EFFECT OF AGEING MICROBIAL POPULATIONS ON SUBSTRATE REMOVAL PATTERNS IN MIXED SUBSTRATE SYSTEMS

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CHAPTER I

INTRODUCTION

A. Basic Concepts and Misconceptions

The initial paper by Gaudy (1) showing sequential removal of glucose and sorbitol by a sorbitol-acclimated heterogeneous microbial population encouraged various researchers in the pollution control field to embark on further investigation of this phenomenon. However, causation for this phenomenon is rather complicated, involving basic metabolic regulatory mechanisms at the level of enzyme synthesis and function, and it has become apparent that some investigators have tended to oversimplify, and indeed ignore, the basic control mechanisms which are operative during substrate interactions. It is felt that this failure to consider the control mechanisms which lead to the manifestation of sequential substrate removal has led to some misinterpretation of experimental results and, in some cases, to incorrect conclusions. It is of the utmost importance to realize that the term "sequential substrate removal," considered by itself and not in context with the parameters of the biological system in which it was obtained, is entirely inadequate to describe the intrinsic behavior of a biological system.

Consider, for example the following illustration. An investigator reports that a given organism pregrown on sorbitol and then placed in a glucose-sorbitol mixture eliminates these substrates sequentially with

the glucose being utilized first. Another investigator using the same organism pregrown on sorbitol reports concurrent utilization. These seemingly divergent results can be completely compatible. To appreciate this fact it is necessary to consider the observed substrate removal patterns in relation to two very important control mechanisms; these are repression of enzyme synthesis and inhibition of enzyme function. If the first investigator had utilized a low initial biological solids concentration and if glucose catabolites completely repressed the formation of the enzymes necessary to remove sorbitol, then the result would indeed have been sequential substrate removal. If, on the other hand, the second investigator had started with a high concentration of biological solids, which consequently would have provided the system with a high initial "enzymatic capability" to remove sorbitol, the compounds would have been removed concurrently provided that the enzyme system necessary for the degradation of sorbitol was not inhibited by glucose or by any of the catabolites from glucose. Therefore, where catabolite repression is exerted but there is no inhibition of preformed enzymes, any pattern of substrate removal, within wide limits, may be obtained, simply by varying the initial biological solids concentration.

If a microbial population is placed in contact with a dual carbon source where the metabolites from one source both repress and inhibit the formation and function, respectively, of the enzyme system necessary for the utilization of the other substrate, a wide range of substrate removal patterns can be obtained. The particular response observed will depend upon the initial biological solids concentration, the initial "enzymatic capability" of the cells to utilize each of the

substrates, and also on both the degree of repression and the degree of inhibition present. Only if an enzyme system necessary for the utilization of one of the compounds is both completely repressed and completely inhibited will it always be possible to observe completely sequential substrate removal in the dual carbon source medium.

One should now appreciate that there are many factors which must be considered in evaluating what is truly meant by a report of sequential substrate removal. Even when the metabolic control mechanisms involved are considered, there has been a failure on the part of some researchers to fully appreciate the basic concepts involved. For example, consider the following statement by Stumm-Zollinger (2):

> Catabolite repression has been observed more frequently than catabolite inhibition. Both responses lead to diauxic growth and sequential substrate utilization.

The inadequacy of the latter part of this statement will be discussed below. Another illustration of this failure to understand control mechanisms is provided by Prakasam and Dondero. They said (3):

> When in a population all of the cells are subject to enzyme repression, the result is to delay the utilization of some available substrate.

Both of these statements indicate that the authors do not realize that repression or inhibition need not be an "all or nothing" phenomenon. A system can be repressed and still produce the enzymes necessary to degrade some compound, but they will be produced at a rate less than that which would be found in an unrepressed system; an enzyme system which is partially inhibited will still function, but not at its maximum rate. Also, a biological population can undergo complete repression but if there is no enzyme inhibition and if the initial biological solids concentration employed provided a relatively large

complement of enzymes necessary to degrade some compound, that compound would still be removed.

An example of how failure to consider the basic control mechanisms can lead to a rather gross misinterpretation of experimental results is provided in an article by Prakasam and Dondero (3). Their experimental results and the conclusions they derived from them will now be presented. Then their results will be analyzed in a manner which this author believes to be more rational and more in keeping with sound metabolic principles.

Their first series of experiments were performed on a heterogeneous "young cell" culture which had been acclimated to sorbitol (adapted sludge) by successive transfers of a small inoculum to fresh sorbitol medium (1,000 mg/l) every 12 hours over a three day period. If one assumes that these adapted cells completely utilized the sorbitol with a 60% yield, the procedure which they used to recover the cells and resuspend them in the experimental units (320 ml of adapted sludge washed thrice and then resuspended in a total volume of 3 liters from which 500 ml portions were dispensed to 1 liter flasks) would have resulted in an initial biological solids concentration (neglecting any losses) of 64 mg/l. This would be the maximum amount of cells which could have been present. Sorbitol COD in the glucose-sorbitol combined unit (initially containing 300 mg/l of each compound) was determined by subtracting the anthrone equivalent COD from the total COD. However, glucose utilization was accompanied by the release of metabolic intermediates, and hence, the subtraction method of calculation provided only an approximation of sorbitol removal. It was concluded that "... the utilization of sorbitol seemed blocked at least partially if not

completely by glucose." (3)

An additional experiment was performed in which 20 μc of ^{14}C-sorbitol were added to 50 ml of a young cell adapted sludge suspension containing 300 mg/l each of glucose and sorbitol. The initial biological solids concentration was 110 mg/l. It was found that $^{14}CO_2$ was produced at the rate of 4×10^4 counts per hour during the first 3.5 hours. This rate then increased, and 13×10^4 counts were recorded between the fifth and sixth hours. Although glucose removal was not measured, they indicated that previous measurements had shown that it would be removed in about 4 hours. From these results the following was concluded (3):

> These results were interpreted as proof that the utilization of sorbitol by the adapted sludge was very largely, although not completely, suppressed in the presence of glucose.

The biological solids for their final experiment were obtained directly from the activated sludge unit of a sewage treatment plant. The experimental reactors contained 5 μc of ^{14}C-sorbitol in a volume of 50 ml; a 150 mg/l concentration each of glucose and sorbitol in the combined unit; 300 mg/l of sorbitol in the sorbitol control unit; and an initial solids concentration of 245 mg/l. Oxygen uptake was measured in a Warburg apparatus with an initial biological solids concentration of 2,450 mg/l being utilized in conjunction with total initial substrate concentrations of 300 mg/l. They found that the $^{14}CO_2$ from the combined system during the first 4 hours produced a total of about 8.5×10^4 counts, and the rate of production was nearly constant. After this time, the rate of $^{14}CO_2$ production increased. Glucose removal in the combined system was not measured. Oxygen uptake occurred at a decreasing rate throughout its measurement (40 hours) in

the combined unit and in the glucose and sorbitol controls. On the basis of this experiment the authors said the following (3):

> In contrast to the suppression of sorbitol metabolism by adapted sludge, the treatment-plant activated sludge utilized the sorbitol in the presence of glucose as readily as in its absence.

In summarizing all of their experiments, they concluded that ". . . although the adapted sludge showed the effects of repression by glucose, the activated sludge was able to attack both substrates immediately and simultaneously." This "difference" in the behavior of the two populations was explained as follows (3):

> The simplification of the population during adaptation provided an explanation for the repression of sorbitol metabolism by glucose.

If one now applies a more rational method of analysis to the results obtained by these investigators, some interesting conclusions can be drawn. Because of the low initial solids concentration (less than 64 mg/l) and because of the indirect method used to estimate sorbitol removal in their first series of experiments, there is no way to ascertain whether the reported blockage resulted from any inhibition of the preformed sorbitol enzyme system. However, their data do seem to indicate that the synthesis of sorbitol-degrading enzymes was completely or very nearly completely repressed in the presence of glucose. Alternatively, there could have been no repression but complete inhibition of any new enzymes synthesized. This would not seem to be too likely an alternative since it would allow the cell to waste energy and amino acids by synthesizing enzymes which it could not use.

In the experiment with the adapted young cell culture utilizing radioactive sorbitol, it would appear that the synthesis of sorbitol-degrading enzymes was completely repressed because the rate of $^{14}CO_2$

production was constant during the time that the glucose was being metabolized. However, since $^{14}CO_2$ was produced, it is obvious that the sorbitol-degrading enzyme system present initially was still functioning and, therefore, was not completely inhibited. In fact, there may have been no inhibition at all. This cannot be determined because a sorbitol control system was not run.

It is surprising that in the experiment employing the activated sludge solids, where the entire purpose of the investigation was to determine the effect of glucose on sorbitol metabolism, that glucose removal was not measured. In an effort to estimate the time required for glucose removal, the data of Stumm-Zollinger (4) have been employed. She found that a field activated sludge taken directly from a domestic wastewater treatment plant had a specific glucose removal rate of 4.6 mg per minute per gram (dry weight) sludge. If one applies this figure to the combined unit of Prakasam and Dondero, it can be seen that the glucose would have been removed in 133 minutes $\left[150/(4.6)(0.245)\right]$. This neglects any additional removal caused by new synthesis of glucose-degrading enzymes. In contrast to this, examination of the $^{14}CO_2$ production in the combined unit shows that the sorbitol was not removed until after 10 to 12 hours.

Although the Warburg studies run in conjunction with the $^{14}CO_2$ measurements should have provided additional information concerning the time required for removal of each of the substrates, the selection of a biological solids concentration in the Warburg flasks of 2,450 mg/l insured that this information would not be obtained. By again utilizing the removal rate reported by Stumm-Zollinger (4), it can be seen that the glucose would have been removed from the combined unit in

13.3 minutes and from the Warburg flask containing only glucose in 26.6 minutes. There is really no accurate way to extrapolate from the $^{14}CO_2$ data to the time required for sorbitol removal in the Warburg sorbitol control. However, the data do indicate that the sorbitol should have been removed within 3 hours or less. It is not too surprising then that they found that oxygen uptake occurred at a continually decreasing rate in all units throughout the 40 hour period of observation.

In summary, very few conclusions can be drawn from the experiment with the activated sludge solids. The synthesis of sorbitol-degrading enzymes may or may not have been completely repressed in the presence of glucose. A comparison of the $^{14}CO_2$ curve from the combined system with that of the sorbitol control system indicates that there was very little if any inhibition.

This extended discussion of the work of Prakasam and Dondero (3) emphasizes the pitfalls encountered when workers attempt to draw generalized conclusions concerning metabolic behavior purely from the observed substrate removal patterns. Indeed, it is apparent that all of the results reported by these authors could have been obtained from a pure culture system which underwent complete repression but not complete inhibition merely by varying the initial biological solids concentrations and the initial cellular content of sorbitol-degrading enzymes.

B. Objectives of This Investigation

The primary objective of this study was to evaluate the extent of substrate interaction in a glucose-sorbitol and a glucose-galactose medium in contact with heterogeneous microbial populations, and to

determine whether an increase in the age of the system would relieve the inhibition and/or repression which was present initially. This was done in an attempt to ascertain whether engineering controls (e.g., possible control of "cell age") could be employed to eliminate the deleterious effects on overall waste treatment efficiency which can occur when certain substrate combinations are encountered.

To eliminate many of the problems which can arise owing to predominance changes, the studies relating cell age to substrate removal patterns were extended to include pure culture systems. Based upon the nature of the substrate removal curves which were obtained during experimentation, estimates of the extent of the repression and inhibition of sorbitol-degrading enzymes by glucose catabolites were also made.

CHAPTER II

LITERATURE REVIEW

Although the previous chapter contained some discussion of various factors which should be considered when interpreting substrate removal patterns, it did not explore in detail all of the factors which contribute to the complexity of the problem. Hence the first part of this chapter will be devoted to a more detailed analysis of a few additional mechanisms which can be of importance. No attempt will be made to review all of the control mechanisms known to be operative in bacteria. An excellent review, for those seeking a summary of these mechanisms, has been given by Grady (5).

