Multicomponent Substrate Utilization by Natural Populations and a Pure Culture of *Escherichia coli*

A. F. Gaudy, Jr., Elizabeth T. Gaudy, and K. Komolrit

Division of Bio-engineering, School of Civil Engineering, and Microbiology Department, Oklahoma State University, Stillwater, Oklahoma

Received for publication 2 November 1962

Abstract

Gaudy, A. F., Jr. (Oklahoma State University, Stillwater), Elizabeth T. Gaudy, and K. Komolrit. Multicomponent substrate utilization by natural populations and a pure culture of *Escherichia coli*. Appl. Microbiol. **11**:157–162. 1963.—A heterogeneous population, typical of activated sludge, and a prototrophic strain of *Escherichia coli* were used to test for sequential substrate removal in a glucose-sorbitol medium. Each culture was preacclimated to sorbitol and was studied in the two-component medium under growing and nonproliferating conditions. In all four systems, glucose blocked sorbitol removal. Since large initial inocula were used, the suppression of sorbitol metabolism could not be totally due to repression of enzyme synthesis. The results indicate that glucose may affect the functioning of an existing enzyme system in addition to its established effect on enzyme synthesis. From an applied standpoint, the results indicate that an activated sludge may be completely and immediately prevented from eliminating a waste constituent to which it is acclimated.

In a previous study (Gaudy, 1962*b*), the importance of the diauxic phenomenon in the operation of biological waste water treatment processes was delineated and its occurrence was demonstrated for a glucose-sorbitol synthetic waste using heterogeneous populations typical of those found in activated sludge. Since sequential substrate removal was shown for a heterogeneous population, the generality of the phenomenon was demonstrated. Its occurrence, then, appeared to be controlled more by substrate than by the particular bacterial species present.

Subsequently, extensive work was conducted using the glucose-sorbitol medium to determine the effect of operational variables such as cell age on the occurrence of sequential substrate removal in heterogeneous populations (Gaudy, Komolrit, and Bhatla, 1962). It was found that with older sludges substrates were concurrently removed. Results with populations of intermediate age indicated that the system behavior was intermediate between the removal patterns found for young and old populations. Also, the results previously presented (Gaudy, 1962*b*) for young cells acclimated to sorbitol, in which glucose completely suppressed sorbitol removal, were repeatedly reproduced using a heterogeneous population developed from sewage seed taken from a treatment plant at an entirely different geographical location, further attesting to the generality, or substrate dependence, of diauxic phenomena. These studies will be reported in detail elsewhere.

Diauxic growth of a sorbitol-acclimated pure culture in a sorbitol-glucose medium was observed by Monod (1947). In his experiments, small initial inocula were used. The phenomenon of diauxic growth has been explained on the basis of repression of enzyme synthesis; i.e., various carbohydrates, including glucose, have been shown to repress the formation of enzyme(s) required for the metabolism of other carbon sources, among them sorbitol. Theories for the mechanism of the repression have recently been reviewed by Magasanik (1961).

In the studies of heterogeneous populations cited above (Gaudy et al., 1962), fairly heavy cell suspensions were employed (150 to 300 mg dry weight per liter). This experimental design was used to test for sequential substrate removal under conditions akin to those employed in the activated sludge process. Under the conditions employed, substrate removal could not have been totally dependent upon the synthesis of new enzyme; therefore, suppression of sorbitol metabolism could not be fully attributed to repression of enzyme synthesis. Since the initial cell suspension had been grown through at least three serial transfers from small inocula in sorbitol medium and all the cells therefore possessed a functioning enzyme system for sorbitol metabolism, the results suggested that the presence of glucose prevents not only the formation but also the function of the sorbitol-metabolizing system.

Most studies of the interaction of substrates in multicomponent media have been concerned with repression of enzyme synthesis in growing cultures, and only a few reports of inhibition of individual reactions in cell suspensions have appeared. Stephenson and Gale (1937) reported partial inhibition by glucose of several amino acid deaminases in resting-cell suspensions but concluded that the principal effect of glucose on deaminases was the inhibition of their formation in growing systems. Raynaud and Macheboeuf (1946) reported inhibition by glucose of the reduction of hydroxyproline in cell suspensions but

found no effect on alanine oxidation. Inhibition by glucose of indole production from tryptophan has been reported in growing cultures by Mastafa (1937) and in resting cells by Silberstein (1941).

