cultures supplemented with blood plus succinate and vitamin K plus blood were found to be severely inhibited by 10^{-2} M cyanide, whereas the culture supplemented with vitamin K plus succinate grew at these concentrations of KCN. Cells grown in twice the normal level of blood were able to grow in its absence when subcultured once, owing to carry-over. Under these conditions, cells were sensitive to 10^{-2} and 10^{-3} M cyanide, thus demonstrating that cyanide inhibition affects intracellular heme. Preliminary spectroscopic analysis failed to detect cytochrome in those cells grown with vitamin K and succinate only.

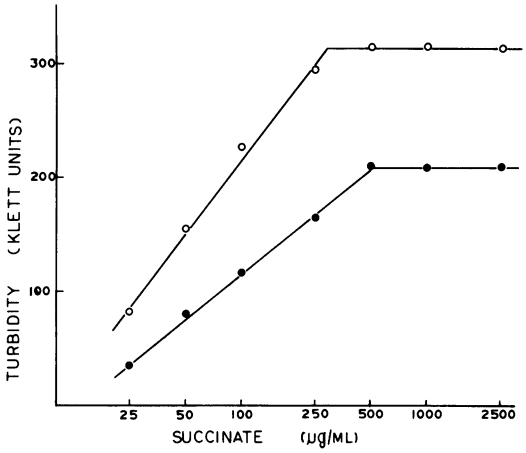

FIG. 2. *Growth response to increasing concentrations of sodium succinate in medium supplemented with vitamin K_1 at 0.1 μg/ml* (○) *and in medium supplemented with blood* (●). *Turbidities were read after 40 hr of incubation when maximum growth was reached.*

The inability to stimulate growth by compounds similar to succinate could be due to the impermeability of *B. melaninogenicus* to these compounds. To examine this possibility further, the uptake of succinate-*2,3*-^{14}C fumarate-*2,3*-^{14}C and acetate-*1*-^{14}C into trichloroacetic acid-precipitable material was examined (Fig. 3). Both succinate, which stimulates growth, and acetate, which is ineffective, were incorporated to a similar degree by *B. melaninogenicus*, whereas fumarate was not incorporated. Approximately 0.2% of cell carbon was derived from succinate in the vitamin K and succinate-supplemented cultures, and approximately 0.6% of cell carbon was derived from succinate in the cultures supplemented with succinate and blood.

DISCUSSION

This investigation has shown that succinate is a growth factor for *B. melaninogenicus*. This fact is based on three observations: succinate significantly increases the growth rate when added to a vitamin K plus blood-supplemented culture, replaces the heme requirement of the microorganisms when added to a vitamin K-supplemented culture, and partially replaces the vitamin requirement when added to a blood-supplemented

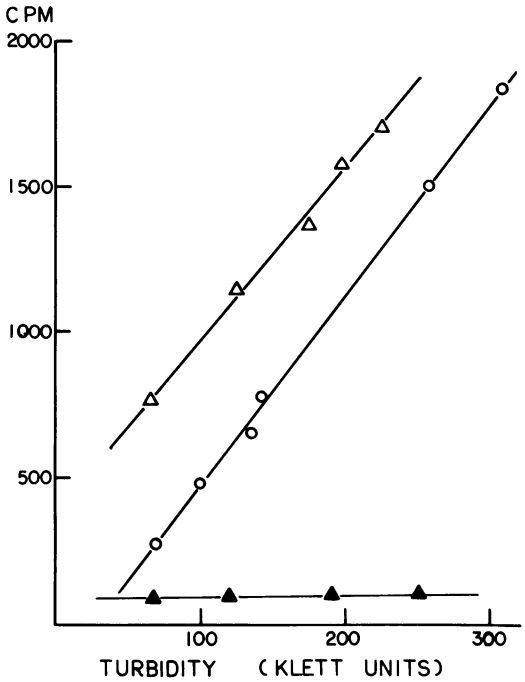

FIG. 3. *Incorporation of ^{14}C-acetate (Δ), succinate-2,3-^{14}C (O) and fumarate-2,3-^{14}C (▲) into trichloroacetic acid-precipitable material by Bacteroides melaninogenicus.*

culture. There appear to be two metabolic pathways through succinate in *B. melaninogenicus* mediated by vitamin K and heme.

The strain of *B. melaninogenicus* used in these experiments forms a cytochrome *b* (*unpublished data*) from the added heme. The inhibitory effect of cyanide for cells grown in the presence of heme but not for cells grown by replacing the heme with vitamin K and succinate may indicate that in the latter cytochrome is not produced. This fact is also indicated by the failure to detect cytochrome by spectroscopic methods.

Succinate is specific in its ability to stimulate the growth of *B. melaninogenicus*, whereas acetate, which is inactive, is taken up by the cells, indicating that permeability of the cell envelope in this case is not the factor responsible for the lack of growth stimulation. As will be shown in a later communication, both acetate and succinate are incorporated into the lipids and phospholipids of *B. melaninogenicus* (Lev and Milford, *unpublished data*). The requirement for succinate is in substrate amounts for maximum growth in contrast to the factors which it can replace (vitamin K and heme), which are required in amounts of 0.1 μg/ml and 6 μg/ml, respectively. Jones and Lascelles (3) have worked with a mutant of *Escherichia coli* which required 4-hydroxybenzoic acid for rapid growth. Succinate which could replace this compound under aerobic conditions was ineffective under anaerobic conditions of growth.

It has been known for some time that volatile fatty acids are growth factors for a number of rumen bacteria and other gram-negative anaerobes, and also that these are incorporated into lipids (1, 4, 10) and phospholipids of cells (G. H. Wegner and E. M. Foster, Bacteriol. Proc., p. 170, 1961). Although several short-chained volatile fatty acids have been described as growth factors, e.g., iso-valerate, a special role for succinate has not to our knowledge been shown previously.

The interest of the succinate-heme and vitamin K-succinate growth conditions lies in the potential usefulness of being able to study the heme-mediated and vitamin K-mediated metabolic pathways in this organism without cross interference, i.e., to study cells grown continuously deprived of either vitamin K or heme, for which there is normally an obligate requirement.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Science Foundation (GB-13590) and the Brown-Hazen Fund of The Research Corporation.

LITERATURE CITED

1. Allison, M. J., M. P. Bryant, I. Katz, and M. Keeney. 1963. Metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. II. Biosynthesis of higher branched-chain fatty acids and aldehydes. J. Bacteriol. **83**:1084-1093.
2. Gibbons, R. J., and J. B. MacDonald. 1960. Hemin and vitamin K compound as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. J. Bacteriol. **80**:164-170.
3. Jones R. G., and J. Lascelles. 1967. The relationship of 4-hydroxybenzoic acid to lysine and methionine formation in *Escherichia coli*. Biochem. J. **103**:709-713.
4. Kanegasaki, S., and H. Takahashi. 1968. Function of growth factors for rumen microorganisms. II. Metabolic fate of incorporated fatty acids in *Selenomonas ruminatium*. Biochim. Biophys. Acta **152**:40-49.
5. Lev, M. 1958. Apparent requirement for vitamin K of rumen strain of *Fusiformis nigrescens*. Nature (London) **87**:203.
6. Lev, M. 1959. The growth promoting activity of compounds of the vitamin K group and analogues for a rumen strain of *Fusiformis nigrescens*. J. Gen. Microbiol. **20**:697-703.
7. Lev, M. 1968. Vitamin K deficiency in *Fusiformis nigrescens*. I. Influence on whole cells and cell envelope characteristics. J. Bacteriol. **95**:2317-2324.
8. Lev, M., and A. F. Milford. 1971. Apparatus for metabolic studies with anaerobes. Appl. Microbiol. **21**:555-556.
9. Sawyer, G. J., J. B. MacDonald, and R. J. Gibbons. 1962. Biochemical characteristics of *Bacteroides melaninogenicus*. Arch. Oral Biol. **7**:685-691.
10. Wegner, G. H., and E. M. Foster. 1960. Fatty acid requirements of certain rumen bacteria. J. Dairy Sci. **43**: 566-568.