A. Additional Concepts Pertaining to Control Mechanisms

1. Permeases

Permease competition or interference with the transport of a substrate molecule into a bacterial cell by another substrate can cause substrate interactions. The necessity of transport systems for various monosaccharides was first suggested by Douderoff et al. (6). They isolated a strain of Escherichia coli capable of normal growth on maltose but unable to utilize glucose. Since all the intracellular enzymes necessary for glucose metabolism were present, it was postulated that a specific transport system for glucose must be present for utilization of an extracellular supply of this compound. The work of Cohen and Monod (7) provided a direct demonstration of the existence of such

selective bacterial transport systems (permeases).

2. Glucose-Galactose Interactions in Escherichia coli

A sufficient amount of information is available pertaining to the effect of glucose on galactose uptake in various strains of E. coli to illustrate, among other things, interference at the level of transport. Horecker et al. (8) studied a mutant which lacked galactokinase and, therefore, could not grow on galactose. However, it did possess a transport system for galactose. Resting cells became saturated when the external galactose concentration reached 2×10^{-5} M, at which point the internal concentration reached 6×10^{-2} M. This was nearly 5% of the total dry weight of the cell.

The uptake of ^{14}C-galactose, when its external concentration was varied from 10^{-4} M to 10^{-6} M, was only slightly affected by the addition of 10^{-4} M (18 mg/l) glucose. However, the addition of 0.005 M (900 mg/l) glucose strongly inhibited the uptake of galactose and caused the rapid and complete loss of ^{14}C-galactose in cells which had been previously equilibrated with this compound in the absence of glucose (8).

Growth of this mutant strain on succinate in the presence of traces of galactose produced cells with a smaller capacity to accumulate galactose than when the cells were grown on succinate alone. This reduced capacity for galactose accumulation was shown to be caused by an increase in the rate of loss of galactose from the cells rather than by a decrease in the rate of galactose uptake. Hence the organism contained an exit mechanism for galactose, different from simple diffusion, which was, at least to some extent, independent of the entry process. When the organism was grown on glucose and traces of galactose, there

was also a reduced capacity to accumulate galactose; however, in this case the rate of exit was unchanged over that found for growth on mannose but the rate of entry was reduced. This would imply that glucose catabolites partially repressed the formation of the galactose transport system required for entry (9).

Rogers and Yu (10) also studied a strain of E. coli which lacked galactokinase; it was believed that it also lacked galactoside permease. It appeared that galactose uptake occurred through a glucose permease system. In the presence of equimolar amounts of glucose or 2-deoxy-D-glucose (0.02 M), galactose uptake was inhibited 33 and 37%, respectively. When the galactose concentration was decreased to 0.005 M the addition of 2-deoxy-D-glucose raised the percentage inhibition to 77%. Galactose uptake was also inhibited by 3-O-methyl-D-glucose. Thus glucose and a variety of nonmetabolizable glucose derivatives inhibited galactose uptake, and the extent of inhibition was a function of their relative concentrations. Since galactose uptake in equimolar concentrations of glucose and galactose need not be significantly affected (8), it would also appear that absolute as well as relative concentrations may influence the extent of interference with uptake manifested. Alternatively, strain variations may have produced the observed differences.

Upon consideration of the results thus far presented, Adhya and Echols (11) felt that glucose inhibition of the induction of the enzymes specific to galactose utilization was exerted mainly at the transport stage, and hence, that the primary mechanism of glucose control was not catabolite repression. When they added glucose to a culture of E. coli at a concentration (10^{-2} M) ten times that of

galactose, the uptake of galactose was virtually stopped. The effect of glucose on the levels of transferase (galactose-1-phosphate uridyl transferase) in four different strains constitutive for the gal enzymes was also investigated. The percentage transferase activity found for growth on glucose was 47%, 43%, 70%, and 54% of that found with each of the respective strains grown on a mixture of glycerol plus fucose (a gratuitous inducer for the gal enzymes). When a strain of E. coli was grown on lactose and glucose, thus providing the galactose intracellularly by virtue of the breakdown of lactose by the intracellular enzyme β-galactosidase, the levels of transferase and epimerase were 40% and 87% of those found with growth on galactose alone. Since these enzymes both belong to the gal operon and hence are coordinately induced or repressed, it is evident that considerable error was present in the enzyme assays. Although the authors were overenthusiastic in trying to prove that catabolite repression was not very significant in repressing the induction of the gal enzymes, it is apparent that glucose inhibition of the formation of the gal enzymes does occur to some extent at the level of inducer transport into the cell.

Further support for glucose-galactose interactions at the transport level has been given by Anraku (12). He has also studied galactose transport in mutant strains of E. coli lacking galactokinase. When the cells were subjected to an osmotic shock there was a reduction of about 50% in the capacity for galactose uptake. A protein factor was released into the shock fluid which complexed with galactose, and when the shocked cells were incubated with the protein factor, active uptake of galactose was restored. In subsequent work (13), the purified protein factor was found to be a galactose-binding protein which would

also bind glucose. Dissociation constants for the binding of these two compounds were nearly equal with a value of approximately 10^{-6} M. A number of other sugars and sugar derivatives were also examined, but none of them were bound by the protein.

3. Glucose-Glycerol Interactions in Escherichia coli

An examination of glucose-glycerol interactions in E. coli illustrates that substrate interactions may arise as the result of the inhibition of a preformed intracellular enzyme system. Lin et al. (14) have demonstrated that E. coli can grow on glycerol only if the enzyme glycerol kinase is present. Further work (15) with several mutants which lacked glycerol kinase showed that no accumulation of ^{14}C-glycerol was possible. However, mutants which possessed glycerol kinase but lacked L-α-glycerophosphate dehydrogenase, when exposed to ^{14}C-glycerol, accumulated large quantities of radioactive material which was present almost entirely as L-α-glycerophosphate. Hence glycerol kinase not only mediates the first reaction in the dissimilatory pathway, but it is also responsible for trapping the glycerol which enters the cell by facilitated diffusion. Thus the membrane transport system is one in which the specific membrane carrier catalyzes the equilibration of intracellular and extracellular glycerol concentrations with no expenditure of metabolic energy (16).

In additional work by Zwaig and Lin (17), a mutant E. coli strain was isolated which was capable of incorporating glycerol while growing on glucose. Studies indicated that the parental cells grown on glucose were replete in an intermediate which severely inhibited glycerol metabolism in wild-type cells but not in the mutant cells. That the difference in behavior between the two types of cells reflected the

properties of their respective glycerol kinases was indicated by the finding that the kinase of the mutant was inactivated at pH 9.5, a condition under which the activity of the wild-type enzyme was nearly maximum. Several glycolytic intermediates were tested for their inhibitory effect on the glycerol kinase isolated from the wild-type cell. Only fructose 1,6-diphosphate was found to be inhibitory. The actual role of fructose 1,6-diphosphate as the physiological inhibitor of glycerol kinase was evident from the fact that the mutant produced an enzyme which was not inhibited by the diphosphate.

4. Glucose-Lactose Interactions in Escherichia coli

An example of the way in which both catabolite repression and interference with transport can affect substrate removal is available from studies with E. coli utilizing lactose and glucose. In 1959, Cohn and Horibata (18) found that the synthesis of β-galactosidase in a wild-type strain of E. coli was completely prevented by glucose when 5×10^{-4} M TMG (methylthiogalactoside) was used as inducer. However, when the TMG concentration was increased to 2×10^{-2} M, the glucose repression was reduced by nearly 50%. It was also observed that pre-induction of β-galactoside permease positive cells would reduce the degree of repression to about 50%. Preinduction of permease negative cells could not reduce the strong repression by glucose.

Subsequent work by Mandelstam (19) with a mutant strain of E. coli constitutive for β-galactosidase (i^-) showed that the organism was still subject to repression by glucose catabolites, and the degree of repression increased as the level of catabolites from glucose was increased in nitrogen-deficient medium. Loomis and Magasanik (20) were able to show that the regulator gene which determines the inducer sensitive region

of the lac operon was different from that which determines the catabolite-sensitive repression of the operon. In mutants which were not subject to catabolite repression (CR^-), the formation of the products of the lac operon was highly insensitive to repression by catabolites derived from diverse carbon sources.

Later work by Loomis and Magasanik (21) with the CR^- strain of E. coli showed that when the cells were suspended in medium containing glucose and lactose, the synthesis of β-galactosidase initially occurred at a low rate which increased with growth in the medium (this was not transient repression). Hence it was evident that glucose had an effect on induction by 10^{-2} M lactose even in CR^- cells. Further work with a lac^+ CR^+ strain of E. coli showed that preinduction of the lac operon for five minutes with 10^{-3} M isopropyl-thio-β-galactoside allowed induction by 10^{-2} M lactose in the presence of glucose. Furthermore, a high concentration of lactose (8×10^{-2} M) was shown to overcome diauxie, suggesting that the lac operon was induced before glucose depletion. Both of these observations were explained as resulting from an increased internal concentration of lactose in the presence of glucose. Thus it became apparent that there was an interference by glucose with the uptake of lactose.

In summary, it is apparent that glucose can affect the expression of the lac operon by at least two distinct mechanisms. One is by reducing the internal concentration of inducer (21), and the other is by catabolite repression of the lac operon depending upon a functional CR gene (20).

B. Substrate Removal Patterns in Heterogeneous Cultures

Now that most of the control mechanisms which can lead to different substrate removal patterns have been introduced, it is appropriate to examine how their operation may be manifested in a manner significant to the engineer. In 1962, Gaudy (1) showed that the mechanisms of induction and repression were not unique to a few species but were of sufficient generality to affect the performance of a heterogeneous population. When a small inoculum of sorbitol-acclimated cells developed from a sewage seed was added to a glucose-sorbitol medium, it was observed that the glucose was utilized in preference to the sorbitol.

Subsequent studies (22) were then undertaken to determine the effect of "cell age" on substrate removal in a glucose-sorbitol medium. Old cells were defined as sludge which had been taken from a batch-operated unit which had been in operation for at least 21 days. Intermediate age cells were developed by taking a small portion of old cells and inoculating a batch unit which was then operated as an old cell unit for 3 days; on the fourth day the cells were harvested for experimentation. Young cells were cells which were maintained primarily in log growth by repeated transfers of a small inoculum to fresh medium. In all experiments, glucose was measured by anthrone, total substrate by the COD analysis, and sorbitol in the combined unit (glucose plus sorbitol) by subtraction of glucose COD from total COD.

Both old and young glucose-acclimated cells were found to remove the glucose from the combined units prior to the utilization of the sorbitol. Examination of the sorbitol removal curves in the sorbitol control units revealed that the cells had no initial ability to utilize sorbitol. Therefore, the results can be explained on the basis of the

complete repression, in the presence of glucose, of those enzymes necessary to degrade sorbitol (22).

Old cells acclimated to sorbitol were found to remove the substrates in the combined system concurrently. The initial solids level employed was such that the sorbitol was removed from the sorbitol control within 3 hours, and substrate removal approximated zero order kinetics. This would mean that very little new synthesis of sorbitol-degrading enzymes occurred, and therefore, even if the glucose in the combined unit had repressed the formation of the sorbitol enzymes, this could not have been detected. Sorbitol removal in the combined unit required 4 hours and followed zero order kinetics. Hence the sorbitol enzymes appear to have been somewhat inhibited by glucose catabolites (22).

Intermediate age cells were also found to remove the substrates concurrently. As long as glucose was present in the combined unit, the rate of sorbitol removal was constant, and, hence, there apparently was a complete repression of any further synthesis of sorbitol-degrading enzymes. The initial COD removal rate in the sorbitol control was about 70 mg/l/hour. The sorbitol removal rate in the combined unit during glucose removal was about 50 mg/l/hour. Hence the preformed sorbitol enzyme system was only subject to about 29% inhibition. The rate of glucose removal per unit of biological solids was somewhat higher with these cells than with the old sorbitol-acclimated cells. The converse was true for the sorbitol removal rate (22).

It appears that large quantities of intermediates were produced when the young sorbitol-acclimated cells were metabolizing glucose (22). Since the sorbitol concentration in the combined unit was

determined by subtraction of glucose COD from total COD, this led to an apparent increase in the sorbitol concentration during glucose metabolism. This rise in the apparent sorbitol COD when the subtraction method of calculation is used has also been noted by others (3, 23). Therefore, it is impossible to estimate the extent of repression or inhibition, and a discussion of heterogeneous young cell systems acclimated to sorbitol will be deferred until somewhat later.

