Growth of Mixed Cultures on Mixed Substrates

I. Continuous Culture

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Received for publication 7 June 1968

Continuous culture on mixed glucose-lactose or glucose-butyrate media inoculated with river water led to a population composed of a pseudomonad and a coliform. The glucose was used preferentially to the other carbon source, and the utilization of the secondary carbon source was greatly reduced at high growth rates. Significant amounts of acetate were excreted even though the cultures were limited by the carbon source, rather than by oxygen or other nutrients. At high growth rates, the pseudomonad dominated the population, whereas at low and moderate growth rates the coliform was dominant. A syntrophic relationship was shown by the fact that the pseudomonad could not grow alone on the glucose-butyrate medium.

When a microorganism is presented with several carbon sources simultaneously, growth will take place first on the best carbon source, then on the second best, and so on. This growth pattern was termed diauxie by Monod (12, 13). One mechanism responsible for this phenomenon has been shown to be the prevention of the synthesis of inducible enzymes required for the catabolism of the less-favored carbon sources. This effect, known originally as the glucose effect, has been demonstrated to be caused not by glucose itself but by metabolites arising from the catabolism of readily available carbon sources, and has been called more appropriately catabolite repression (10).

Another possible mechanism responsible for diauxic growth is catabolite inhibition (2-4, 8, 16), in which the function, rather than the synthesis of catabolic enzymes, is inhibited by either carbon sources or their intermediary catabolites.

The pattern of diauxic growth in batch culture represents a sequence in which growth takes place successively on one or more substrates, all of which are present at the beginning of the growth process but are consumed successively during the growth period. On the other hand, if two or more substrates are contained in the medium feed in continuous culture, the situation differs from the batch case in that the microorganisms are always exposed to all substrates and might metabolize all of them simultaneously or might attack only one or another, depending on the growth or dilution rates.

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It has been shown (11) that, when fructose and glucose were the growth-limiting nutrient, pure cultures of either Escherichia coli or Pseudomonas fluorescens consumed both sugars at low dilution rates, but the less-preferred carbon source (fructose) was attacked slightly or not at all at high dilution rates. Preliminary results were also obtained showing that a similar pattern was obtained for a natural microbial flora grown continuously with a mixed glucose-lactose feed.

This paper reports an extension of the investigation on the metabolism of other carbon sources by natural mixed cultures, and also considers changes that occur in the population after prolonged continuous culture.

Materials and Methods

Microorganisms. Mixed cultures were obtained by inoculating 500 ml of the medium to be used in continuous culture with water from the Charles River, a local polluted stream. Incubation was at room temperature (21 to 25°C) and the culture was aerated by means of a sparger. Subcultures were made at intervals of several days with large inocula.

Media. When sugars were used as carbon sources, the medium contained (g/liter of tap water): glucose and/or lactose, 1.0; KH2PO4, 3.5; Na2HPO4, 5.7; (NH4)2SO4, 1.0; MgSO4·7H2O, 0.5; Difco yeast extract, 0.02; and Dow P-2000 antifoam, 0.05. When butyric acid was included, the medium was modified to contain (g/liter of tap water): butyric acid, 1.0; KH2PO4, 1.8; and Na2HPO4, 6.7, with the other components remaining the same. The pH of the medium after sterilization was 6.8 to 7.0. The carbon sources were sterilized separately and added to the sterilized
basal medium in the reservoir. In all experiments the carbon source(s) were the growth-limiting nutrient.

Growth. All continuous culture experiments were carried out in baffled fermentors holding 310 ml of medium. The temperature was maintained at 30 ± 0.5 °C by a water bath. The medium was agitated by an external stirrer, and humidified air was introduced into the bottom of the fermentor at the rate of 500 ml/min. No attempt was made to ensure pure culture conditions, and the fermentor was not sterilized before use.

Dissolved oxygen partial pressure was measured with a dissolved oxygen probe (Lee Scientific Corp., Cambridge, Mass.) and never fell below 0.08 atmospheres. A constant feed of medium was obtained by using a Sigmamotor peristaltic pump and by minimizing changes in the hydraulic head between the reservoir and the pump. Broth was removed from the fermentor by overflow, and measurement showed that the overflow had the same cell and substrate composition as the bulk fermentor broth. The cell suspension was homogeneous and no clumping was observed.

Analyses. At least five residence times were allowed at any dilution rate before samples were taken. Samples were withdrawn from the overflow into a test tube immersed in ice and were centrifuged immediately in a refrigerated centrifuge to remove cells.

