
Substrate Interactions in Natural Microbial Populations

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When a bacterial culture is placed in a medium containing two energy sources, it is faced with two alternatives: the utilization of the two compounds concurrently or the utilization of one compound preferentially over the other. The alternative that is employed is the result of the metabolic control mechanisms operative within the organisms, for those control mechanisms govern the rates of the various reactions. Because enzymes are the catalysts of living systems, the reaction rates will depend upon both the quantity and activity of the enzymes present. The former is controlled by induction and repression of enzyme synthesis, and the latter by the inhibition of enzymes present in the system.

Enzyme induction is "the increase in the specific rate of enzyme synthesis upon addition of some nutrient, usually the substrate of the enzyme" (Pardee, 1961), and the inducer is the specific substance causing the synthesis. Enzymes found in large amounts in the absence of inducer are constitutive. Enzyme repression is defined as "a decrease, resulting from the presence in cells of a given substance (repressor), in the rate of synthesis of a particular enzyme or group of metabolically related enzymes" (Maas & McFall, 1964). If the repression acts on catabolic enzymes and if it is produced by intermediates of carbohydrate metabolism, it is termed "metabolite" repression. Metabolite repression is the main topic of this paper.

Before the term "metabolite repression" was coined (McFall & Mandelstam, 1963), the phenomenon was referred to as the glucose effect because the ability of glucose to repress enzyme synthesis was so widespread. However, the effect is not entirely specific for glucose, and any compound which can serve efficiently as a source of energy and intermediate metabolites may repress the synthesis of certain enzymes. Much work has been performed on metabolite repression using pure cultures of microorganisms, with the histidine- and lactose-degrading systems receiving the most attention.

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Because multicomponent carbon systems are encountered in biological waste treatment systems, work was begun by Gaudy and coworkers (1962, 1963a and b, 1964) to investigate the occurrence of metabolite repression and other control mechanisms in heterogeneous populations. These studies have demonstrated the existence of metabolite repression in heterogeneous populations and have also lead to the proposal of an inhibition effect in catabolic pathways. Recent work by Stumm-Zollinger (1966) has confirmed the existence of both enzyme inhibition and repression in heterogeneous populations.

In an effort to extend the investigation of control mechanisms in natural bacterial populations, studies are now in progress in the interactions of mixtures of carbohydrates and amino acids. This paper is concerned with a portion of that work, namely, the effect of glucose on the utilization of glutamic acid, aspartic acid, and histidine.

MATERIALS AND METHODS

The heterogeneous populations used in each experiment were developed from the effluent of the primary clarifier of the sewage treatment plant in Stillwater, Oklahoma. The sewage was used to seed shaker flasks, with each 250-ml volume Erlenmeyer flask containing 5 ml of sewage seed, 20 ml of amino acid stock solution, and 25 ml of standard dilution water. The concentration of the amino acid stock solution was prepared to give 1000 mg/l of COD in the shaker flasks. The standard dilution water was prepared so that the cultures would contain the concentrations of the compounds listed in Table I. The flasks were aerated at room temperature (approx. 22 C) on a reciprocal shaker at 100 strokes/minute for 24 hr. After 24 hr, 5 ml were removed and placed in fresh medium. The procedure was repeated for three days; then replicate systems were placed on the shaker and after approximately 18 hr were used to seed the experimental units.

The standard experiment consisted of inoculating three aerated growth tubes, total volume 1500 ml, with 100 ml of acclimated seed, following growth by optical density, and removing samples periodically for analysis. Tube No. 1 was the glucose control and contained glucose in concentration to give 500 mg/l of COD, 100 ml of seed acclimated to the amino acid being studied, and enough standard dilution water, Table I, to give a total volume of 1500 ml. Tube No. 2 was the amino acid control and was the same as No. 1 except that it contained the amino acid instead of glucose. Tube No. 3 contained glucose (500 mg/l COD) plus amino acid (500 mg/l COD) to give a total COD of 1000 mg/l. The seed and dilution water in No. 3 were the same as the controls. The temperature was controlled by a water bath at 25 C.

At zero hour the substrates were added to the units and 40-ml samples were removed from each unit and centrifuged for 25 min at 18,400 RCF in a Sorval high-speed centrifuge, Model SS-1. At the same time samples were removed for optical density determination at 540 $m\mu$ using 19-mm tubes in a Bausch and Lomb Spectronic 20. After centrifugation, the supernatant was carefully removed from the centrifuge tubes, 25 ml being used for COD analysis and 10 ml being frozen for later analysis. This procedure was repeated at each sample point.

The COD procedure employed was the alternate procedure for 50-ml sample size given in paragraph 4.5, Section IV, of the 12th Edition of Standard Methods (APHA, 1965). Mercuric sulfate was used only in the histidine experiments for the histidine was in the mono-hydrochloride form. Silver sulfate was used at all times. A check of the procedure for all substrates employed validated the applicability of the COD test.

The frozen samples were analyzed for glucose by the anthrone test (Gaudy, et al., 1963b), and by the enzymatic Glucostat test (Worthington

TABLE I. STANDARD DILUTION WATER

Compound	Quantity in Reaction Unit
1.0 M Potassium phosphate buffer, pH 7.0	10 ml/l
(NH ₄) ₂ SO ₄	500 mg/l
MgSO ₄ ·7 H ₂ O	100 mg/l
FeCl ₃ ·6 H ₂ O	0.5 mg/l
MnSO ₄ ·1 H ₂ O	10.0 mg/l
CaCl ₂	7.5 mg/l
Tap water	100 ml/l
Distilled water	to volume

Biochemical Corp., Freehold, N. J.). The results from the two methods were similar, and therefore only the anthrone curves are presented in the figures.

Amino acid determinations were made by two techniques. In the first ten experiments descending paper chromatography was employed to determine when the amino acid had been removed from solution, and to estimate the time course of the removal. Samples of 20 μ l were applied to Whatman No. 1 sheets in such a manner as to maintain a spot size less than 7 mm diameter. The sheets were developed overnight, using a butanol:acetic acid:water (4:1:1) solvent system. They were then dried in a draft-free cabinet at room temperature and sprayed with ninhydrin spray made by dissolving 200 mg ninhydrin in 200 ml of a mixture of 95% butanol and 5% 2*N* acetic acid. The sheets were again dried at room temperature and then heated for 5 min at 100 C.

In experiments after No. 10 a modified ninhydrin test proposed by Lee and Takahashi (1966) was used to analyze for amino acid quantitatively. Because ammonia will react with ninhydrin it was necessary to pretreat the samples to remove it. This was accomplished by placing 5-ml samples in calibrated test tubes and adding 0.02 ml of 20% KOH to each to raise the pH above 9.0. The tubes were placed in a water bath at 80 C for 10 min, then, while still in the water bath, they were aerated at a rapid rate for 20 min using a 36-tube manifold. The tubes were cooled and evaporation losses were compensated with distilled water. In order to insure applicability of blanks and standards, they were made with the standard dilution water and subjected to the same procedure. Each rack of 36 tubes contained one blank, 5 standards, and 30 samples. The pretreated samples were then subjected to the test of Lee and Takahashi (1966) except that all volumes were doubled. Six standard curves are shown in Figure 1.

All curves were plotted as equivalent COD. The conversion factors were: glucose, 1.00 mg/l glucose = 1.06 mg/l COD; glutamic acid, 1.00 mg/l glutamic acid = 0.978 mg/l COD; aspartic acid, 1.00 mg/l aspartic acid = 0.721 mg/l COD. For experiments 1-10 the amino acid removal curves were calculated using the COD removal curves and glucose removal curves after correcting for intermediate production as evidenced by the control curves. Amino acid COD = (Total COD in mixture) - (Glucose COD in mixture) - (Intermediate COD in mixture). The interme-

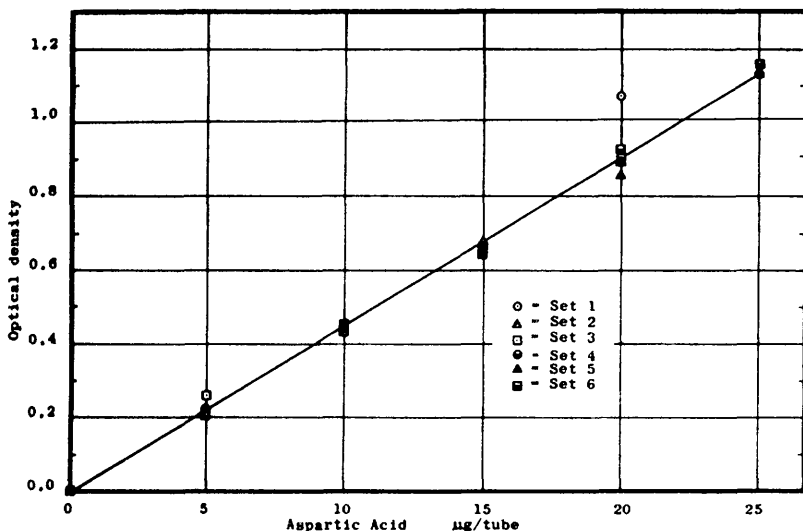


Figure 1. Standard curves for ninhydrin test after removal of ammonia by stripping

diate COD was determined using the assumption: Intermediate COD = (Total COD in glucose control) — (Glucose COD in glucose control) at the time when the glucose COD in the control was the same as the glucose COD in the mixture. The calculations were made on the basis of the anthrone test because it was more sensitive to glucose at low concentrations than was the Glucostat test.

RESULTS

Glutamic Acid—Two experiments were run using a mixture of glutamic acid and glucose with the culture acclimated to glutamic acid. The responses in the two experiments were essentially the same so that data will be presented for only one experiment. The curves for the control units, 1 and 2, are presented in Figure 2. During preparation of the stock solutions a miscalculation was made in the amount of glutamic acid required, which accounts for the high COD due to glutamic acid. Growth on glucose was delayed slightly but no difficulty was encountered in its utilization. An amount of metabolic intermediates was excreted into the medium as shown by a comparison of the COD removal curve and the carbohydrate removal curve. These intermediates were subsequently used by the cells, and cessation of growth corresponds to removal of the majority of COD from the medium. The rate of glutamic acid removal in Unit 2 was slightly faster than the rate of COD removal, again showing intermediate production. These intermediates were evidently not used by the cells since COD removal and glutamic acid removal stop at the same time. Figure 3 presents the growth curve and COD removal curves in Unit 3, the mixture of glucose and glutamic acid. The dashed curves are the specific substrate removal curves of the control units and are reproduced in Figure 3 for ease of comparison. These curves show that the presence of glutamic acid enhanced glucose removal. The substrates appear to be removed concurrently. The residual COD in the mixture is approximately equal to the sum of the residual COD's in the controls (compare COD control curves in Figure 2 with total COD in mixture, Figure 3) indicating

that the production of intermediates was not changed appreciably. The fact that glutamic acid utilization continued in the presence of glucose indicates that the enzymes necessary for glutamic acid utilization were still being synthesized.

Aspartic Acid—The effects of glucose on aspartic acid removal were also investigated. Duplicate experiments indicated similar results, there-

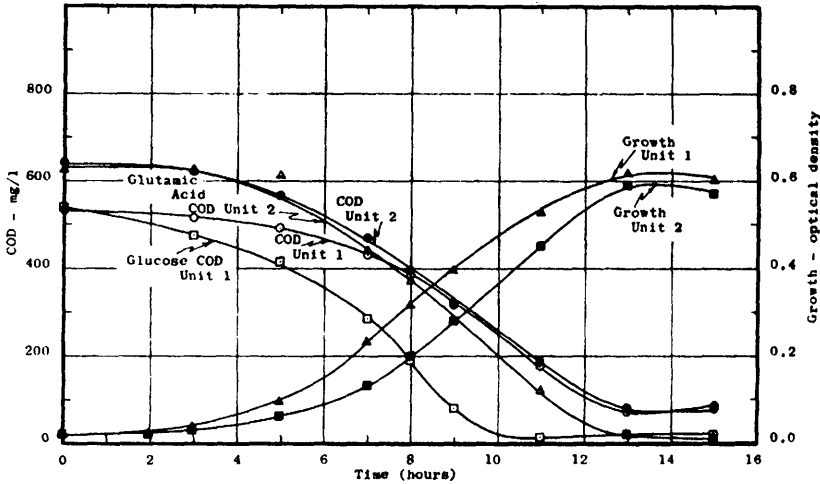


Figure 2. System performance in control units—cells acclimated to glutamic acid

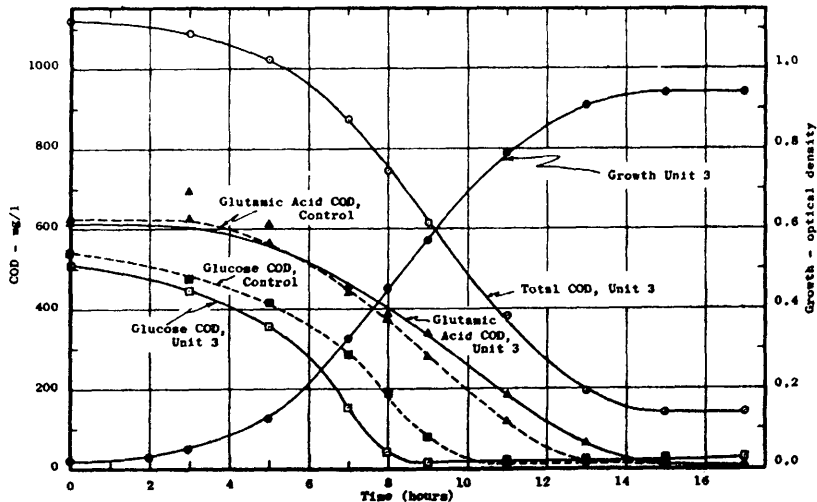


Figure 3. System performance in mixed unit with comparison to controls, cells acclimated to glutamic acid

fore one experiment is presented herein. Figure 4 shows the growth and COD removal curves for the control units. It should be noted that there was no intermediate production in the glucose unit and that growth was considerably slower than in the aspartic acid unit. There was considerable production of intermediates in the glucose control for the duplicate experiment which is not shown, but the growth curve was similar to this one, as were the overall effects. It was confirmed by paper chromatography that aspartic acid removal followed the COD removal in Unit 2. Figure 5 shows results for Unit 3, the mixture of substrates, and it is seen that glucose was removed much faster than in the control, while aspartic acid removal was retarded. It was confirmed by paper chromatography that aspartic acid removal followed the curve obtained by calculation. It is seen from Figure 5 that while glucose retarded aspartic acid removal, it did not stop it, indicating that the organisms were still capable of synthesizing enzymes required for aspartic acid utilization.

Histidine—Two experiments were run with histidine and glucose, and the results were essentially the same. The results for one experiment are presented in Figures 6 and 7. It is seen in Figure 6 that a period of acclimation was required before the cells were able to use glucose, and that there was intermediate production during growth. Growth on histidine was fairly rapid with histidine removal stopping at 8 hr with a residual COD still in the medium. No histidine was detected in the medium at 8 hr by paper chromatography, and the technique employed was sensitive enough to register concentrations as low as 50 mg/l. Figure 7 shows that glucose had no effect upon histidine removal in Unit 3. The calculated histidine curve follows closely the histidine (COD) removal curve of the control. A comparison of the chromatograms for Units 2 and 3 revealed no detectable difference in histidine removal. Since a small inoculum was used for these experiments, there is an indication that the enzymes of histidine removal were synthesized in the presence of glucose. Glucose removal was faster than in the control, but the shape of the COD removal curve and the rise of the calculated histidine curve after 9 hr indicates greater intermediate production in the mixture.

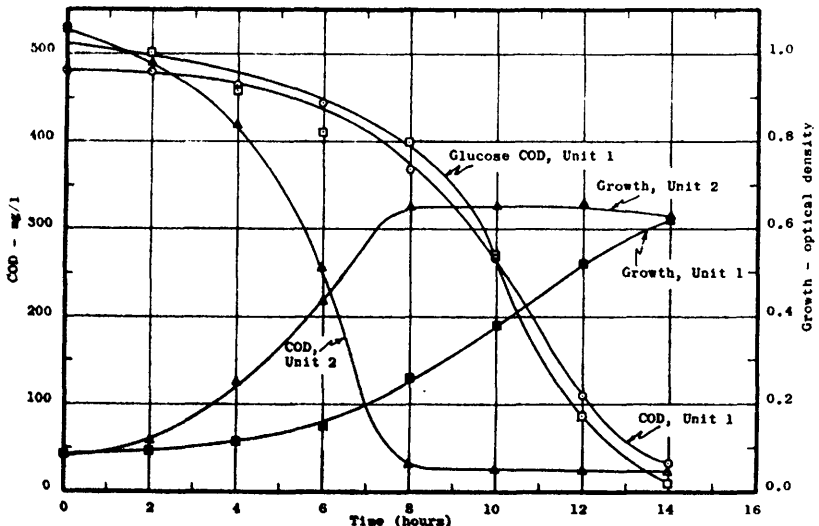


Figure 4. System performance in control units—cells acclimated to aspartic acid

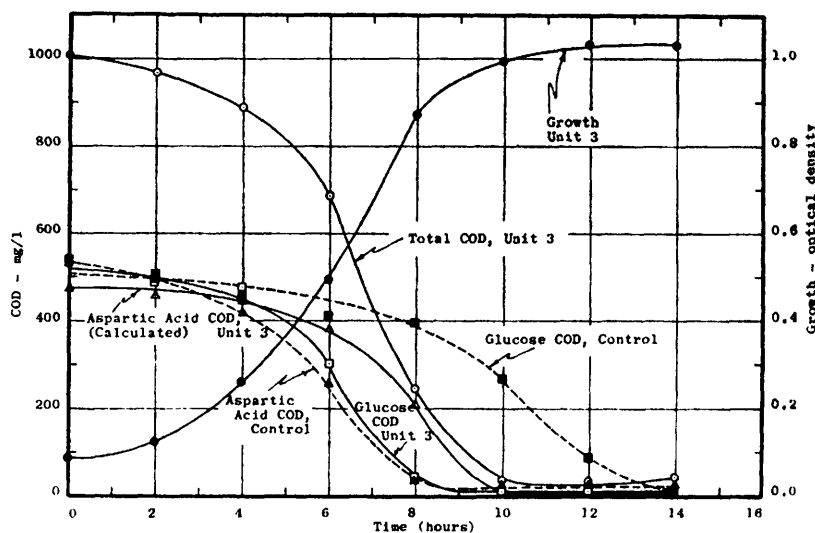


Figure 5. System performance in mixed unit with comparison to controls, cells acclimated to aspartic acid

DISCUSSION

Glutamic Acid—The main method of glutamic acid degradation in bacteria is initiated by the activity of *L*-glutamic acid dehydrogenase which oxidatively removes the α -amino group yielding α -ketoglutarate and ammonium ion. Glutamic acid also takes part in transamination, again yielding α -ketoglutarate when the α -amino group is transferred to another α -keto acid. Many amino acids are obtained from this reaction, including valine, alanine, aspartic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and tyrosine (Cohen & Sallach, 1961). The α -ketoglutarate formed in either reaction can go directly to the TCA cycle. *L*-glutamic acid dehydrogenase can also operate in the reverse direction, in which case it is responsible for the fixation of ammonia from the medium. Then by use of transamination, the amino group is transferred to other carbon chains, resulting in the amino acids required for protein synthesis. The participation of glutamic acid in amino acid synthesis is largely responsible for its rapid utilization for growth of cells. Even if no glutamic acid dehydrogenase were in the system, glutamic acid utilization would continue because the glutamic acid would serve as the source of nitrogen in amino acid synthesis, thus making α -ketoglutarate available for the TCA cycle. Jacoby (1964) found that cells of *Pseudomonas fluorescens* grown in a glucose-glutamic acid medium had less than one-third the ability to oxidize glutamic acid than those grown in a glutamic acid medium. Bowles and Segal (1965), on the other hand, reported that *Mycobacterium tuberculosis* utilized glucose and glutamic acid concurrently, with the rate of glutamic acid removal being the same in the unit with glucose as in the one without glucose. The results presented here indicate concurrent removal of glucose and glutamic acid by a natural bacterial population but with a slight decrease in glutamic acid removal rate after the glucose had been removed. This decrease could be explained in light of Jacoby's findings that ability to oxidize glutamic acid is decreased by growth in a glutamic acid-glucose mixture.

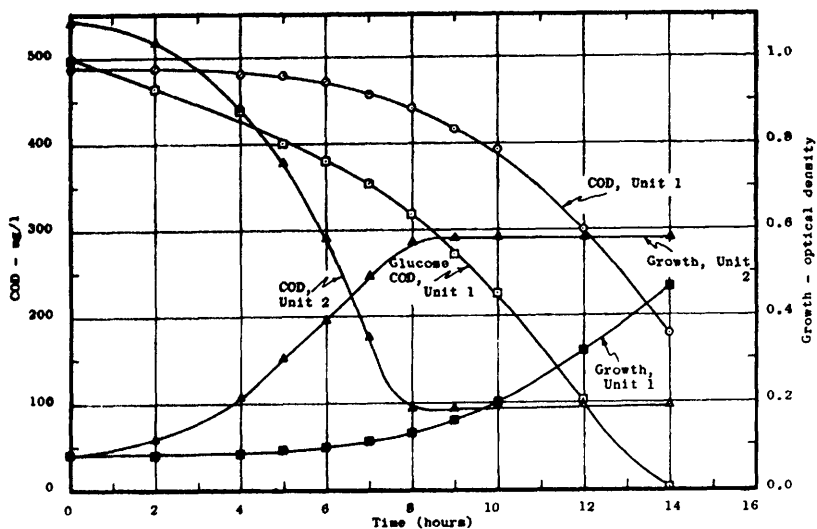


Figure 6. System performance in control units—cells acclimated to histidine

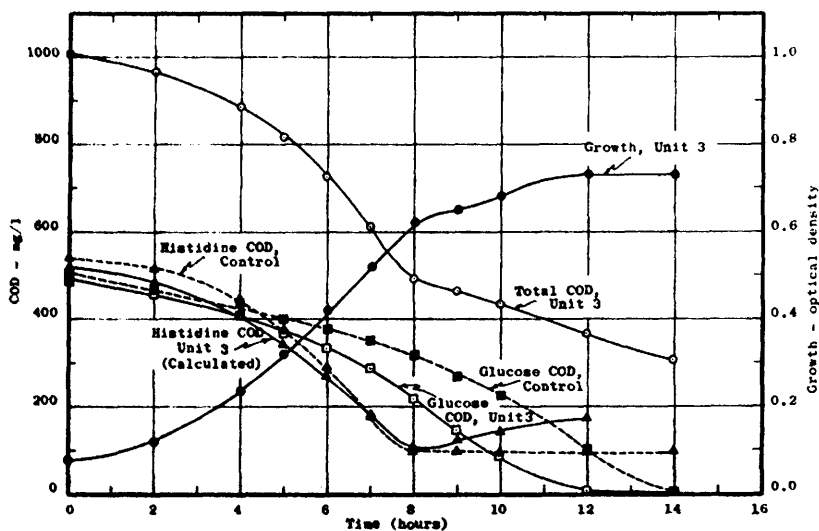


Figure 7. System performance in mixed unit with comparison to controls, cells acclimated to histidine

Aspartic Acid—The major pathway of aspartic acid degradation is by transamination with α -ketoglutarate to yield oxaloacetate + glutamic acid. The oxaloacetate enters the TCA cycle and the glutamic acid is deaminated to regenerate α -ketoglutarate. The deamination of glutamic acid would occur as discussed earlier. Another possible mechanism of aspartic acid utilization would be through an L-amino acid oxidase yielding oxaloacetate. As with glutamic acid, the many possible reactions available for utilization of aspartic acid indicates that the repression of one enzyme would result only in a decrease in rate and not in a complete nonutilization. Jacoby (1964) has shown a threefold decrease in the rate of oxidation of aspartic acid in the presence of glucose. As with glutamic acid, the results presented here show that glucose decreases the rate of aspartic acid utilization but does not completely block its usage.

Histidine—Histidine is degraded in *Aerobacter aerogenes* by a conversion through four steps to L-glutamic acid and formamide (Magasanik, et al., 1965). The pathway in *Pseudomonas fluorescens* is the same except for the last step which yields glutamic acid, formic acid, and ammonia. The first two enzymes of the pathway, L-histidine ammonia-lyase and urocanase, are induced by histidine and urocanate in *Aerobacter aerogenes* (Magasanik, et al., 1965) and in *Pseudomonas fluorescens* (Jacoby, 1964), and are repressed by glucose and other metabolites. The data presented herein show no evidence of metabolite repression of histidine-degrading enzymes in the two natural populations used. Jacoby (1964) does state that glucose did not interfere with the transport of histidine into the cell; however, more than just transport is involved here since the histidine removal curve for the mixture followed the histidine removal curve for the control so closely. Also, the growth curves for the control and the mixture follow each other very closely, indicating that the early growth in the mixture was due to catabolism of histidine. Other pathways of histidine degradation in bacteria are known to exist (Thorne, 1956), so it is possible that another pathway not subject to metabolite repression was followed. Another possible explanation is that glucose itself is not the repressor, but that some catabolite is. Since glucose utilization was slow, quantities of histidine-degrading enzymes could have been synthesized by the cells before the repressor level became high enough to stop synthesis. This appears unlikely, however, because the rate of histidine removal continued to increase until histidine became limiting. If enzyme production had ceased, this would not have been the case. Further study is required before it can be determined whether or not enzyme repression occurred and, if it did, which alternative pathway was followed.

CONCLUSIONS

The studies presented here indicate that glucose should cause no trouble to a natural population degrading glutamic acid or aspartic acid, though it will cause a slight decrease in the rate of the amino acid removal. These studies also indicate that in natural populations glucose exerts no effect upon histidine removal as it does in pure cultures. Further study is needed to determine the cause of this response.

ACKNOWLEDGEMENT

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