Subsequent work by Gaudy et al. (24) used a periodate oxidation followed by reaction of the formaldehyde produced with chromotropic acid to measure sorbitol concentrations. Since glucose reacts at only 1/10 the rate of sorbitol, it is possible to correct for the glucose without a significant error in the sorbitol determination. However, periodate selectively cleaves a molecule between adjacent carbons which have any combination of hydroxyls, aldehydes, ketones, or primary amine groups (25). Therefore, whenever large quantities of intermediates are produced from glucose there exists the possibility that they can also react with periodate (shown repeatedly in Chapter IV). Hence, whenever the periodate reaction is employed to measure sorbitol in the presence of glucose and if there are no provisions taken to measure the interference, if any, from glucose intermediates, the results are implicitly dependent upon the assumption that no reactive intermediates were produced.

Further studies of old cell systems acclimated to various compounds were conducted by Gaudy et al. (24). When an instantaneous shock load of glucose was added to a culture removing sorbitol (measured directly), there appeared to be a complete repression of any further synthesis of the enzymes necessary to remove sorbitol. However, there was only a

slight inhibition of preformed sorbitol enzymes. When the experiment was repeated in the absence of an exogenous nitrogen source, the effect of glucose on sorbitol removal was essentially the same. In an interesting experiment with old mannitol-acclimated cells under nonproliferating conditions, it was found that the cells had lost the ability to utilize glucose, and the glucose did not interfere with mannitol removal; this means that glucose did not affect mannitol transport. Studies on an old cell system acclimated to glycerol (26) showed that glucose was unable to produce any inhibition of the preformed glycerol enzymes.

When a young cell heterogeneous population acclimated to sorbitol was examined by Gaudy et al. (27) it was found that approximately 130 mg/l of sorbitol were removed in the presence of glucose. The glucose control unit was analyzed for total COD, anthrone-reactive material (glucose plus carbohydrate intermediates), and periodate-reactive material. Small amounts of intermediates were produced, including a total of 30 mg/l of periodate-reactive material. If the same amount of periodate-reactive material was produced from glucose in the presence of sorbitol, the amount of sorbitol actually removed in the combined unit would have been 160 mg/l. If there had been complete repression but no inhibition of the existing sorbitol enzyme system, the data presented for the sorbitol control indicates that 275 mg/l of sorbitol would have been removed from the combined unit in the presence of glucose. If one assumes complete repression of enzyme synthesis, then the existing sorbitol enzyme system was inhibited by about 42%. If repression was less than complete, the degree of inhibition would have been higher. Since the figure from which this data were obtained

has not been published, it has been reproduced in the appendix.

When glucose was added instantaneously to a young cell culture actively metabolizing sorbitol (24), it was found that sorbitol removal was completely blocked until the glucose was removed. This result could indicate that the enzymes necessary for sorbitol utilization were completely inhibited. On the other hand, if 30 mg/l of the periodate-reactive intermediates had been produced from the glucose which was introduced (as was the case in the control system of the preceding experiment (27)) the sorbitol enzymes could have been inhibited by 77%. If as much as 130 mg/l of periodate-reactive intermediates had been produced from the added glucose, the resulting mode of sorbitol removal could have ensued because of complete repression of further sorbitol enzyme synthesis without any inhibition of preformed enzymes. Hence small quantities of periodate-reactive intermediates can have a serious effect on the observed degree of blockage.

Bhatla and Gaudy (28) also extended the investigation of glucose-sorbitol interactions to dilute systems. When a young cell system acclimated to glucose was used as seed in a BOD bottle containing 5 mg/l glucose and 5 mg/l sorbitol, it was observed that the glucose was removed prior to the sorbitol and oxygen uptake was diphasic. When a sorbitol-acclimated seed was used, glucose was nearly exhausted before sorbitol removal began, but in this case there was no diphasic oxygen uptake. Thus it would appear that glucose catabolites caused repression of the synthesis of the enzymes required for sorbitol degradation even at low concentrations.

Additional studies with young cell systems acclimated to compounds other than sorbitol and then exposed to glucose have also been made.

Krishnan and Gaudy (26) found that when glycerol-acclimated cells were placed in a glucose-glycerol medium, no glycerol was removed prior to glucose removal. The preformed enzyme system for the degradation of glycerol was shown to be completely inhibited. Komolrit and Gaudy (29) found that when a shock load of glucose was added to a young cell culture metabolizing dulcitol, there was a partial inhibition of the enzymes required for dulcitol metabolism. Grady and Gaudy (30) have investigated the effect of glucose on the utilization of various amino acids. In the cases of glutamic acid and aspartic acid, amino acid removal was slightly retarded when mixed with glucose. Glucose was found to have no effect on histidine removal.

Yu (31) has recently completed a study encompassing both batch and continuous flow experiments. From the results of the batch studies using a variety of combined carbon sources, he concluded that when growth on one of the compounds as a sole carbon source proceeds at a significantly faster rate than growth on another compound or when growth on one compound leads to the accumulation of a significantly higher level of metabolic intermediates, the presence of that compound in a combined substrate system will retard the removal of the other substrate. Cells were also harvested for batch experiments from a continuous flow reactor fed glycerol and operated at various dilution rates ranging from 0.25 hr^{-1} to 0.0052 hr^{-1}. It was found that it was impossible to make generalized statements concerning the effect of cell age on the substrate removal patterns in glucose-glycerol medium.

It would appear, however, that one facet of Yu's work (31) is subject to some criticism. At no time was the production of periodate-reactive intermediates from glucose investigated, even though the

periodate reaction was used to measure glycerol in the presence of glucose (32). Although it is obvious that this was generally not an important consideration (certainly not important enough to invalidate any of the conclusions obtained from overall consideration of the entire series of experiments in glucose-glycerol medium) there were a few times when it should have been considered. The most prominent example can be found by examination of Figure 141. It was observed that the reduction of total COD in the glucose control during the first 8 hours was 100 mg/l and the intermediate level at this time was 420 mg/l; all 520 mg/l of glucose COD had just been removed. During the same time period the COD removal in the combined unit was 225 mg/l, glucose removal 500 mg/l, "glycerol" COD had increased by about 40 mg/l, and the "intermediate" COD level was 225 mg/l. The author concluded that glycerol removal was not initiated until the glucose was exhausted from the medium. If this was the case, why was the amount of COD removal in the combined unit more than double that of the glucose control, and why was the "intermediate" level in the combined unit about half of that in the glucose control? One logical explanation of the results of this experiment is that periodate-reactive intermediates were indeed produced from glucose, and the level of production would seem to be nearly 195 mg/l (420 - 225). In this event, the glycerol concentration in the combined unit would actually have decreased by 155 mg/l during the course of glucose removal.

Su (33) studied the utilization of mixtures of carbohydrates by investigating a total of 18 different substrate combinations. Experiments were run with cells acclimated to each substrate in each pair, and four different substrate removal patterns were observed. These

were: 1) concurrent removal accompanied by monophasic growth, 2) sequential removal accompanied by diphasic growth, 3) sequential removal accompanied by monophasic growth, and 4) concurrent removal accompanied by diphasic growth caused by inhibition of enzymes by metabolic intermediates. Of the 33 experiments in which he characterized the growth patterns, 20 were of type 1, 11 of type 2, 1 of type 3, and 1 of type 4. Since almost all of the experiments were conducted with a relatively low initial biological solids concentration, this was primarily a study of the repression of enzyme synthesis caused by various substrate combinations.

Grady (5) performed an extensive study on the metabolic control mechanisms operative in heterogeneous microbial populations which were able to degrade L-lysine. In all batch experiments, glucose retarded the removal of lysine due to metabolite repression of lysine-degrading enzymes. This effect was also exerted by excreted metabolic products of glucose degradation and was not relieved until the concentration of those products was reduced. Preformed enzymes were subject to only minor inhibition. Preinduction offered only a small degree of protection against the repression caused by glucose and the omission of ammonia nitrogen from the system did not overcome it. Fructose retarded lysine removal only slightly and caused no inhibition of enzyme activity. Ribose was found to produce a slight increase in the rate of lysine removal due to greater efficiency in the production of enzymes. Under all conditions studied in the continuous flow experiments (carbon or magnesium limited), glucose and fructose caused a significant degree of repression of synthesis of the enzymes for lysine degradation. The initial response to ribose addition was an increase

in the enzymatic capability for lysine removal, but once ribose degradation began there was a slight repression effect.

Chian and Mateles (34) operated a continuous flow reactor fed a mixture of glucose and butyric acid (1 gm/l each) along with a small amount of yeast extract. As the dilution rate was increased to about 0.3 hr^{-1}, increasing quantities of butyrate were present in the effluent. There was a discontinuity, however, and at slightly higher dilution rates (0.35 hr^{-1}) all of the butyrate was metabolized. At higher dilution rates, butyrate was again released into the effluent. It was not until the dilution rate reached 0.85 hr^{-1}, that glucose was first detected in the effluent, and at this time only about 4% of the incoming butyrate was being utilized. When a mixed feed of glucose and lactose was used, a similar pattern of preferential use of glucose was observed, but in this case there was no discontinuity in the release of lactose into the effluent.

The final series of articles dealing with substrate interactions in heterogeneous populations which will be considered are those of Stumm-Zollinger (2, 4). A number of criticisms pertaining to her work have already been given by Grady (5), and since this author is in complete agreement with him, not all of these criticisms will be repeated here.

Several experiments to determine the effect of glucose on galactose utilization were performed (2). When galactose-acclimated cells were exposed to galactose, the rate of substrate removal was 2.5 mg/l/minute. When the cells were exposed to galactose in the presence of glucose, the rate of galactose removal was 0.66 mg/l/minute. Hence glucose inhibited the activity of preformed galactose enzymes by about

75%. From another experiment, it was concluded that activation of galactose enzymes takes place within 50 minutes and that the presence of glucose does not repress their formation. However, this was concluded from an experiment in which the same time was required for the initiation of galactose removal in the galactose control as in the combined unit, and during this time the glucose was exhausted from the combined system. Therefore, the possibility that glucose did repress galactose enzyme formation certainly exists. When a low solids inoculum was used for experimentation, it was observed that no removal of galactose occurred during the 11 hours required for glucose removal. When all of these results are considered together, one serious question is raised. If galactose enzymes are produced in the presence of glucose within 50 minutes, and if they are only subject to 75% inhibition in the presence of glucose, why was no galactose removed from the combined growth system during the 11 hour period of glucose removal? It would seem that glucose did, indeed, repress the formation of a galactose-degrading enzyme system.

Additional experiments with glucose-galactose systems were again performed by Stumm-Zollinger (4). However, it appears that the legend given for Figure 3 applies to Figure 3a only, and that the galactose removal curves depicted in Figures 3b, 3c, 3d, and 3e are opposite to the legend. For example, Figure 3b (according to her legend) clearly shows that the rate of galactose elimination is enhanced in the presence of glucose. Yet she states that Figure 3b shows that the rate of galactose removal is reduced in the presence of glucose. Since Figure 3a was reproduced from previous work (2), the most obvious conclusion is that she changed symbols for the galactose systems

without realizing it. Hence the following paragraph will be stated under this assumption.

Because Stumm-Zollinger (4) was quite concerned over the loss of diversity of species obtained during acclimation to a particular compound, she isolated 50 bacteria in a nonselective manner from river water. Each inoculum was then cultured for several days on nutrient broth supplemented with glucose and galactose. The isolates were then recombined and used for experimentation. She concluded that ". . . the rate of galactose elimination is not affected by the presence of glucose in this mixed culture." This is then followed by the statement that "for unknown reasons, the onset of galactose elimination is delayed by 80 minutes in the presence of glucose." To illustrate that galactose removal was not affected by glucose, she found it necessary to ignore the first six data points which she obtained for the galactose concentrations in the combined system. Had the galactose removal curve been drawn in a manner somewhat related to all of the data points, it would be obvious that glucose did, indeed, inhibit galactose removal.

C. Substrate Removal Patterns in Pure Cultures

Numerous investigators have studied substrate interactions in pure culture systems operated under both batch and continuous flow conditions. However, only two reports, which are directly related to the results to be presented later, will be discussed here.