Glucose was measured by the Glucostat micro-method (Worthington Biochemicals Corp., Freehold, N.J.). Determinations for chemical oxygen demand (COD) were run according to Standard Methods for the Examination of Water and Wastewater (American Public Health Assn., New York) except that silver sulfate catalyst was not used. Lactose was determined by subtracting the glucose concentration expressed as COD from the total COD value. Volatile fatty acids in the medium were determined by gas chromatography by direct injection of broth onto a 6 ft × 1/8 inch (1.83 m × 0.32 cm) column of 20% n-pentyl-glycol succinate and 2% H3PO4 on 60- to 80-mesh firebrick; acetate was identified by comparison to a known solution. An F & M model 1609 gas chromatograph with hydrogen flame detector was used. Quantitation was by incorporation of a known concentration of m-cresol into the broth that was being analyzed. Ghosting was prevented by use, in the injection system, of a stainless-steel sponge treated with 15% H3PO4.

Observations to identify the dominant organisms were made according to the Manual of Microbiological Methods (McGraw-Hill Book Co., Inc., New York, 1957) or the Difco Manual (Difco Laboratories, Detroit, 1953). Colony appearance, the gram reaction, oxidase reaction (9), and cell motility and morphology were determined on nutrient broth or agar. Pigment formation was determined on fluorescein and pyocyanin agars (7). Sugar dissimilation and hydrogen sulfide production were determined on triple sugar-iron broth, indole formation on 1% tryptone broth, methyl red formation and the Voges-Proskauer test on methyl red-Voges-Proskauer medium, citrate utilization on Simmons citrate agar, 12-hr urease production on urea broth, and the coliform differential test was performed on Levine EMB agar and in Durham tubes with Brilliant Green bile broth. Carbohydrate dissimilation was also determined by the method of Hugh and Leifson (6). Flagella were observed in electron micrographs of cultures grown on tryptcase soy + 0.5% yeast extract agar.

Plate counts to determine the percentage of each type of colony were carried out by dilution of samples to a concentration of 50 to 350 cells per 0.1 ml. An 0.1-ml sample was spread on nutrient agar plates in triplicate, incubated at 30 C for 24 hr, and counted. The numbers of translucent and opaque colonies were determined and the ratio of each to the total was calculated.

RESULTS

A series of continuous cultures was made with a mixed feed of glucose and butyric acid (Fig. 1). The culture was inoculated with a mixed batch culture and allowed to grow; continuous feed was started. Beginning at a low dilution rate, at least five mean residence times were permitted to elapse before the dilution rate was increased. Thus, the points representing low dilution rates were obtained early in the run, whereas those representing...

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FIG. 1. Substrate utilization curves for a heterogeneous population with a mixed feed of glucose and butyrate. Acetate production is shown.
high dilution rates were obtained after the culture had been run for several days.

Figure 1 shows results of continuous cultures made on glucose and butyrate which are typical in several respects. (i) The glucose is the preferred substrate and butyrate utilization is greatly reduced at high dilution rates. (ii) Substantial excretion of acetate was observed at moderate and high dilution rates. (iii) A discontinuity in butyrate utilization was observed. As the dilution rate was raised from 0.2 hr⁻¹ to 0.4 hr⁻¹, the butyrate utilization increased, thus resulting in a new curve more or less parallel to the curve obtained at lower dilution rates. Figure 2 presents data on butyrate concentration from another such run, showing a similar discontinuity.

When samples from the continuous culture were plated on nutrient agar, the colonies appeared to be of two types, translucent and opaque. Several colonies of each type were picked and purified, and the characteristics were determined (Table 1). Isolate A was identified as a pseudomonad; isolate B was found to be a coliform.

Further taxonomic study was not undertaken. Another run, with glucose-butyrate mixed feed (Fig. 3) showed that the coliforms were the predominant organisms at dilution rates below 0.8 hr⁻¹; above this dilution rate, the pseudomonads became increasingly dominant.