In view of the lack of definitive information concerning substrate interactions in systems where substrate removal is not totally dependent upon enzyme synthesis and the potential importance of such interactions in biological treatment, further studies were undertaken. Both a heterogeneous population and a pure culture, *Escherichia coli*, were studied in glucose-sorbitol media with and without exogenous nitrogen.

MATERIALS AND METHODS

Methods of analysis. Optical density was determined at 540 mμ using a Coleman (model D-6) colorimeter (Coleman Instruments, Inc., Maywood, Ill.). Biological solids were measured by the membrane filter technique (Millipore Filter Corp., Bedford, Mass.; HA 0.45 μ). The membrane filtrate was used for determination of total organic material remaining in the medium, by the chemical oxygen demand (COD) test (*Standard Methods for the Examination of Water, Sewage, and Industrial Wastes*, American Public Health Association, 1960). The filtrate was also used for measurement of glucose remaining in the medium, using the anthrone method (Morris, 1948). The protein and carbohydrate contents of the cells were determined as previously reported (Gaudy, 1962*a*). In all anthrone determinations, the volume of anthrone reagent was increased to 9 ml. This modification has no effect on the sensitivity of the method and has been found to prevent clouding of samples which sometimes occurs with smaller volumes of the reagent.

Glucose concentrations were converted to COD values by multiplying by the ratio of concentrations obtained by the two methods (COD and anthrone) for a standard glucose solution (1.0 mg/liter of glucose = 1.03 mg/liter COD).

Heterogeneous population studies. Primary clarifier effluent from the municipal sewage treatment plant at Stillwater, Okla., was used as seed for development of a sorbitol-acclimated activated sludge. A synthetic waste of the following composition was used (concentrations in mg/liter): sorbitol, 1,000; $(NH_4)_2SO_4$, 500; $MgSO_4 \cdot 7H_2O$, 100; $FeCl_3 \cdot 6H_2O$, 0.5; $MnSO_4 \cdot 1H_2O$, 10; $CaCl_2$, 7.5. These components were dissolved in 0.01 M potassium phosphate buffer (pH 7.0) containing 100 ml of tap water per liter.

A batch system was employed, using a total volume of 1.5 liters in an aeration tube 20 in. high and 4 in. in diameter. This was immersed to a depth of 10 in. in a water bath maintained at 25 C. Air was supplied through a porous carborundum diffuser at a rate of 4,000 ml/min.

At 24-hr intervals, 500 ml of the cell suspension were removed and discarded. The remaining suspension was allowed to settle for 1 hr and 500 ml of the supernatant were removed. The synthetic waste was then added to the 1.5-liter mark and aeration was resumed. The heterogeneous population developed and maintained by this procedure was used as inoculum for substrate removal studies which were carried out as follows. A sorbitol medium containing the same constituents as the synthetic waste used in the batch activated sludge system, but with double the concentration of each salt and a triple concentration of buffer, was placed in Erlenmeyer flasks and inoculated with a small volume of cell suspension from the batch system. These flasks were incubated in a water bath shaker at 25 C for 24 hr. A small inoculum was then transferred to a flask containing fresh medium. After three such serial transfers, a large number of identical flasks were inoculated from the third flask culture and were incubated on the shaker for 18 hr. This procedure was employed to obtain a young culture completely acclimated to sorbitol.

The cells were harvested by centrifugation, washed twice with 0.05 M potassium phosphate buffer (pH 7.0), and resuspended in 2 liters of buffer-salts solution. This solution was of the same composition as the growth medium used for flask cultures except that it contained no sorbitol. For the experiment using nonproliferating cells, the $(NH_4)_2SO_4$ was also omitted. A sample was removed for determination of initial biological solids. Glucose and sorbitol were then added (8 ml of a 10% solution of each) to an initial concentration of approximately 400 mg/liter of each carbon source. A sample was removed immediately for determination of initial COD and glucose concentrations. The cell suspension was then placed in an aeration tube identical to that described above and aerated at a rate of 4,000 ml/min. At intervals, samples were withdrawn for measurement of the COD and glucose remaining in the medium.

Pure-culture studies. A prototrophic strain of *Escherichia coli* (supplied by the Department of Microbiology, Oklahoma State University) was grown on the shaker in sorbitol medium as described above for the heterogeneous culture. Cells were harvested near the end of the log growth phase (14 hr after inoculation), washed, and resuspended in 2 liters of buffer-salts solution without $(NH_4)_2SO_4$. A sample was removed for determination of biological solids. A 40-ml sample was also removed and placed in a Warburg flask for measurement of endogenous respiration. The suspension was then divided into two parts and $(NH_4)_2SO_4$ added to one portion. Glucose and sorbitol were added to both portions to give an initial concentration of approximately 400 mg/liter of each substrate, and samples were removed for determination of initial concentrations of COD and glucose. The cell suspensions were then placed in 140-ml Warburg flasks (40 ml of suspension per flask) and placed on the Warburg respirometer. The center well of each flask contained 1.5 ml of 20% KOH. Flasks were shaken at 105 oscillations/min and the temperature was maintained at 25 C. Manometer readings were made at 15-min intervals. Flasks

were removed periodically; the contents were withdrawn by pipette and used for measurement of optical density, cell protein and carbohydrate content, and COD and glucose remaining in the medium.

RESULTS

Heterogeneous population (growth system). The course of substrate removal using a heterogeneous population is shown in Fig. 1. The dotted-line curve labeled "sorbitol COD" was obtained by subtracting the glucose concentration, as measured by the anthrone test and calculated to a COD value, from the total COD removal curve. It is clearly seen that glucose was removed first even though the biological mass was acclimated to sorbitol. This experiment substantiates results on similar systems previously cited. It is emphasized that the sorbitol COD curve must be considered as an apparent sorbitol concentration since it was not obtained by direct analysis. The possible significance of the rise in the "sorbitol COD" curve is discussed below.

Heterogeneous population (nonproliferating cell system). The course of substrate removal for a sorbitol-acclimated heterogeneous population in a glucose-sorbitol medium devoid of an exogenous source of nitrogen is shown in Fig. 2. It is seen that, under nonproliferating conditions, glucose retained its ability to suppress sorbitol utilization. In this case, as with the growth system herein reported in Fig. 1 and all other studies using young cell systems, the results can best be described as an *en masse* response of all the cells present. The major differences in response between the systems with and without nitrogen were the slower substrate elimination rate and the absence of a pronounced rise in the apparent sorbitol COD curve in the nonproliferating system. The major similarity is the ability of glucose to block sorbitol removal in both systems. This sequential removal in a nonproliferating system is indicative of interference with function of the sorbitol-metabolizing system rather than with synthesis of enzymes