Gaudy et al. (35) studied a young culture of *E. coli* acclimated to sorbitol. Sorbitol removal in the combined unit (glucose plus sorbitol) was determined by subtracting glucose COD from total COD. When the cells were placed in the glucose-sorbitol medium, the substrate removal patterns indicated that glucose catabolites completely

repressed any further synthesis of sorbitol-degrading enzymes. The degree of inhibition of the preformed sorbitol enzyme system cannot be stated with certainty, but the high initial biological solids concentration employed (255 mg/l) would suggest that the inhibition was nearly complete. When a similar experiment was performed under non-proliferating conditions, there appeared to be no sorbitol removal during the course of glucose removal, and this was once again caused by the inhibition of the preformed sorbitol enzymes.

Tsay (36) recently completed a study utilizing two strains of E. coli and a strain of Achromobacter sp. exposed to various substrate combinations. While this author is in general agreement with most of her results, there are several points which merit discussion. A periodate reaction was used to measure sorbitol in the presence of glucose. The production of periodate-reactive intermediates from glucose was found to be insignificant when this was checked using young cell cultures. No further checks were made in the ageing experiments, and thus it was assumed that these intermediates were never produced. However, results to be presented later clearly show that increased ageing of a pure culture can lead to the production of periodate-reactive intermediates. With one strain of E. coli (presented in Chapter IV) it was found that the periodate-reactive intermediates went from none with young cells to nearly 40% (measured as sorbitol COD) of the added glucose COD with aged cells. Hence, the possibility of these intermediates must be considered when interpreting the results given for the ageing experiments.

Finally, this author must disagree with the extent of the reported counterflow of sorbitol. For example, Figure 5a shows that 175 mg/l of

"sorbitol" were released due to the presence of glucose. The initial optical density was 0.103 and this corresponded to a viable count of 5.3×10^8 (37). Assuming that the wet weight of each cell was 10^{-12} gm and that the moisture content was 80% (38) this would correspond to an initial biological solids concentration of 106 mg/l. The initial sorbitol concentration in the combined unit was 1,000 mg/l, and at the point of maximum "counterflow" the extracellular "sorbitol" concentration increased to 1,175 mg/l. Since this was more sorbitol than was present initially, it could only have come from unmetabolized sorbitol "carried over" in the inoculum. However, this would require the 106 mg/l of initial biological solids to have an internal storage of sorbitol sufficient to yield an exogenous concentration of 175 mg/l. This is impossible. A comparison of Figure 5a (sorbitol counterflow 175 mg/l at the time of glucose removal) with Figure 6a (sorbitol counterflow 20 mg/l at the time of glucose removal) with Figure 25a (sorbitol removal 165 mg/l at the time of glucose removal) for cultures of Achromobacter sp. subjected to the same experimental and pre-experimental conditions clearly illustrates that experimental error played a more important role than was considered in the analysis of results. This is further illustrated in Figure 13 in which sorbitol removal during the first hour of experimentation varied from 100 mg/l (initial glucose concentration 1,000 mg/l) to a counterflow of 55 mg/l (initial glucose concentration 200 mg/l). Therefore, it appears that the extent of sorbitol counterflow reported must be viewed with caution.

This rather extended discussion of the results reported by Tsay (36) should not be construed as invalidating the work which she performed or the conclusions (with the possible exception of the extent of

sorbitol counterflow) which she made. Indeed, it was shown that increased ageing of E. coli or Achromobacter sp. did not lead to a decrease in the extent of enzyme inhibition found with young cell cultures of these organisms. Substrate interactions arising as a result of the combination of numerous different compounds were also shown. In addition, a means of utilizing substrate removal curves for the estimation of the metabolic control mechanisms which were operative during the course of substrate interactions was also given.

The preceding review of the literature illustrates that substrate interactions can readily be understood on the basis of the metabolic control mechanisms involved. Sequential substrate removal can arise from catabolite repression or catabolite inhibition of preformed enzymes or interference with transport or from other causes or from any combination of several mechanisms. Merely looking for the presence or absence of sequential substrate removal and failing to consider the metabolic control mechanisms which were involved has been shown to be an inappropriate way to attempt to evaluate the environmental factors which may affect substrate interactions.

CHAPTER III

MATERIALS AND METHODS

A. Microbial Populations

1. Heterogeneous Populations

The heterogeneous populations which were utilized in this study were developed from an inoculum of primary clarifier effluent from the municipal sewage treatment plant at Stillwater, Oklahoma.

2. Pure Cultures

The pure cultures employed in this study were obtained from the Department of Microbiology at Oklahoma State University. The organisms utilized were as follows.

Escherichia coli, strain K-12

Escherichia coli, strain 45

Serratia marcescens

An organism tentatively identified as *Escherichia intermedia*. This was the same strain as that studied by Bustamante (39).

An unidentified organism which will hereafter be called Blue organism. This was also the same strain as that studied by Bustamante (39).

B. Daily Growth Conditions

1. Heterogeneous Populations

a. Sorbitol-Acclimated Cells. Seventy-five ml of sewage were

added to a reactor containing (total volume 3 liters) the following medium: sorbitol, 2,000 mg/l; $(NH_4)_2 SO_4$, 1,000 mg/l; $MgSO_4 \cdot 7H_2O$, 200 mg/l; $MnSO_4 \cdot H_2O$, 20 mg/l; $FeCl_3 \cdot 6H_2O$, 1 mg/l; $CaCl_2$, 15 mg/l; 1 M potassium phosphate buffer (pH 7.0), 20 ml/l; and tap water 100 ml/l. This medium will hereafter be referred to as the standard medium. Filtered air was provided through a single diffuser at a rate of 5,000 ml per minute. Temperature control was not provided; room temperature was 22 ± 2° C.

When a dense microbial population had developed, the culture was defined as being 1 day old, and it was then subjected to the following mode of operation. One liter of mixed liquor was withdrawn, and the remaining liquor was allowed to settle quiescently for 1 hour. An additional 1 liter of "supernatant" was then withdrawn. Aeration was then restarted, and concentrated standard medium plus distilled water were added to yield the volume and concentrations previously given. This feeding procedure was repeated daily, and each succeeding day of batch operation was defined as increasing the cell age by one day. It was from this reactor that the cells which were used for periodic experimentation were taken. Observation of the settling characteristics and color were made and recorded daily.

Because the term "cell age" presents various connotations to different researchers, it is emphasized that the definition of cell age which was presented in the preceding paragraph is merely an operational definition. Whenever a biological population is aged by batch operation, there is no direct relationship between chronological time and physiological time, and such a direct relationship is not implied by the use of the term "cell age." Each day of batch operation was

defined as increasing the cell age by one day merely because this was felt to be the simplest method of describing the chronological age of the system.

b. Galactose-Acclimated Cells. The galactose-acclimated cells were grown in a manner identical to that given for the sorbitol-acclimated cells except that the standard medium contained galactose as the sole carbon source at a concentration of 2,000 mg/l. In addition to daily observation of color and settling characteristics, spread plates on Difco nutrient agar were also made several times during the period of operation of the unit. The plates were incubated at 25° C for 48 hours and were then stored at 2° C to permit further differentiation of the colonies which had developed.

2. Pure Cultures

a. Apparatus. The reactor and other equipment which were utilized to perform one particular batch ageing experiment with a pure culture of *E. coli* are shown in Figure 1. Since the actual "set-up" varied considerably from experiment to experiment, only the general features of the equipment will be described. The 4 liter reactor (Sargent #34530) was fitted with a ground glass top which contained 4 ground glass joints. In all cases, sterile air, which had been passed through two glass wool filters, was supplied to a diffuser through one joint and escaped through another. The actual use of the other 2 joints depended upon the number of feed reservoirs and upon the manner of withdrawing cells. In all cases, the effluent from the reactor was passed through a "free-fall" cylinder before it was exposed to the atmosphere. Connections necessary to add feed or to withdraw cells were never "broken" (except where a large enough feed reservoir could not be used)

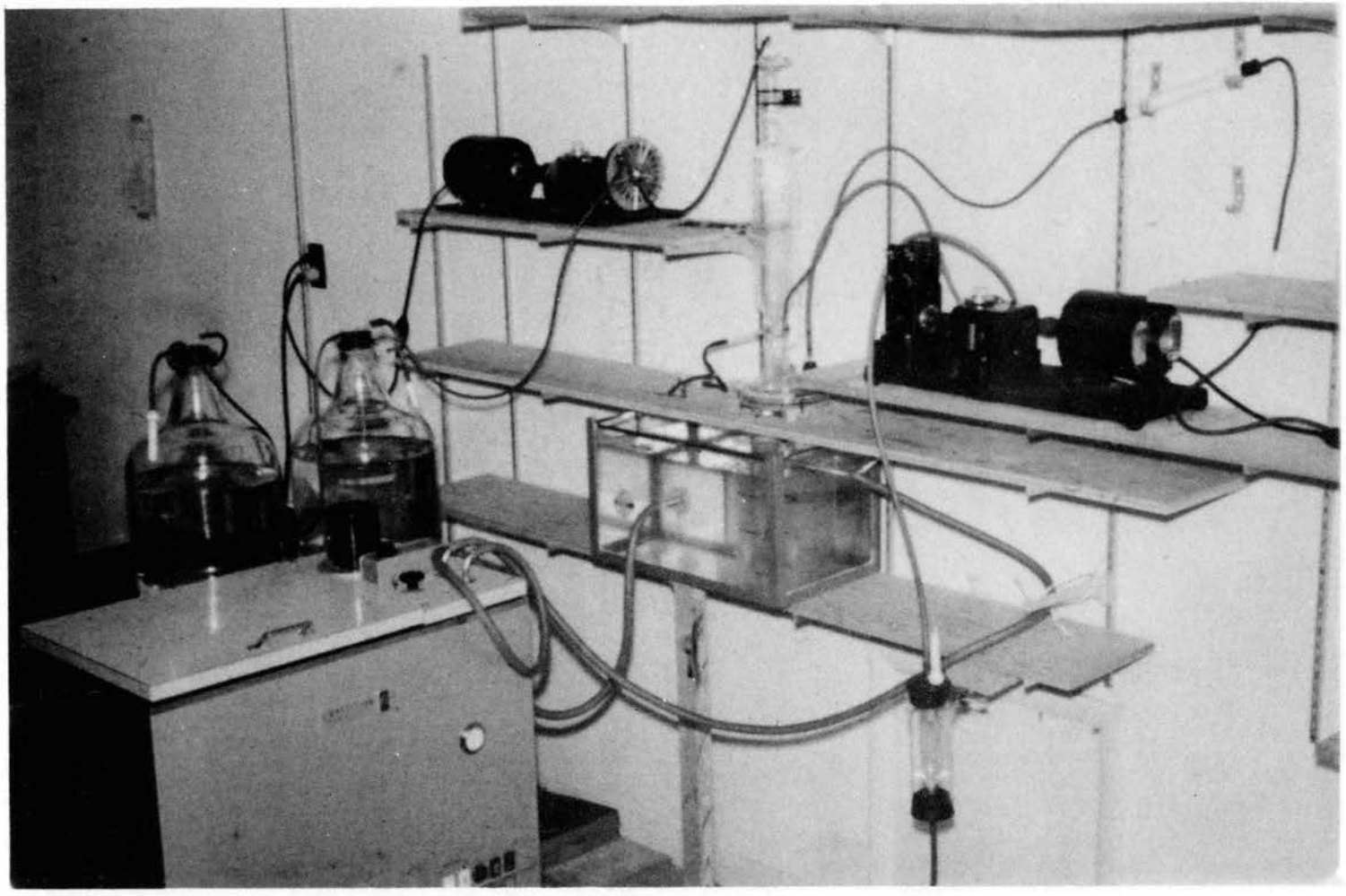


Figure 1. Photograph of equipment used to age a pure culture of Escherichia coli.

during the course of an experiment. Prior to assembly, all fittings and joints were carefully wrapped and the components of the reactor and the feed reservoir(s) were sterilized at 15 psi and 121° C for a period of 2 hours. Once the reactor was assembled and inoculated, there was little chance of contamination.

b. Ageing of Blue Organism. The ageing of a pure culture of Blue organism in essentially the same apparatus as shown in Figure 1 was performed with the standard medium using the daily operating procedure previously described for the heterogeneous populations with the exception that feeding occurred only once every 48 hours. The unit was fed for the first time 48 hours after it had been inoculated, and the culture was defined as being 2 days old at that time. Each 24 hour period, thereafter, was defined as increasing the age by one day. Aeration was supplied at the rate of 5,000 ml/minute, and no temperature control was employed (room temperature was 22 ± 2° C). Spread plates (Difco nutrient agar) were made each time the unit was fed. Since this organism has no distinct morphological characteristics when grown on an agar plate, any colony which appeared "different" from the rest was used to inoculate a sterile 250 ml flask containing 50 ml of standard medium, and this was then aerated on a reciprocal shaker. The formation of a blue color due to excreted metabolic products was accepted as an indication of purity. Microscopic examinations of India ink slides were also frequently made. At no time was any contamination detected.

c. Growth and Ageing of the Other Pure Cultures. Various methods were employed for the growth or ageing of the other pure cultures studied. Therefore, the actual method of ageing or growth will be

described when the results obtained with these organisms are presented. In all cases the ageing was performed under aerobic conditions. Whenever Escherichia coli or Escherichia intermedia was studied, spread plates on EMB agar were always made to check for possible contamination. The purity of cultures of Serratia marcescens was checked by incubation of spread plates on Difco nutrient agar at 25° C; development of red colonies was taken as an indication of purity. At no time was an experiment performed with a pure culture without spread plates being made and examined for contamination.