In an attempt to understand the reason for the discontinuity, which suggests the development of a culture better able to utilize butyrate in the presence of glucose, extended runs were carried out with a mixed glucose-butyrate feed at constant dilution rates of 0.3 hr⁻¹ and 0.4 hr⁻¹. These results are presented in Fig. 4 and 5, and indicate that, even after 10 to 15 residence times (residence time equals the reciprocal of the dilution rate), the system is not in a stable steady state. This compares to the usual experience with pure cultures on single substrates in which steady states are readily achieved in three to six residence times. Although the steady states obtained in the experiments reported herein were not as stable as those from pure cultures, it was still possible to obtain useful data, although the variations with time were fairly large (±10 to 20%). The data in Fig. 4 and 5 do not indicate any large changes in population. For both dilution rates, the percentage of coliforms increased from about 20% at the time of inoculation to about 90% at the time a steady state was reached. Figure 4 does not show any large changes in butyrate utilization once the steady state was reached, but Fig. 5, with a dilution rate of 0.4 hr⁻¹, does show a large increase in butyrate utilization with a parallel increase in culture density at a time when the organism population showed no further changes. This suggests that the improvement in utilization shown in Fig. 1-3 is caused by changes within the coliform population rather than by a change in the ratio of coliforms to pseudomonads. Such changes, leading to the selection of mutants with an enhanced ability to metabolize a secondary carbon source in the presence of glucose, have been observed in pure cultures of E. coli growing on mixed substrates (Silver and Mateles, in preparation).

When attempts were made to grow the purified pseudomonad or coliform alone in batch culture in glucose-butyrate medium, the pseudomonad did not grow. However, when a mixed inoculum was used, both organisms grew, with the pseudomonad accounting for 70 to 90% of the total population. Thus, in batch culture with high levels of glucose corresponding to the higher levels obtained in continuous culture at high dilution rates, the pseudomonad dominated the population as it did in continuous culture at high dilution rates. The behavior of the mixed culture under batch growth conditions on mixed substrates is
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Isolate A</th>
<th>Isolate B</th>
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<tbody>
<tr>
<td>Colony appearance</td>
<td>Translucent</td>
<td>Opaque</td>
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<tr>
<td>Colony diameter</td>
<td>1-2 mm</td>
<td>2-3 mm</td>
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<tr>
<td>Cell morphology</td>
<td>Short rod</td>
<td>Short rod</td>
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<tr>
<td>Gram stain</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>Kovacs oxidase (192)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Hugh and Leifson (6)</td>
<td>Oxidative, acid</td>
<td>Fermentative gas, acid</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
<td>Motile</td>
</tr>
<tr>
<td>Electron microscope</td>
<td>Polar flagella</td>
<td>No flagella (fimbriae)</td>
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<tr>
<td>Fluorescein agar</td>
<td>Diffusible yellow-green pigment</td>
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<tr>
<td>Pyocyanin agar</td>
<td>Diffusible yellow-light brown pigment</td>
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<td>Triple sugar iron agar</td>
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<td>Methyl red</td>
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<td>Voges-Proskauer</td>
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<td>Indole formation</td>
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<td>Citrate utilization</td>
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<td>Urease formation</td>
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<td>EMB agar</td>
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Acid, gas, H₂S
(+)
(-)
(+)

± Sparse growth
(−)
Brown-centered colonies, sometimes metallic sheen
(−)

The pattern of preferential use of glucose in the presence of a secondary carbon source, which was studied in pure cultures of *E. coli* and *P. fluorescens* (11), has been shown to occur also in natural mixed populations growing on mixed substrates in continuous culture. This appears to be a general
phenomenon, not limited to a particular type of bacterial organism.

Although excretion of acetate has been reported in continuous cultures of *Neisseria gonorrhoeae* limited by nitrogen or nutrients other than the energy source (1) or in cultures of *Aerobacter aerogenes* limited by oxygen (14), it was surprising to find amounts as large as 10 to 20% of the carbon source consumed being excreted under conditions of carbon source limitation. This phenomenon, which is now being explored further under carefully defined conditions in pure culture, may account, at least in part, for the drop in yield reported at dilution rates approaching the maximal growth rate (5). Its discovery in this particular instance occurred only because gas chromatography was being used to assay butyrate, and an extra unknown peak appeared.

The relatively unstable steady state which was attained much more slowly than in continuous pure cultures on single carbon sources has also been observed in continuous culture studies with mixed known cultures (15; H. R. Bungay, Abstr. Am. Chem. Soc. Meeting, 152nd, New York, 1966; D. E. Contois and L. D. Yango, Abstr. Am. Chem. Soc. Meeting, 148th, Chicago, 1964). Population changes, whether of one species replacing another or selection of mutants within a species, are not likely to be obvious when conventional counting and identification techniques are used, but must be assumed to be taking place unless careful study excludes such a possibility.

**ACKNOWLEDGMENTS**

This investigation was supported in part by grant NsG-496 from the National Aeronautics and Space Administration to the Massachusetts Institute of Technology. S. K. Chian was a predoctoral trainee under training grant ES 00063 from the Public Health Service.

**LITERATURE CITED**

6. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative