Sensitivity of a Bacteroides melaninogenicus Strain to Monosaccharides: Effect on Enzyme Induction

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The inhibition of growth in Bacteroides melaninogenicus by sugars is described. Monosaccharides such as D-glucose, D-galactose, D-mannose, and D-fructose are inhibitory at low concentrations, whereas the disaccharides sucrose and lactose are not inhibitory even at high concentrations. The major inhibitory effect of the sugar is found during the transition of lag to logarithmic growth phases. There was no primary effect of D-glucose on protein, ribonucleic acid, or deoxyribonucleic acid synthesis on cells in transition from lag to logarithmic growth. However, the addition of glucose or galactose completely abolished the induction of 3-ketodihydrosphingosine synthetase by vitamin K in vitamin K-depleted cells. Furthermore, in cells which were not vitamin K depleted, the level of this enzyme was drastically reduced by the addition of the sugar. Cyclic adenosine 5' monophosphate was unable to reverse the growth inhibition produced by glucose. In actively growing cultures, addition of sugar slows the growth rate. In these experiments the level of 3-ketodihydrosphingosine synthetase fell only after the cells had assumed the slower rate of growth. There were two indications that D-galactose was more inhibitory than D-glucose; in the presence of 0.1% D-galactose cells in lag phase did not show the increase in turbidity found in similar cells placed in medium with 0.1% D-glucose, and also D-galactose caused a greater decrease in the growth rate of actively growing cultures than was found with D-glucose. These studies suggest that the inhibitory effect of monosaccharides in lag → logarithmic growth transition can be ascribed to an effect on enzyme induction. On the other hand, the ability of many monosaccharides to inhibit growth, and the greater inhibitory property of D-galactose compared with D-glucose, suggests that other mechanisms may be operative as well.

Many sugars, especially monosaccharides, are utilized rapidly by a wide variety of bacteria as a source of carbon and energy. The inhibition of growth by low concentrations of sugars is therefore of interest, especially when this inhibition appears in wild-type microorganisms. In Salmonella typhi, the addition of rhamnose causes a partial inhibition of growth (15). This inhibition may be due to the accumulation of rhamnulose-1-phosphate (4). Other studies have shown that certain mutants of Escherichia coli lacking one enzyme of the Leloir pathway are inhibited by D-galactose (7, 18), and also that strains of M mutants of Salmonella are lysed by added D-galactose or form spheroplasts in hypertonic medium (5, 16). In these mutants D-galactose affects growing cells only and D-glucose reverses the inhibition. During studies on the uptake of D-[¹⁴C]glucose by Bacteroides melaninogenicus, it was found that a severe growth inhibition was produced by the addition of the sugar to the medium. The type of inhibition produced by this and other sugars differs in several respects from those previously described, and a study of this type of inhibition is presented.

MATERIALS AND METHODS

Bacterial strains. The strain of B. melaninogenicus was a vitamin K- and heme-requiring strain isolated from the bovine rumen and used previously in studies on vitamin K metabolism (8, 12). Succinate is a growth factor for this microorganism and can replace the heme requirement (9). Experiments were also performed with a mutant strain S which requires vitamin K but not heme. This mutant arose spontaneously in a culture of B. melaninogenicus which normally requires supplementation with both vitamin K and 2 × 10⁻⁴ M sodium succinate. Strain S was isolated from a control tube supplemented with vitamin K only.

Medium and growth conditions. The basal medium, consisting of 3.0% Trypticase (Baltimore Bio-
logical Laboratories, Inc., Baltimore, Md.), 0.3% yeast extract (Difco), and 0.5% NaCl was adjusted with NaOH to pH 7.4. After autoclaving, vitamin K₁
(Nutritional Biochemical Co., Cleveland, Ohio) was added to 0.1 μg/ml final concentration. In experiments with the parent strain of B. melaninogenicus, horse erythrocytes laked in distilled water were also added to a final concentration of 1:4,000. Cultures were incubated in anaerobic jars in an atmosphere of 95% H₂ and 5% CO₂ at 37 C.

Sugars of purest commercial grade were sterilized as 10% solutions in distilled water by autoclaving (121 C, 10 min) or by filtration through a membrane filter (Millipore Corp.). To assay a growth inhibitory property, various concentrations were added to 10 ml of basal medium (in duplicate) supplemented with vitamin K₁ and blood, or with vitamin K₁ only when strain S was used. Test media were seeded with 0.1 ml of a 1:10 dilution of a 2-day culture. After mixing, the cultures were incubated for 24 h, and turbidities were read on Kläi-Summerson colorimeter (red filter). A turbidity reading of 120 is equivalent to 0.4 g (dry weight) of cells per liter.