C. Experimental Protocol

Regardless of the origin of the cells to be used for experimentation, i.e., heterogeneous or pure cultures, the experimental protocol was the same. However, one point should be emphasized. Although the pure culture cells were not handled aseptically once they had been harvested for experimentation, the experimental reactors in which they had been grown or aged were always operated under aseptic conditions. Since fairly high solids concentrations were used and experiments of relatively short duration were performed, it was felt that there was no need to protect against the possibility of minute contamination from outside sources once the cells had been harvested.

Immediately prior to performing an experiment, the cells were collected either from that portion of the mixed liquor which was wasted each time a batch unit was fed or from a reactor containing cells which had been expressly grown for the particular experiment to be performed. The cells were then centrifuged for 15 to 20 minutes; the supernatant was discarded, and the cells were resuspended in 0.05 M potassium phosphate buffer. The cells were then used to seed reactors of 1 liter

volume which contained the following:

a. Glucose control. This reactor contained glucose as the sole carbon source.

b. Sorbitol or galactose control. This reactor contained sorbitol or galactose as the sole carbon source.

c. Combined unit. This reactor contained glucose and sorbitol or galactose as the carbon sources.

d. Shocked unit. This unit initially contained sorbitol or galactose as the sole carbon source. After growth was underway for some time, glucose was added as an instantaneous shock load. A shocked unit was run for most but not all experiments.

The inorganic constituents of the experimental reactors were always the same (except for one experiment described later) as those present during the growth or ageing of the cells to be used for experimentation. In all cases, this provided a ratio of inorganics to carbon source in excess of that found in the standard medium. Sterile carbon sources were always added to the reactors, and the air supplied to the diffusers was first passed through glass wool filters. All glassware was carefully cleaned and heat dried prior to use. These precautions minimized opportunities for contamination. The temperature during experimentation was always the same as that which was used to grow or age the cells.

At the start of each experiment, equal portions of the cells were added to the experimental reactors and samples were then immediately removed and centrifuged. For many experiments samples were also withdrawn for optical density readings. The centrifuged samples were then passed through 0.45 μ membrane filters. A portion of the filtrate was

immediately added to a COD flask containing potassium dichromate. The remaining filtrate was frozen for later analysis. This sampling procedure was repeated frequently during the course of each experiment.

D. Analytical Techniques

1. Biological Solids

For some experiments, biological solids were determined gravimetrically by filtration through 0.45 μ membrane filters as described in Standard Methods (40). Biological solids were also determined in some cases by optical density readings at 540 mμ with a Bausch and Lomb Spectronic 20. Optical density readings on samples diluted 3 parts of sample to 8 parts distilled water were also frequently made. For some experiments, all three methods of solids determination were utilized. For any given experiment, the biological solids concentrations have been presented either as optical density readings (in which case the designation "Optical Density of Biological Solids" has been employed) or as gravimetric measurements.

2. Chemical Oxygen Demand

The COD test was performed exactly as described in Standard Methods (40).

3. Glucose

In all cases, the glucose concentrations were measured by the enzymatic Glucostat test; method 1-A was used for all analysis (41). Glucose standards were run each time the test was performed, and the results are expressed as equivalent COD values.

4. Galactose

Galactose concentrations were determined by the enzymatic Galactostat test according to the procedure given by the Worthington Biochemical Corporation (42). Galactose standards were run each time the test was performed, and the results are given as equivalent COD values.

5. Sorbitol

The procedure for the determination of sorbitol was a slight modification of that previously presented by Komolrit (43). Proof that the method herein reported yields results identical to those obtained by the method of Komolrit will be presented in Chapter IV. When the procedure herein reported was checked using sorbitol concentrations varying from 50 to 250 µg, the linear correlation coefficient between sorbitol concentration and optical density was found to be 0.9997. The procedure is as follows.

a. Place an aliquot containing 50-250 µg of sorbitol in a test tube.

b. Make up the volume to 1.0 ml with distilled water.

c. Add 0.1 ml of 10 N H_2SO_4.

d. Add 3 ml of 0.0167 M (0.9583 gm/250 ml) periodic acid and mix well.

e. Allow the reaction to proceed for exactly 10 minutes, and then add 3 ml of 0.167 M (5.4167 gm/250 ml) sodium arsenite and shake vigorously for 2-3 seconds.

f. Wait 10 minutes.

g. Add 2.9 ml of distilled water to make up the total volume to 10 ml.

h. Take 1 ml of the above solution and place in a test tube.

i. Add 10 ml of chromotropic acid solution.

j. Heat for 30 minutes in a boiling water bath in the dark.

k. Cool to room temperature and read optical density at a wavelength of 570 mμ.

The chromotropic acid reagent was prepared as described by Komolrit (43). Duplicate blanks and standards were run with each analysis, and a correction for glucose was applied as previously described (43). All sorbitol concentrations are expressed as equivalent COD values.

CHAPTER IV

RESULTS

A. Heterogeneous Populations

1. Galactose-Acclimated Cells

The results which were obtained using the batch heterogeneous population acclimated to galactose are presented in Figures 2-9. In all cases, a description of the characteristics of the batch unit from which the cells were taken for experimentation (a portion of the wasted mixed liquor) and a description of the colonies which developed on the spread plates which were made are also presented. The glucose control units in Figures 3, 4 and 5 and all of the units presented in Figures 6, 7 and 8 contain a curve which has been designated as apparent sorbitol COD. These units were the only ones which were examined for periodate-reactive compounds, and the absence of these curves in the remainder of the experiments is due to the fact that a periodate analysis was not performed. These curves represent periodate-reactive intermediates excreted into the medium during the metabolism of glucose and/or galactose. The periodate analysis was performed on 1 ml samples of filtrate exactly as described by the method given for sorbitol analysis in Chapter III. The reason, and it is emphasized that this is the only reason, that it has been designated as "apparent sorbitol COD" is because sorbitol was used as the standard in the preparation of the standard curve relating optical density to concentration. It should be

Figure 2. Metabolic response of a 1 day old culture of galactose-acclimated cells.

The microbial population in the batch unit had not completely developed at the time of this experiment; hence, the cells were harvested for experimentation while actively growing. An examination of 12 spread plates revealed that about 80% of the population consisted of two different species. In all, about 6 different species of organisms were detected.

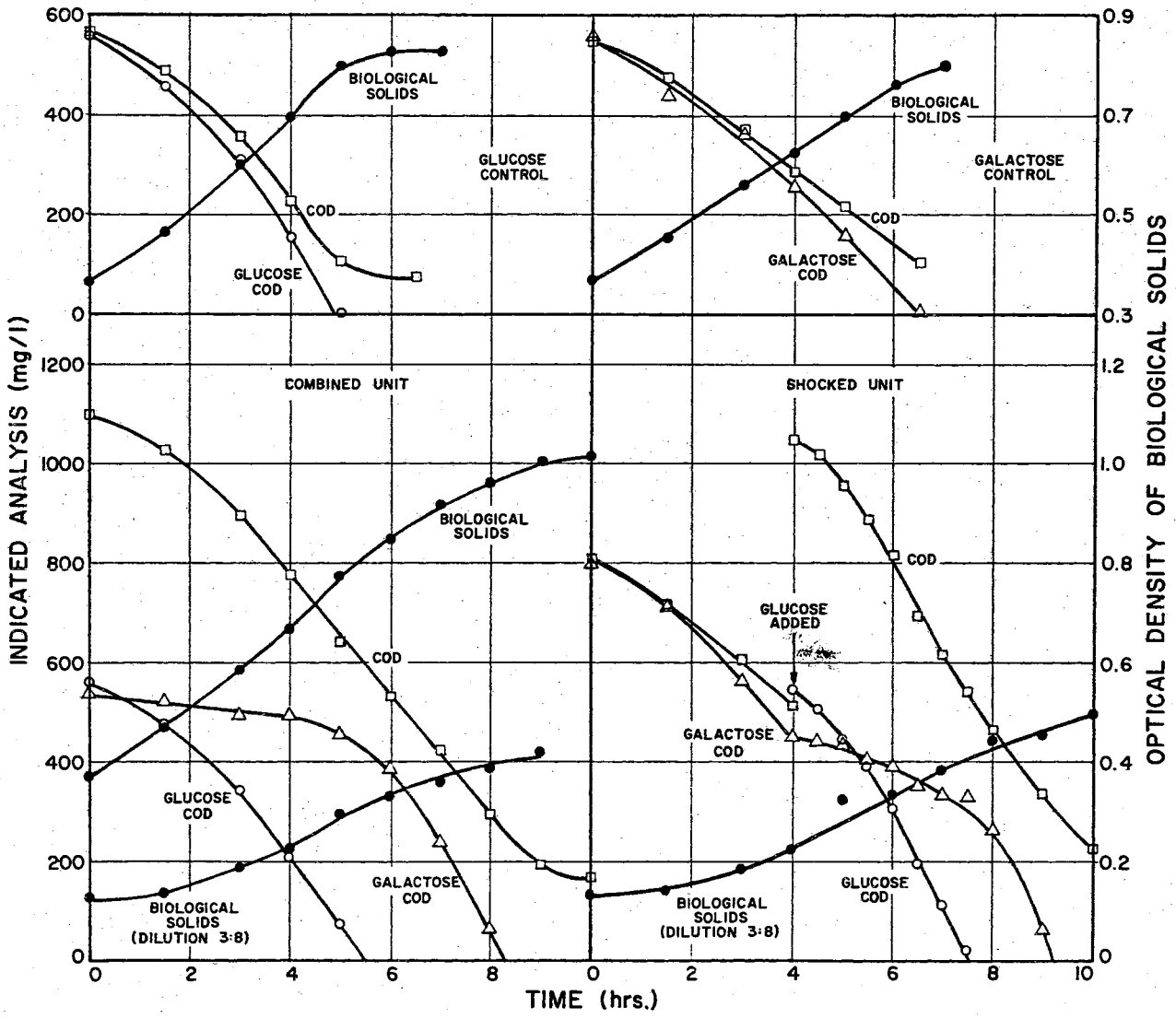

INDICATED ANALYSIS (mg/l)
OPTICAL DENSITY OF BIOLOGICAL SOLIDS
TIME (hrs.)
GLUCOSE CONTROL
BIOLOGICAL SOLIDS
COD
GLUCOSE COD
GALACTOSE CONTROL
GALACTOSE COD
COMBINED UNIT
SHOCKED UNIT
GLUCOSE ADDED
BIOLOGICAL SOLIDS (DILUTION 3:8)

Figure 3. Metabolic response of an 8 day old culture of galactose-acclimated cells.

By the time this experiment was performed the cells in the unit had started to flocculate; however, the supernatant was still not clear. The color of the mixed liquor was yellow-green. An examination of 2 spread plates revealed that about 50% of the population consisted of a single species not detected in the previous experiment. The other predominating organisms appeared to be the same as the 2 predominating species of the previous experiment. In all, about 6-7 different species were present.