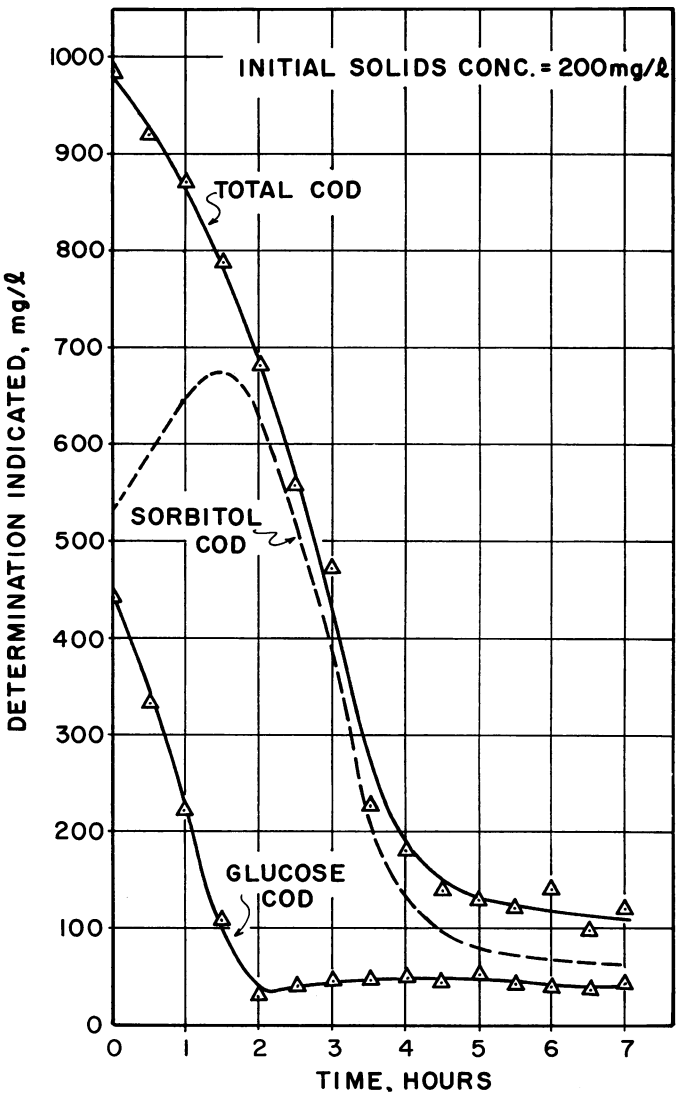

FIG. 1. *System response—heterogeneous population, under growing conditions, two-component carbon source, glucose (400 mg/liter) plus sorbitol (400 mg/liter). Sludge acclimated to sorbitol.*

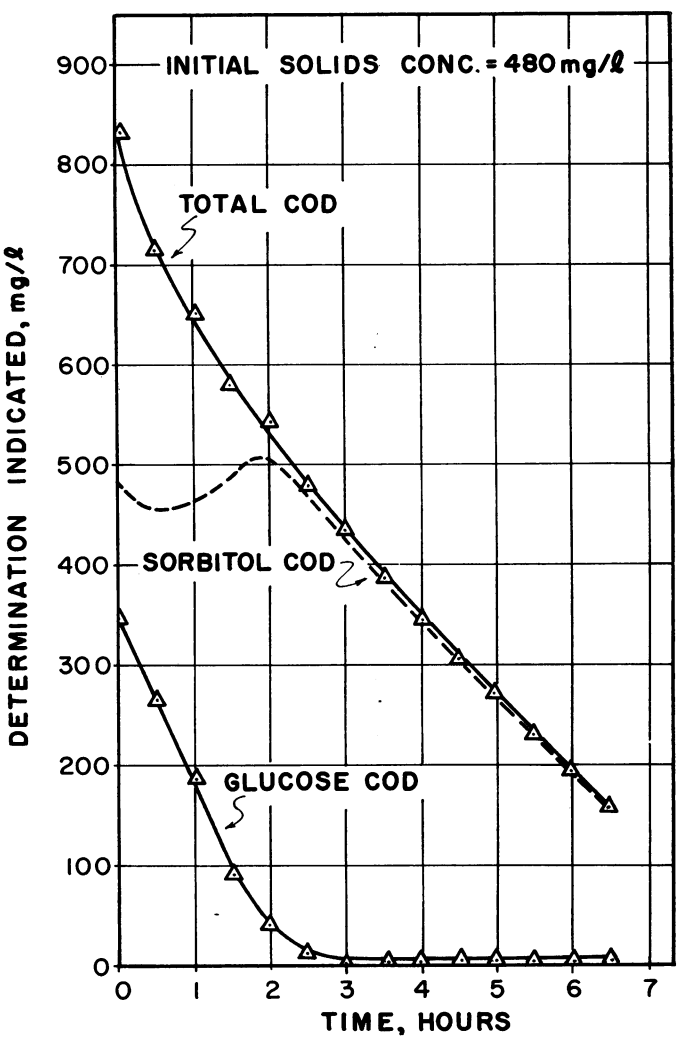

FIG. 2. *System response—heterogeneous population under nonproliferating conditions, two-component carbon source, glucose (400 mg/liter) plus sorbitol (400 mg/liter). Sludge acclimated to sorbitol.*

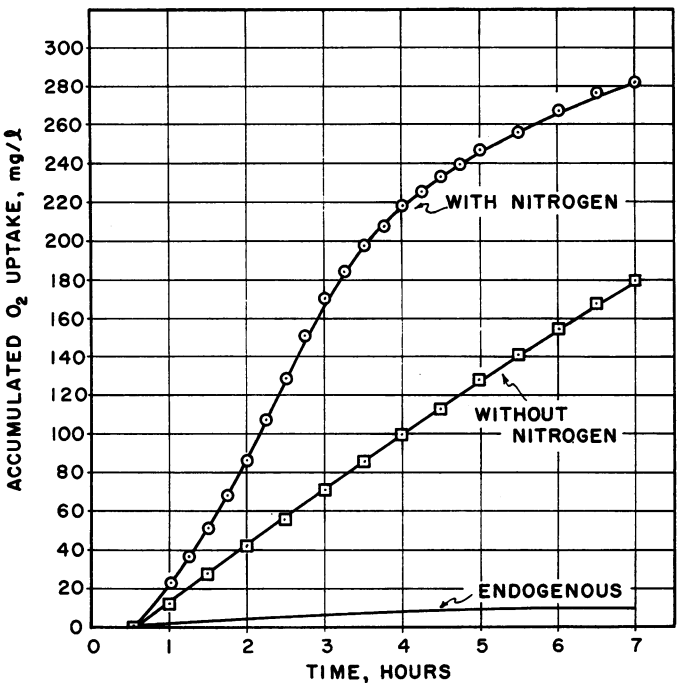

FIG. 3. *Accumulated oxygen uptake, E. coli, two-component carbon source, glucose (400 mg/liter) plus sorbitol (400 mg/liter). Cells acclimated to sorbitol.*

required to metabolize sorbitol. A large cell concentration (approximately 500 mg/liter) was used to insure that the system initially possessed a high degree of sorbitol-metabolizing ability; hence, sorbitol metabolism did not depend on the synthesis of new enzyme protein. Withholding the exogenous nitrogen source prevented synthesis of new enzyme (except that which could arise due to turnover of endogenous protein).

Pure-culture studies. Although the use of heterogeneous (or natural) populations is highly desirable from an applied standpoint as related to bioengineering aspects of waste-water purification, the demonstration of the glucose effect in a nitrogen-starved system and its consequent suggestion of interference with enzyme function in addition to the well-established effect on enzyme synthesis is of significance from the basic standpoint as well. Because of the possibility of introduction of unknown variables by the use of heterogeneous populations, it was felt that similar studies should be undertaken using a pure culture. Accordingly, a prototrophic strain of *E. coli* was grown on sorbitol. Equal portions of cells were concurrently studied under growing and nonproliferating conditions in glucose-sorbitol medium. In addition to the analytical parameters employed for the heterogeneous

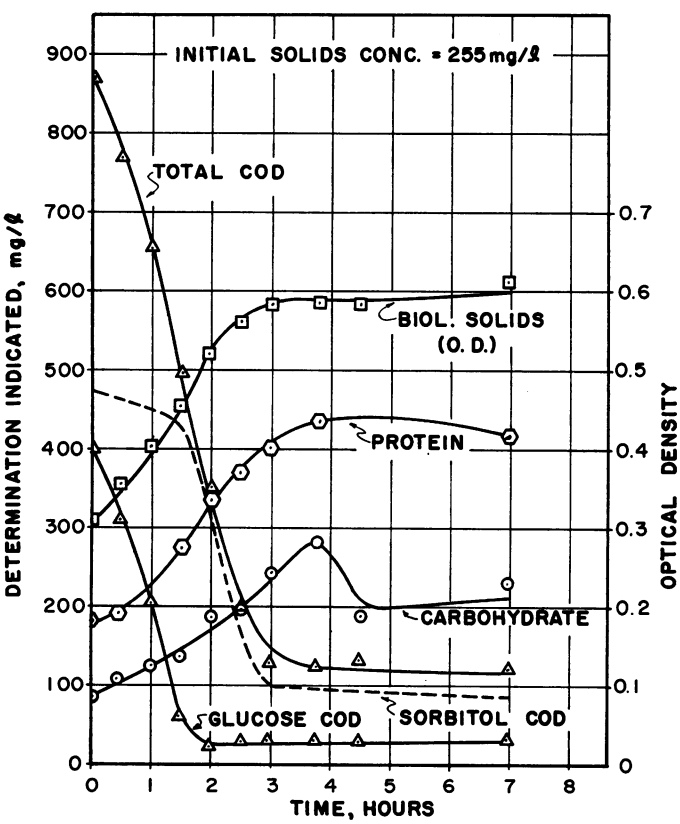

FIG. 4. *System response—E. coli under growing conditions, two-component carbon source, glucose (400 mg/liter) plus sorbitol (400 mg/liter). Cells acclimated to sorbitol.*

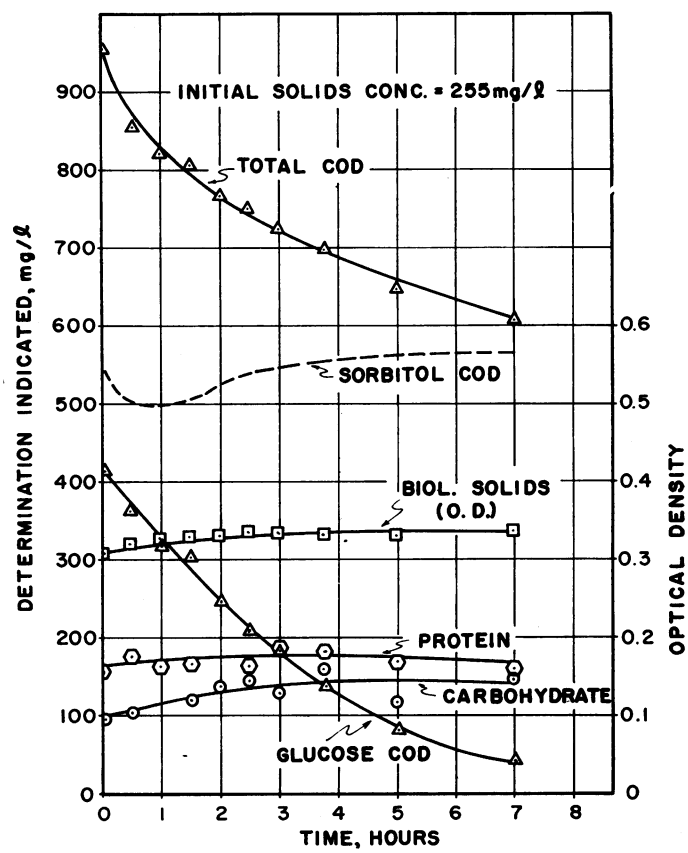

FIG. 5. *System response—E. coli under nonproliferating conditions, multicomponent carbon source, glucose (400 mg/liter) plus sorbitol (400 mg/liter). Cells acclimated to sorbitol.*

populations, the protein and carbohydrate content of the cells was also determined.

Oxygen uptake under growing and nonproliferating conditions is shown in Fig. 3. O_2 uptake was not measured during the first 0.5 hr of the experiment; therefore, the curves initiate at this point on the abscissa. The results are seen to be those typically expected for growing and nonproliferating systems metabolizing a sole carbon source; the oxygen-uptake curve yielded no evidence of diphasic substrate removal.

The results for parameters other than oxygen uptake for the growing system are shown in Fig. 4. Those pertaining to system growth (biological solids, COD removal, protein, and carbohydrate) are typical of those usually observed for a culture growing on a single carbon source to which it is acclimated. However, analysis of the substrate-removal curves (total COD, glucose COD, and sorbitol COD by difference) indicates that sorbitol was not actively metabolized until glucose was eliminated from the system.

Analysis of the same experimental parameters under nonproliferating conditions (Fig. 5) indicates no net synthesis of protein and only slight carbohydrate synthesis. Substrate removal was much slower than in the growing system, but it is seen that all substrate removal is attributable to glucose metabolism.

Discussion

Previous work with young heterogeneous populations (Gaudy et al., 1962) gave some indication that prevention of sorbitol removal by glucose could be caused by interference with the functioning of the enzyme system required to metabolize sorbitol as well as by repression of enzyme synthesis. The basis of such a conclusion was that a fairly large initial cell concentration was used and, since these cells already possessed a functioning system for sorbitol metabolism, its elimination from the medium would not depend entirely upon the synthesis of new enzymes. Results of the present study of heterogeneous populations yield much stronger evidence implicating glucose as a suppressor of sorbitol metabolism. In the nonproliferating system, the initial solids concentration was purposely made high (480 mg/liter) and substrate removal was shown to be sequential, with glucose accounting for all of the COD removed during the first cycle (2 hr). A functioning enzyme system for sorbitol was present in these cells, since COD removal continued after glucose was eliminated. The most logical conclusion is that glucose prevented the operation or function of the sorbitol-metabolizing system. The same result was obtained in the pure-culture studies. It cannot be unequivocally stated on the basis of these results that no sorbitol was removed during the period of glucose metabolism. The increase in "sorbitol COD" (noncarbohydrate COD), which is particularly pronounced in Fig. 1, can be explained by release of partially oxidized intermediates. Since sorbitol concentration was not determined directly, the possibility exists that some sorbitol may have been metabolized along with glucose and that the intermediates released may have originated from the metabolism of either glucose or sorbitol or both. However, the evidence indicates that sorbitol was not utilized during the first cycle of substrate removal. Increases of noncarbohydrate COD of similar magnitude have been obtained in identical experiments in which glucose was the sole carbon source. Further studies are planned to establish the nature of the intermediates and their origin. Preliminary experiments using semiquantitative paper chromotography of the filtrates indicate that sorbitol does not begin to decrease in amount until glucose has disappeared from solution.

It is felt that the results obtained are significant from both an applied and a basic standpoint. From an applied standpoint it is seen that, regardless of the nitrogen content of the waste, a new substrate introduced into the waste stream may completely prevent the utilization of a compound to which the cells are acclimated. The prevention of utilization of such a compound appears to be caused not only by repression of synthesis of the inducible enzyme(s) required for acclimation, but also by suppression of existing enzyme function, thereby causing an immediate response which can completely change the behavior pattern of the treatment process. Although a synthetic waste consisting of glucose and sorbitol is not representative of the complex mixture of components found in some wastes, the use of such a model waste allows more definitive study of the mass response to change in substrate by natural populations. Other synthetic wastes and aspects of phasic substrate removal are presently under investigation. The major point to be delineated herein is that the phenomenon occurs in nonproliferating as well as growing systems for both a pure culture and a completely heterogeneous population such as that found in biological waste treatment processes.

Concerning the nature of the control mechanism for prevention of utilization of a carbon source, repression of enzyme synthesis is generally cited as the major mechanism involved for substrates participating in energy-yielding pathways. Repression of enzyme synthesis has also been shown to be an important mechanism of control in biosynthetic pathways. Many of the pathways possess, in addition, a second control mechanism which allows more rapid and flexible control of enzyme activity. This mechanism, feedback inhibition, involves inhibition, by the end product of the pathway, of an initial reaction in the biosynthetic sequence. The results obtained in these studies indicate that a similar control, based on inhibition of enzyme activity by a product of an energy-yielding pathway, possibly a common intermediate, may operate in carbohydrate metabolism in multicomponent media.

ACKNOWLEDGMENT

This work was supported by the Water Supply and Pollution Control Division of the U.S. Public Health Service (research grant WP-75).

LITERATURE CITED

GAUDY, A. F., JR. 1962*a*. Colorimetric determination of protein and carbohydrate. Ind. Water Wastes **7**:17–22.

GAUDY, A. F., JR. 1962*b*. Studies on induction and repression in activated sludge systems. Appl. Microbiol. **10**:264–271.

GAUDY, A. F., JR., K. KOMOLRIT, AND M. N. BHATLA. 1962. Substrate removal in heterogeneous populations. (Presented at the 35th annual meeting of the Water Pollution Control Federation Toronto, Canada. Oct., 1962)

MAGASANIK, B. 1961. Catabolite repression. Cold Spring Harbor Symp. Quant. Biol. **26**:249–256.

MASTAFA, A. 1937. Production of indole by indole-forming bacteria and its relation to the kind of peptone used. Compt. Rend. Soc. Biol. **124**:450–451.

MONOD, J. 1947. The phenomenon of enzymatic adaptation and its bearings on problems of genetics and cellular differentiation. Growth **11**:223–289.

MORRIS, D. L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science **107**:254–255.

RAYNAUD, M., AND M. MACHEBOEUF. 1946. Inhibitory action of glucose on Strickland's reaction in *Clostridium sporogenes*. Compt. Rend. **222**:694–696.

SILBERSTEIN, W. 1941. Cleavage of tryptophan by resting bacteria, a rapid method to prove the formation of indole. Bull. Fac. Med. Istanbul **4**:2042–2052.

STEPHENSON, M., AND E. F. GALE. 1937. Factors influencing bacterial deamination. I. The deamination of glycine, *dl*-alanine and *l*-glutamic acid. Biochem. J. **31**:1316–1322.