Metabolic studies were performed using a specially adapted anaerobic jar (10) which permits removal of sequential culture samples for turbidity and radioactivity determinations without disturbing the Eₐ of the medium. All inoculations and removal of samples were made via a syringe.

Protein synthesis was studied by the addition of 40 μCi of L-[¹⁴C]leucine per 200 ml of medium (New England Nuclear Corp., Boston, Mass.; 32.7 mCl/ mmol) to test and control cultures. Samples were removed, and 0.5 ml was immediately mixed with an equal volume of 10% trichloroacetic acid. The precipitate was collected on a membrane filter (Millipore Corp.), washed five times with cold 5% trichloroacetic acid, and counted in a dioxane-based scintillation fluid.

Ribonucleic acid and deoxyribonucleic acid synthesis was investigated in a similar manner by the addition of 100 μCi of [³H]uridine or [³H]thymidine (New England Nuclear Corp., Boston, Mass; 27.9 and 50.8 Ci/mmol, respectively) to sugar-treated and control cultures. The incorporation of acetate into lipids was determined by the addition of 40 μCi of L-[¹⁴C]acetate (Schwarz/Mann, Orangeburg, N.Y.; 51 mCi/mmol) to test and control cultures. Samples (15 ml) were removed, and the cells were sedimented and washed three times with phosphate buffer (0.05 M, pH 7.4). The cells were resuspended in buffer, and the lipids were extracted by the method of Bligh and Dyer (3). Radioactivity was determined on samples of the extracted lipid. To determine incorporation of glucose in various cell fractions, cultures were grown in medium containing D-[¹⁴C]glucose (25 μCi/200 ml) (International Chemical and Nuclear Corp., Irvine, Calif.; 4.08 mCi/mmol) or labeled glucose (100 μCi/200 ml) plus 0.1% unlabeled glucose and fractionated by the method of Roberts et al. (17). In other experiments cells were grown with the low level of [¹⁴C]glucose, and the nucleic acids were extracted and purified by the cetyltrimethylammonium bromide method (2).

The activity of 3-ketodihydrosphingosine synthetase and the induction of this enzyme by vitamin K₁ were determined as described previously (13). Cells were sedimented and washed three times with 0.05 M sodium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol and then suspended (1 g [wet weight] in 9 ml of buffer) and sonicated three times for 15 s with a 1-min interval between sonication. The sonicated cells were centrifuged at 27,000 × g for 15 min and the sediment was discarded. Enzyme activity was determined by measuring the incorporation of [¹⁴C]palmitoyl coenzyme A (New England Nuclear Corp., Boston, Mass.; 58.2 mCi/mM) into 3-ketodihydrosphingosine. The reaction mixtures (37 C, 20 min) contained L-serine (3 μmol), and [¹⁴C]palmitoyl coenzyme A (0.2 μmol, 0.1 μCi) and bacterial extract (0.5 ml) were vigorously stirred during incubation at 37 C. The reaction was terminated by the addition of 9.5 ml of chloroform-methanol (2:1 vol/vol). Suspended protein was removed by centrifugation, and unreacted acyl coenzyme A and other water-soluble components were removed by a modification of the partitioning procedure of Folch et al. (6). The lower phase was evaporated to dryness in a stream of nitrogen, dissolved in 0.2 ml of chloroform-methanol, and applied as a 2-cm band to a thin-layer plate.

Thin-layer chromatography was performed using a 0.5-mm layer of Silica Gel G (Merck & Co., Rahway, N.J.) activated at 110 C for 90 min prior to use. Plates were developed in paper-lined tanks using chloroform-methanol-water-ammonia (280:70:6:1, vol/vol/vol/vol) (12). Labeled compounds were detected by radioautoradiography using Kodak X-ray film. Radioactive bands corresponding to the standard were scraped off the plate and counted.

RESULTS

Inhibition of growth from the lag phase. The inhibition by D-glucose of the initiation of growth of B. melaninogenicus is illustrated in Fig. 1. A wide zone of inhibition was found at 2 days of incubation. The edge of the inhibitory zone was marked by a dense ring of growth. The growth response of the organism cultured in the presence of various concentrations of D-glucose is shown in Fig. 2. After 24 h of incubation, complete inhibition was found at a level of 0.01% (6 × 10⁻⁴ M), whereas a decrease in inhibition was found over the range 0.0025 to 0.0005% glucose.

A number of monosaccharides were inhibitory, with a potency approximately equal to that of glucose. These included D-fructose, D-galactose, L-arabinose, D-mannose, 2-deoxy D-glucose, and 2-deoxy D-galactose. D-Fucose, D-arabinose, D-mannitol, D-sorbitol, M-inositol, potassium gluconate, and substituted sugars such as glucosamine and methyl α- and β-glucoside were slightly inhibitory at a concentration of 0.1%. Disaccharides such as sucrose, lactose, and cellobiose were not inhibitory at a 1% level.
formed of D-glucose. Colony counts examined after 3
days of incubation were identical on both me-
dia, indicating that individual cells overcame
the inhibition of the sugar; the colonies on the
sugar-containing plates were considerably
smaller than those on the control agar.

No gross effects on the appearance of glucose-
inhibited cells was detected on phase-contrast
microscopy or staining. No evidence was ob-
tained for lysis or spheroplast formation by
inhibitory sugars. Attempts were made to re-
verse the inhibition due to glucose by the
supplementation of the medium with a number
of compounds such as dibutyryl cyclic-adeno-
sine 5'-monophosphate (5 x 10^{-3} M) acetate,
malate, or succinate at 10^{-1} M. Only succinate,
which is a growth factor for this microorganism
(9), partially reversed the effect of glucose
inhibition.

To examine the possibility that the sugars
inhibit by affecting uptake of vitamin K by the
cells, cultures were grown with a high level of
vitamin K (2.0 μg/ml); the cells were washed
twice with medium and plates were uniformly
seeded with these cells in medium unsupple-
mented with vitamin K. Sugar sensitivity was
determined by placing a disk containing D-
glucose on the plates. The result of this exper-
iment was identical to that shown in Fig. 1,
indicating that cells preloaded with vitamin K
are equally sensitive to sugars.

The distribution of glucose in inhibited and in
noninhibited cells was determined by growing
cultures with D-[14C]glucose at a non-inhibitory
level of 0.0005% and with glucose at an inhibi-
tory level of 0.1%. The majority of radioactivity
was found in the alcohol (18%) and in the hot
trichloroacetic acid-soluble fractions (65%). In
the scheme of Roberts et al. nucleic acids are
extracted by hot trichloroacetic acid. However,
after purification (2) nucleic acids from glucose-
labeled cells showed only traces of radioactivity.
Incorporation of glucose carbon into polysac-
charide could account for the high activity of
the hot trichloroacetic acid-soluble fraction.

Although only limited growth of the cells
occurred in the presence of 0.1% glucose (62
Klett units final turbidity), a considerable
amount of the sugar was incorporated—459
nmol/mg of cells (dry weight). Cells grown to an
approximately similar turbidity with the low
level of D-glucose incorporated 20.6 nmol/mg of
cells.

**Metabolic experiments.** Early experiments
had indicated that the major inhibitory effect of
the sugar was on the lag phase cells. The effect
of sugars on the metabolism of cells in transition
from lag to logarithmic phases of growth was

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**Fig. 1. Growth inhibition of B. melaninogenicus by D-glucose.** A sugar differentiation disk (Difco)
containing 20 mg of glucose was placed on the surface
of an agar plate containing basal medium supple-
mented with blood and 10 μg of vitamin K, per ml.
The plate was uniformly seeded with an overnight
culture of B. melaninogenicus. A wide zone of inhibi-
tion was observed after 2 days of incubation.

**Fig. 2. Growth response of B. melaninogenicus strain S to varying concentrations of D-glucose.** Dilu-
tions of D-glucose were added to duplicate tubes of
basal medium supplemented with vitamin K, (0.1
μg/ml) only. Turbidity determinations were per-
fomed after 24 h of incubation.

The growth inhibition by monosaccharides is
overcome on prolonged incubation for 2 to 3
days. To determine whether this was due to the
ability of individual cells to overcome the ef-
fects of the sugar or due to the selection of a few
resistant cells, decimal dilutions of a mid-
logarithmic phase culture of strain S were
plated by the method of Miles and Misra (14)
on agar containing no sugar and on agar plus
0.5% D-glucose. Colony counts examined after 3
days of incubation were identical on both me-
dia, indicating that individual cells overcame
the inhibition of the sugar; the colonies on the
sugar-containing plates were considerably
smaller than those on the control agar.
determined as follows. Cells grown to mid-logarithmic phase were centrifuged at 5°C, resuspended in fresh medium, and inoculated into 200 ml of prewarmed and prereduced medium supplemented with vitamin K₁, contained in the specially adapted jar (10), to give a final turbidity of 50 to 70 Klett units. The test culture received sugar added to a final concentration of 0.1%. Cultures were stirred during the experiment, and samples were removed at intervals for growth, uptake, and enzyme determinations.

In these experiments, the logarithmic phase of growth of the control cultures apparently commenced 3 to 4 h after the initiation of the experiment. Logarithmic growth was not attained in the glucose-treated cultures. The effect of 0.1% glucose on protein synthesis as determined by the incorporation of L-leucine into trichloroacetic acid-precipitable material is shown in Fig. 3. The incorporation of leucine by the sugar-treated culture paralleled that of the control culture until the logarithmic growth phase was reached. However, incorporation reflected the cell mass of the culture as indicated by turbidity. Thus, there was no primary effect of glucose on protein synthesis.

In a similar manner, ribonucleic acid and deoxyribonucleic acid synthesis determined by uridine or thymidine incorporation (Fig. 4 and 5) showed that there was no primary effect of glucose on the synthesis of these compounds, and likewise the incorporation of acetate into lipids followed the increase in cell mass of both control and glucose-treated cultures (Fig. 6).

We have shown previously that the addition of vitamin K₁ to cells of B. melaninogenicus which have been depleted of vitamin K induces an enzyme involved in sphingolipid synthesis, i.e., 3-ketodihydrosphingosine synthetase (13). This is the only inducible enzyme system known in B. melaninogenicus. The effect of the presence of 0.1% glucose on the induction of this enzyme by vitamin K₁ is shown in Fig. 7. The glucose-treated and control cultures show an approximate parallel increase in turbidity in the first 1 to 4 h after the initiation of the experiment. In the control culture, induction of the enzyme begins 2 h after growth in the presence of vitamin K₁ and reaches a maximum at 4 h. In the presence of 0.1% glucose, the ability of the cells to induce 3-ketodihydrdoshingosine synthetase is almost completely abolished. In other experiments we have shown that, when cells which are not vitamin K-depleted are inoculated into fresh medium, the level of this enzyme falls in the first 1 to 3 h.

![Fig. 3. Effect of 0.1% D-glucose on protein synthesis in cultures during transition from lag to early logarithmic growth. An overnight culture of strain S was centrifuged, resuspended in fresh medium, and injected into 200 ml of prewarmed, prereduced medium supplemented with vitamin K₁ and contained in a specially adapted anaerobic jar. At time 0 glucose + [¹⁴C]leucine were added to the test jar; the control received [¹⁴C]leucine only. Sequential samples were removed by syringe and processed as described in the text. Symbols: O, control culture; •, glucose-treated culture.](http://jb.asm.org/)

![Fig. 4. Effect of D-glucose on ribonucleic acid synthesis in cultures during transition from lag to early logarithmic growth. Conditions were similar to those of Fig. 3. Test and control cultures received [³H]uridine. Symbols: O, Control culture; •, glucose-treated culture.](http://jb.asm.org/)
FIG. 5. Effect of 0.1% D-glucose on deoxyribonucleic acid synthesis in cultures during transition from lag to early logarithmic growth. Conditions were similar to those of Fig. 3. Test and control cultures received [3H]thymidine. Symbols: O, Control culture; ●, glucose-treated culture.

FIG. 6. Effect of 0.1% D-glucose on lipid synthesis. Conditions were similar to those of Fig. 3. Test and control cultures received [14C]acetate. Samples were removed, and the cells were sedimented and washed with phosphate buffer (0.1 M, pH 7.4). Lipids were extracted and a sample was removed for radioactivity determination. Symbols: O, Control culture; ●, glucose-treated culture.

FIG. 7. Effect of 0.1% D-glucose on the induction by vitamin K of 3-ketodihydrosphingosine synthetase in B. melaninogenicus strain S. Cells were starved of vitamin K by overnight growth in medium supplemented with 2 × 10⁻³ M sodium succinate. They were sedimented, resuspended in fresh medium, and transferred to prerduced, prewarmed medium supplemented with 0.1 µg of vitamin K₁ per ml contained in the specially adapted anaerobic jar. Samples (15 ml) were removed, and the enzyme was assayed as described in the text. Symbols: O, Control culture; ●, glucose-treated culture.

(unpublished observation). In the experiment shown in Fig. 8, where the control culture was inoculated into medium supplemented with vitamin K at time zero, the level of the synthetase drops after the first hour and then rises quickly. In the presence of glucose, this subsequent rise in enzyme activity is completely abolished. D-Galactose had a similar effect on 3-ketodihydrosphingosine synthetase activity. Neither glucose nor galactose have any effect in vitro on the activity of the synthetase.

Effect of sugars on growing cultures. The addition of D-glucose or D-galactose to a growing culture of strain S resulted in a slowing of the growth rate apparent 1 h after the addition of sugar (Fig. 9A). Cultures to which D-galactose had been added showed a greater retardation of growth than did cultures treated with D-glucose. Enzyme levels showed no diminution 1 h after sugar addition, although later samples showed a marked reduction in enzyme levels in the sugar-treated cultures (Fig. 9B).

This marked reduction in enzyme activity indicates that the apparent inactivation of the enzyme could be due to the loss of the enzyme.
into the medium under the influence of the sugar. In an experiment similar to that shown in Fig. 9B, supernatants from cells treated for 2 h with 0.1% D-glucose or 0.1% D-galactose were concentrated and assayed for enzyme activity. No 3-ketodihydrosphingosine synthetase activity was detected in these supernatants.

Although the tube dilution assay showed that the sensitivities to glucose and galactose were similar, there are some indications that the inhibitory property of galactose is greater than that of glucose. For example, in the preceding experiments the turbidity of the culture increases in the presence of glucose (e.g., Fig. 4); in the presence of 0.1% glucose for 5 h the cultures showed an increase in turbidity of 43 Klett units. In similar experiments in the presence of 0.1% galactose, no increase in turbidity was found. Secondly, as noted above, in actively growing cultures galactose caused a slightly greater diminution in growth rate than did glucose (Fig. 9A).

**DISCUSSION**

The sugar inhibition described in this study differs from those described previously in several important respects: (i) the observed inhibitory effects are found in the wild-type organism; (ii) a number of monosaccharides rather than a specific sugar are inhibitory; and (iii) the major effect of the sugars is the inhibition of growth of cells in transition from lag to logarithmic growth. The sugars had less of an effect on...
actively growing cultures, resulting in a slower growth rate.

The fundamental question is why cells of *B. melaninogenicus* are sensitive to sugars. This sensitivity cannot be due to an influence of the sugar on uptake of vitamin K, since cells which are preloaded with vitamin K are as sensitive to inhibition as are cells which require vitamin K for growth. Using cells in transition from lag to logarithmic growth, it appears from our results that the sugar has no primary effect on macromolecular synthesis or the incorporation of acetate into lipids. However, the induction by vitamin K of 3-ketodihydrosphingosine synthetase is inhibited; the effect of sugar is greater than that found with puromycin or rifampin (13). Even in cells not depleted of vitamin K, glucose or galactose abolishes synthetase activity.

Thus, the growth inhibition of sugars in cells in transition from lag to logarithmic growth can be ascribed to an effect on enzyme induction of which the inhibition of 3-ketodihydrosphingosine synthetase activity is one example. In actively growing cultures, the retardation of growth rate would not appear to be due to the activity of this enzyme, since levels of the synthetase in sugar-treated cultures correspond to that of the control culture 1 h after addition of the sugar. At this time the growth rate of the sugar-treated cultures is retarded. It is also possible that other enzymes are affected by the inhibitory monosaccharides, contributing to a retardation of the growth rate. The dramatic decrease in 3-ketodihydrosphingosine synthetase activity found in growing cultures treated with glucose and galactose could suggest that the enzyme was released into the medium by the effect of the sugar. However, no enzyme activity could be found in concentrates of supernatants of sugar-treated cultures. The mechanism of the inactivation of 3-ketodihydrosphingosine synthetase by sugar is unknown. It is of interest to note that sugars do not inhibit enzyme activity in vitro.

With respect to this inhibition of growth by sugars, it should be noted that *B. melaninogenicus* is an obligate anaerobe. The strains used in this study do not require carbohydrate as an energy or carbon source and are regarded as asaccharolytic (1). However, glucose is actively metabolized; in the limited growth occurring in the presence of 0.1% D-glucose, a large amount of the sugar is incorporated by the cells. The recovery of most of the 14C derived from glucose in the alcohol and hot trichloroacetic acid-soluble fractions of cells is consistent with lipid and polysaccharide as the major end products of glucose metabolism in *B. melaninogenicus*.

Thus, the active metabolism of glucose and the effect of glucose and galactose on enzyme induction would suggest a particularly severe form of enzyme repression, resulting in the observed growth inhibition. A unique aspect of this repression is the relatively large number of monosaccharides which are effective.

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**LITERATURE CITED**