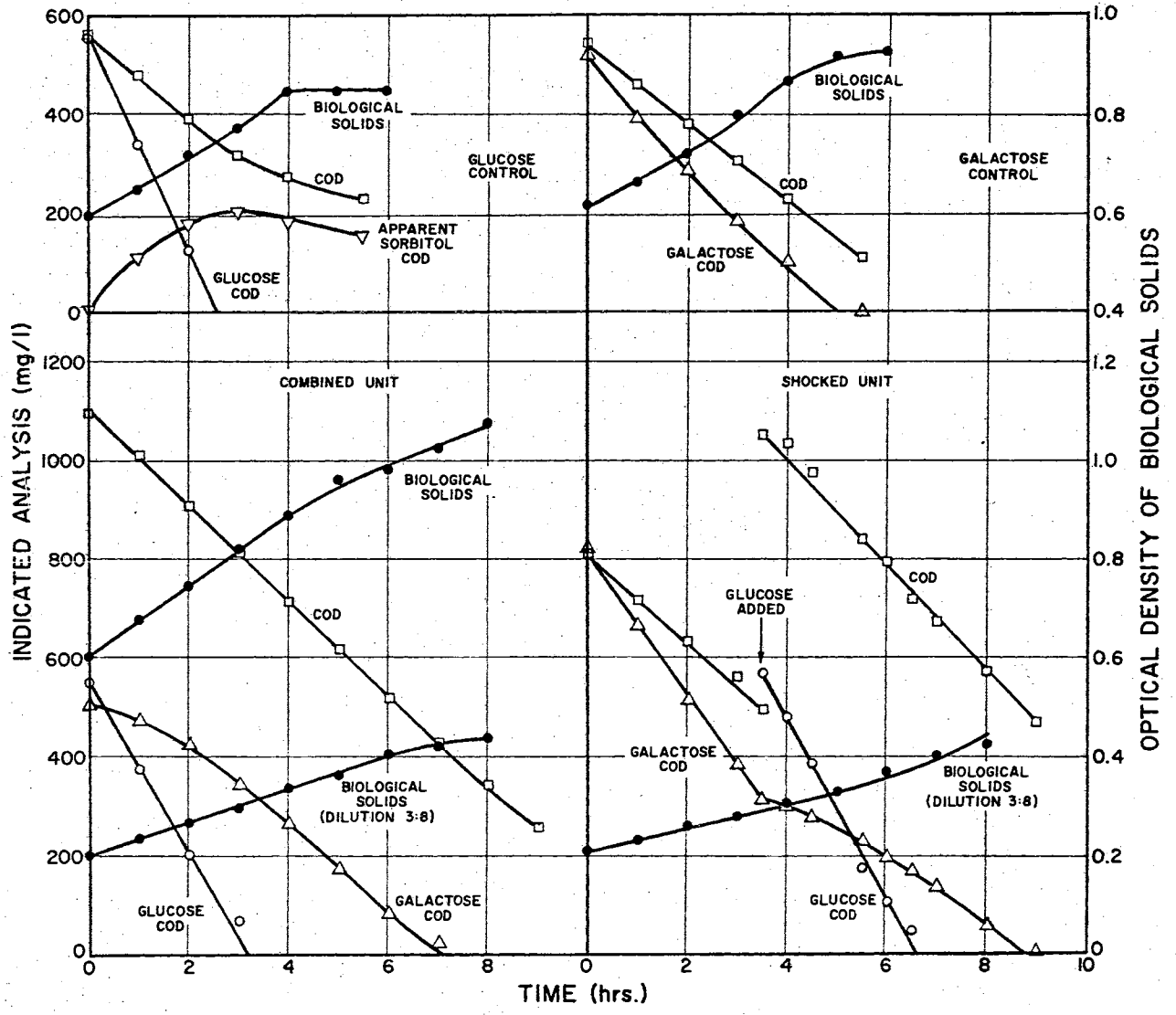

INDICATED ANALYSIS (mg/l)
OPTICAL DENSITY OF BIOLOGICAL SOLIDS
TIME (hrs.)
GLUCOSE CONTROL
BIOLOGICAL SOLIDS
COD
APPARENT SORBITOL COD
GLUCOSE COD
GALACTOSE CONTROL
GALACTOSE COD
COMBINED UNIT
BIOLOGICAL SOLIDS (DILUTION 3:8)
SHOCKED UNIT
GLUCOSE ADDED

Figure 4. Metabolic response of a 15 day old culture of galactose-acclimated cells.

The cells in the batch unit were flocculated and the mixed liquor was green-brown in color. An analysis of 4 spread plates revealed that about 9 different species were present, and there was no one species which could be considered predominant. A very few colonies identical in appearance to those which predominated in the previous experiment were present.

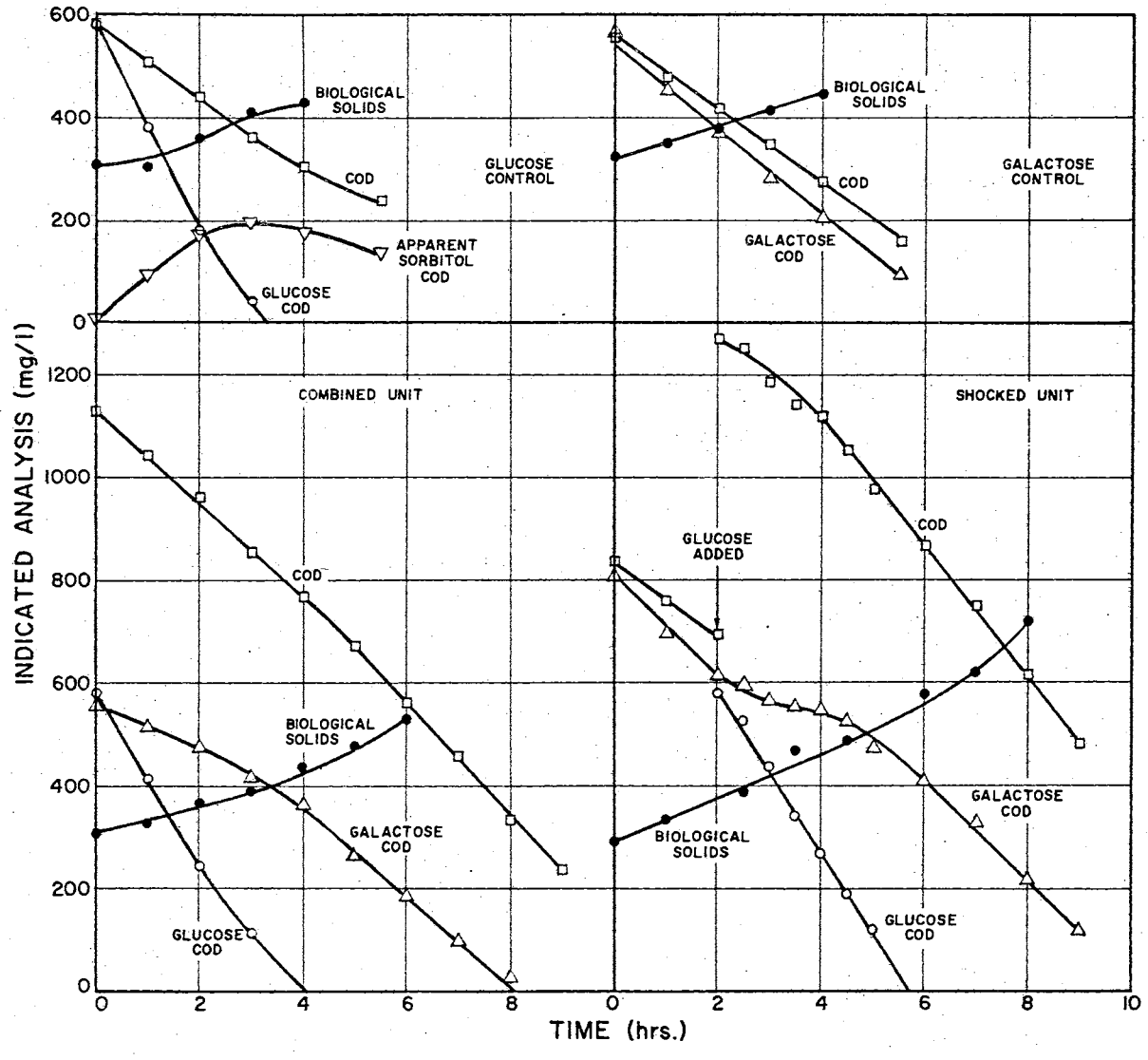

INDICATED ANALYSIS (mg/l)
TIME (hrs.)
GLUCOSE CONTROL
BIOLOGICAL SOLIDS
COD
APPARENT SORBITOL COD
GLUCOSE COD
GALACTOSE CONTROL
GALACTOSE COD
COMBINED UNIT
SHOCKED UNIT
GLUCOSE ADDED

Figure 5. Metabolic response of a 21 day old culture of galactose-acclimated cells.

The appearance and settling characteristics of the mixed liquor were essentially the same as in the previous experiment. An examination of 3 spread plates revealed that one of the species which was present in the preceding experiment comprised about 50% of the population. The remaining population consisted of an approximately equal distribution of 4 different species.

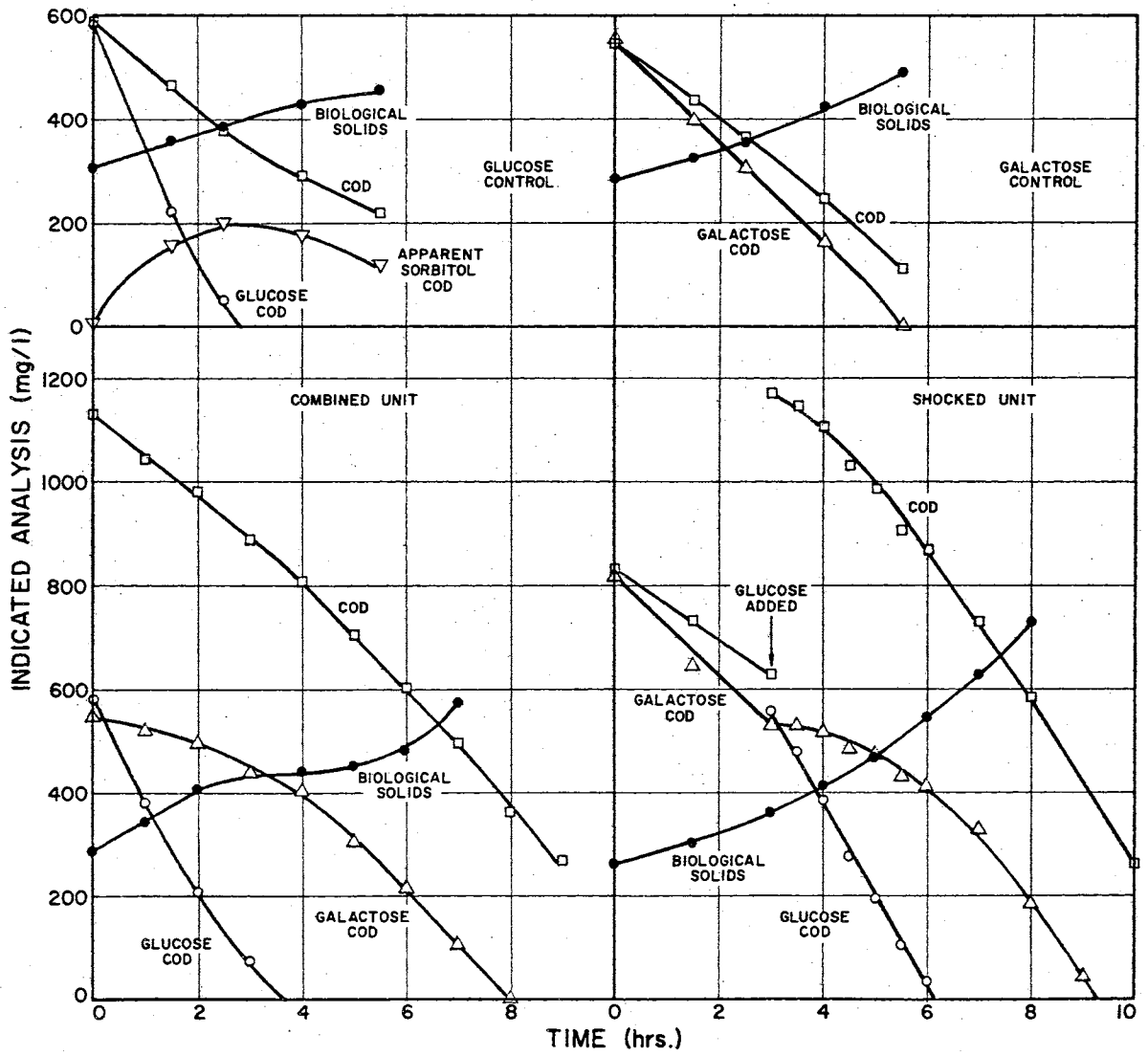

INDICATED ANALYSIS (mg/l)
TIME (hrs.)
GLUCOSE CONTROL
BIOLOGICAL SOLIDS
COD
APPARENT SORBITOL COD
GLUCOSE COD
GALACTOSE CONTROL
GALACTOSE COD
COMBINED UNIT
SHOCKED UNIT
GLUCOSE ADDED

Figure 6. Metabolic response of a 28 day old culture of galactose-acclimated cells.

At the time this experiment was performed, the sludge had started to bulk, and the mixed liquor was dark green in appearance. An examination of the colony characteristics on 4 spread plates revealed that the organism which predominated in the previous experiment accounted for only about 25% of the total population. The remaining organisms consisted of a relatively even distribution of 4-5 different species.

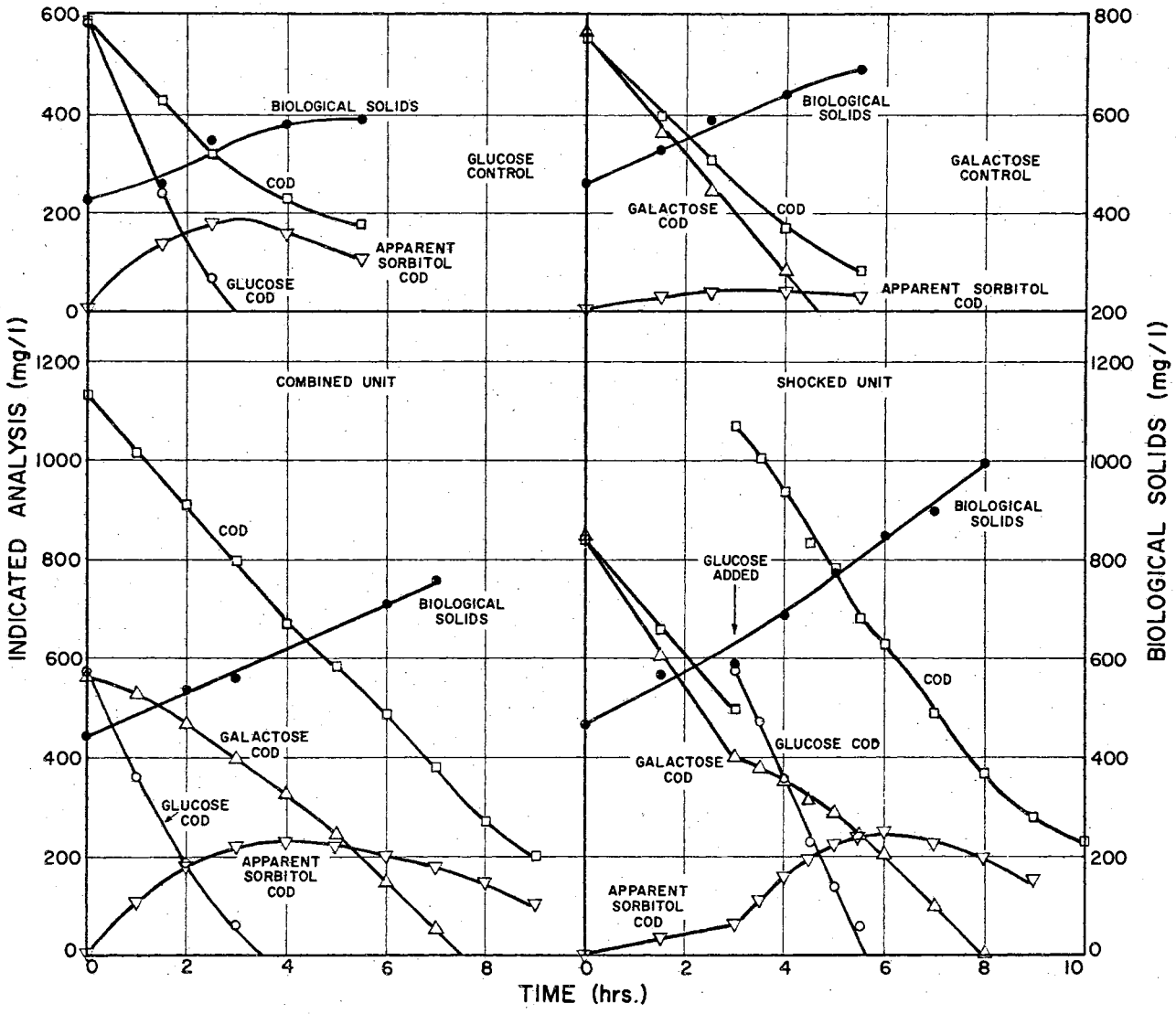

INDICATED ANALYSIS (mg/l)
BIOLOGICAL SOLIDS (mg/l)
TIME (hrs.)
GLUCOSE CONTROL
BIOLOGICAL SOLIDS
COD
APPARENT SORBITOL COD
GLUCOSE COD
GALACTOSE CONTROL
GALACTOSE COD
COMBINED UNIT
SHOCKED UNIT
GLUCOSE ADDED

Figure 7. Metabolic response of a 35 day old culture of galactose-acclimated cells.

When the culture was 31 days old it was very badly bulked and virtually no settling occurred. At the time of this experiment, however, the bulking was greatly reduced and settling had almost returned to normal. The color of the mixed liquor was still dark green. An examination of 4 spread plates revealed that about 75% of the population consisted of a single species. The appearance of the colonies of this species was identical to that of the predominating species in the 8 day old culture. The remainder of the population consisted of 5-6 different organisms.

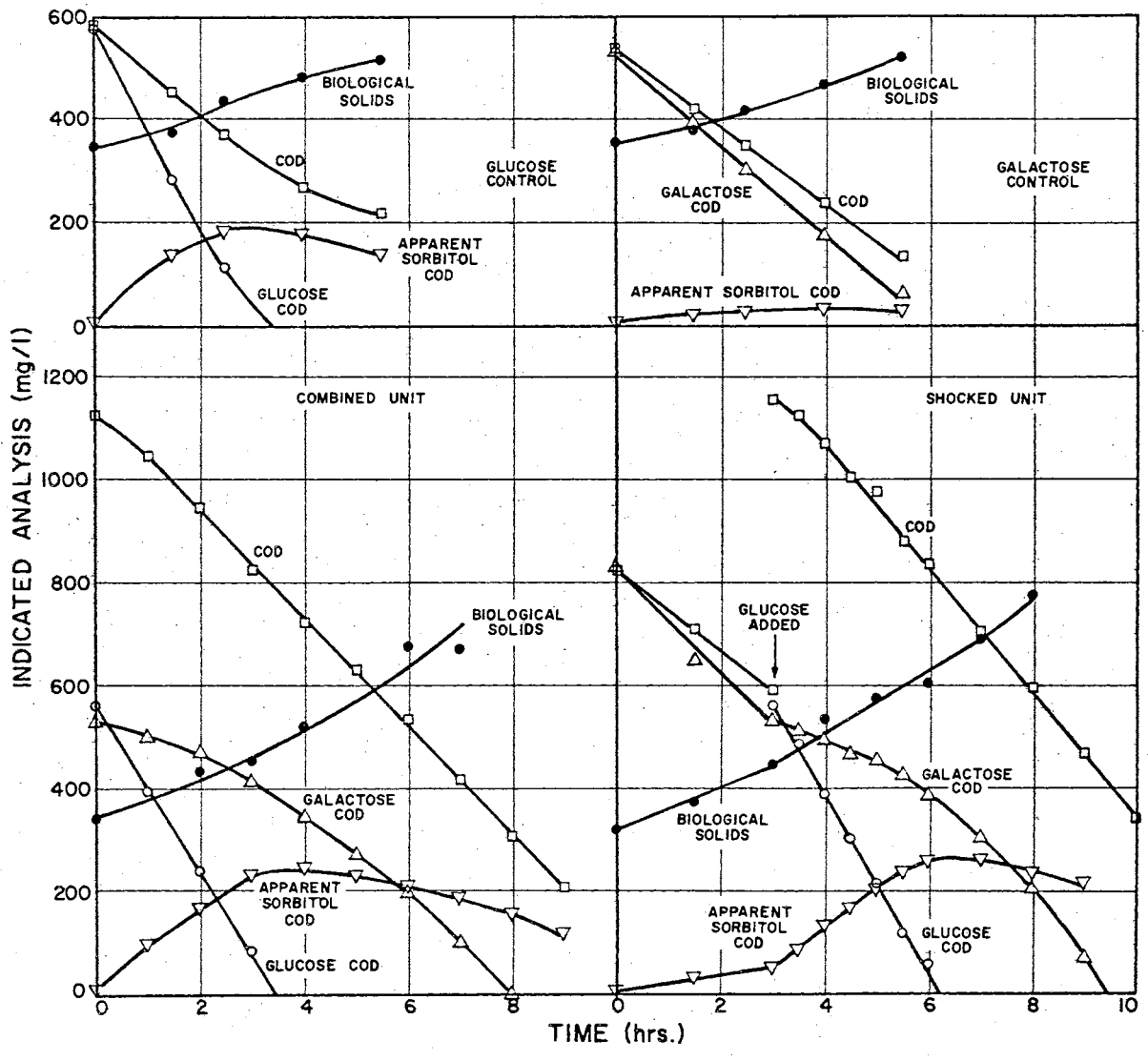
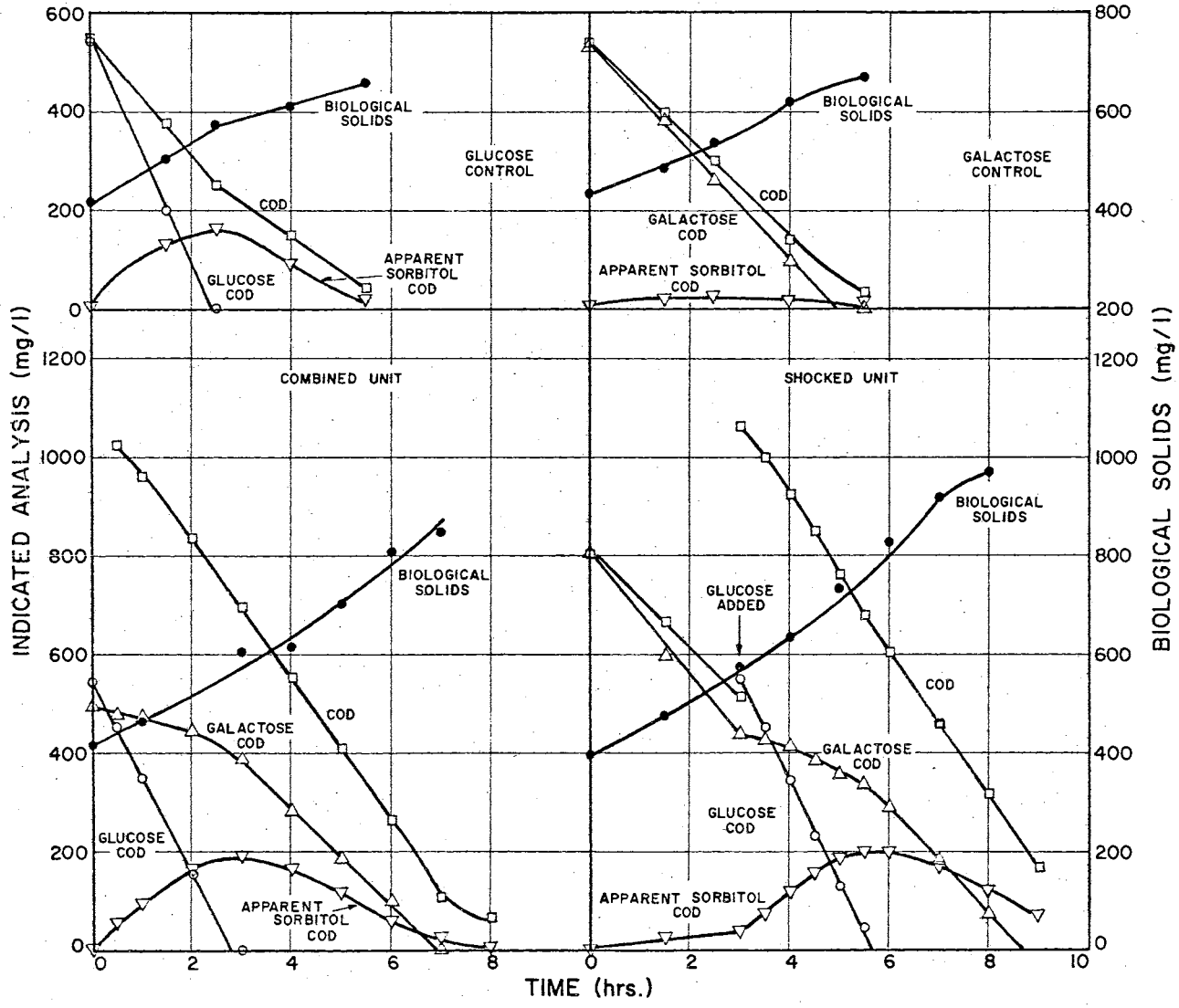

INDICATED ANALYSIS (mg/l)
TIME (hrs.)
GLUCOSE CONTROL
BIOLOGICAL SOLIDS
COD
APPARENT SORBITOL COD
GLUCOSE COD
GALACTOSE CONTROL
GALACTOSE COD
COMBINED UNIT
SHOCKED UNIT
GLUCOSE ADDED

Figure 8. Metabolic response of a 42 day old culture of galactose-acclimated cells.

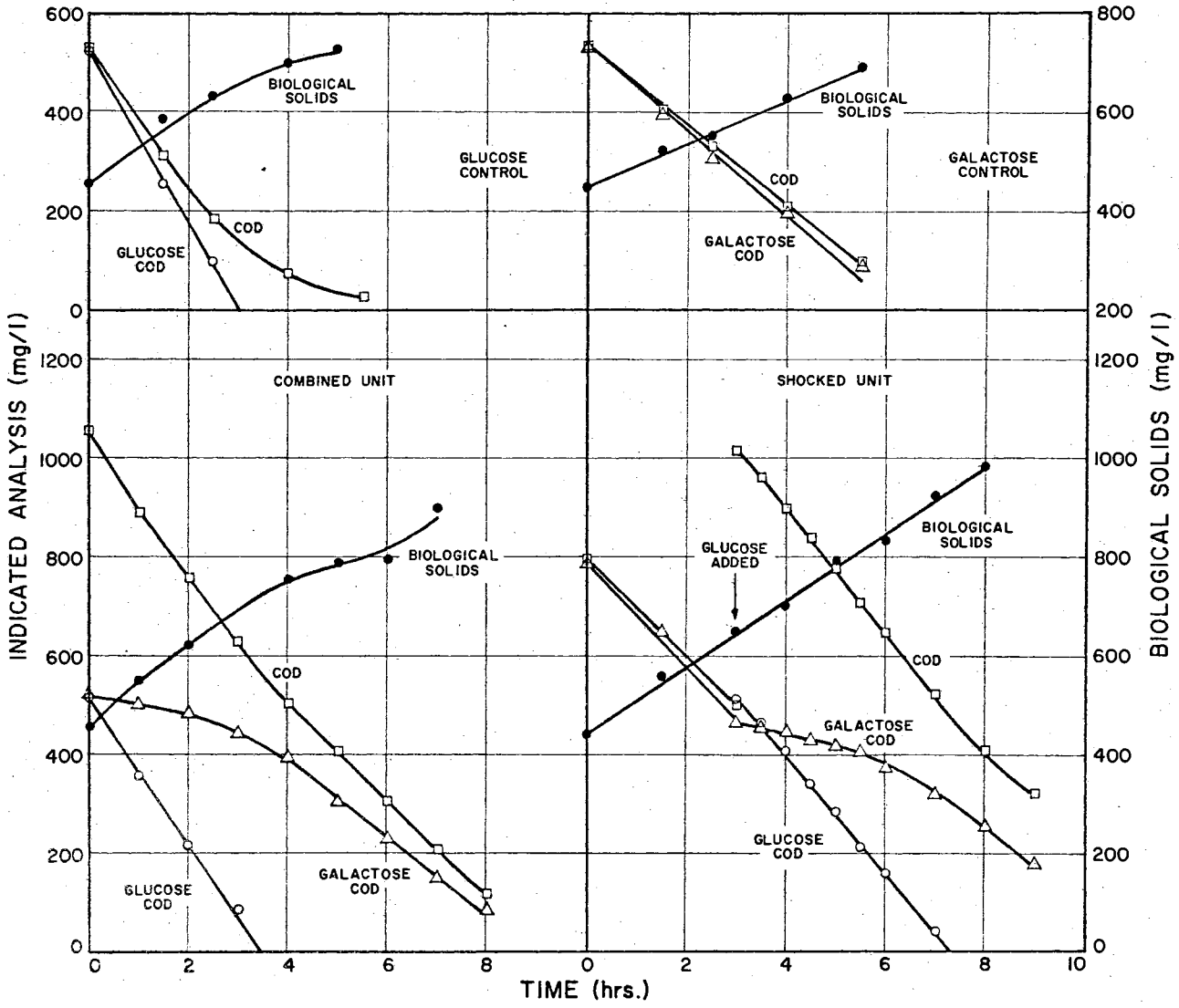
At the time this experiment was performed, the settling characteristics were very good, and the color of the mixed liquor was green-brown. The organism which predominated in the previous experiment still represented about 50% of the total population. The remainder of the population consisted of an approximately equal distribution of about 5 different types of organisms.


INDICATED ANALYSIS (mg/l)
BIOLOGICAL SOLIDS (mg/l)
TIME (hrs.)
GLUCOSE CONTROL
BIOLOGICAL SOLIDS
COD
GLUCOSE COD
APPARENT SORBITOL COD
GALACTOSE CONTROL
GALACTOSE COD
COMBINED UNIT
SHOCKED UNIT
GLUCOSE ADDED

cc

Figure 9. Metabolic response of a 48 day old culture of galactose-acclimated cells.

By the time this experiment was performed the color of the mixed liquor had changed to yellow. Settleability was still good. An examination of 3 spread plates revealed that a minimum of 9 different species were present, and there was no species which could be considered predominant.


GLUCOSE CONTROL
GALACTOSE CONTROL
COMBINED UNIT
SHOCKED UNIT
BIOLOGICAL SOLIDS
COD
GLUCOSE COD
GALACTOSE COD
GLUCOSE ADDED
INDICATED ANALYSIS (mg/l)
BIOLOGICAL SOLIDS (mg/l)
TIME (hrs.)

recalled that periodate selectively cleaves a molecule between adjacent carbons which have any combination of hydroxyls, aldehydes, ketones, or primary amine groups, and, therefore, there is no reason to suspect that these intermediates were actually sorbitol. The necessity for performing this analysis will become apparent when the results obtained from the heterogeneous population acclimated to sorbitol are presented, and, hence, a discussion of these curves will be deferred until that time.

An examination of the results presented in Figures 2-9 reveals several characteristics which were common to most or all experiments. In every case, the glucose was removed from the glucose control prior to the time required for galactose removal in the galactose control. The observation that there was never any lag in glucose removal strongly suggests that the cells possessed constitutive enzymes for glucose. Intermediates were always produced during the catabolism of glucose, and large quantities of these compounds were produced in the glucose controls represented in Figures 3-8. In every experiment, the addition of glucose as an instantaneous shock load resulted in the inhibition of galactose removal. This inhibition could have resulted from catabolite inhibition and/or transport interference. Galactose removal in each of the control units represented in Figures 3-9 was essentially linear. This indicates that the galactose was being removed by preformed enzymes and that no additional synthesis of a galactose degrading enzyme system occurred. Therefore, it is logical to conclude that there was also no synthesis of the galactose enzymes occurring in the combined unit during the course of glucose removal, and, hence, the mode of galactose removal was apparently only the

result of the inhibition of the preformed enzymes necessary for galactose removal. The magnitude of this inhibition can be discerned simply by comparing the galactose removal curves in the combined units with the corresponding curves in the control units.

It is only in the galactose control unit represented in Figure 2 that galactose removal occurred at a continually increasing rate. This indicates that there was a continual increase in the content of the enzymes required for galactose utilization. Therefore, the difference between the galactose removal curves in this unit and the combined unit of Figure 2 cannot be attributed only to inhibition of preformed enzymes because in addition to whatever enzyme inhibition occurred in the combined unit, the nature of the galactose removal curve suggests that there was also a complete or very nearly complete repression of synthesis of the galactose enzymes during the course of glucose removal.

The results which were obtained from each of the combined units are presented in Table I. In this table, the rates of galactose removal in the combined unit are compared with the rates of galactose removal in the galactose control for various times after the start of the experiment. The ratio of the galactose removal rate in the combined unit to the rate in the control unit has been calculated for each time period. The rates given for the data of Figure 2 represent both the effect of inhibition and repression. Since there apparently was no synthesis of the galactose enzymes during glucose removal, the rates for the remainder of the experiments represent only the inhibition of the preformed enzymes necessary for galactose utilization. In each case, the inhibition was strongest during the early stages of the experiment, and as increasing quantities of glucose were removed the rates of galactose

TABLE I

COMPARISON OF THE GALACTOSE REMOVAL RATE IN THE COMBINED UNIT WITH THE CORRESPONDING RATE IN THE GALACTOSE CONTROL

Fig. No.	Age days	Time hrs.	Glucose in comb. unit mg/l	Galactose control rate mg/l/hr.	Combined unit rate mg/l/hr.	Ratio of rate in comb. to control
		0.5	535	50	12	.240
		1.5	476	70	12	.172
2	1	2.5	391	82	12	.146
		3.5	277	86	12	.140
		4.5	140	95	31	.326
		0.5	463	115	28	.244
3	8	1.5	289	112	55	.491
		2.5	118	102	73	.715
		3.5	0	97	85	.876
		0.5	494	82	41	.500
4	15	1.5	325	82	41	.500
		2.5	172	82	51	.610
		3.5	57	82	66	.805
		0.5	472	96	20	.208
5	21	1.5	288	96	32	.333
		2.5	129	96	43	.448
		3.5	13	96	57	.594
		0.5	462	119	33	.277
6	28	1.5	267	119	60	.504
		2.5	109	119	72	.605
		3.5	0	119	72	.605
		0.5	483	86	27	.314
7	35	1.5	318	86	33	.384
		2.5	157	86	52	.605
		3.5	0	86	67	.779
		0.5	447	108	24	.222
8	42	1.5	253	108	29	.268
		2.5	61	108	57	.528
		3.5	0	108	95	.880
		0.5	438	86	17	.198
9	48	1.5	291	86	19	.221
		2.5	143	86	36	.419
		3.5	0	86	56	.651

removal in the combined units increased.

The response of each of the shocked units to the instantaneous addition of glucose is summarized in Table II. The rates of galactose removal immediately prior to the addition of glucose are given, and they are compared with the rates of galactose removal for various times after the addition of glucose. The ratios of the various rates after the addition of the shock are also shown. It can again be seen that in all cases glucose inhibited the removal of galactose, and once again the trend was toward less inhibition as increasing quantities of glucose were consumed.

The data presented in Table III provide a more direct means of making an "overall" comparison of the results obtained from each experiment. Although there was a small variation in the initial glucose and galactose concentrations from experiment to experiment, these initial concentrations were sufficiently close so that the method of calculation chosen does provide for a meaningful comparison. Columns 4 and 5 refer to the time given in column 3. The data in column 7 refer to the time given in column 6. A glucose concentration of 100 mg/l was chosen as the "reference point" merely for convenience; the selection of this value made it possible to accurately determine the times given and still provide for removal of over 80% of the glucose. The results presented in columns 10 and 11 are both indicative of the extent of interference of galactose removal in the presence of glucose. It is obvious from these results and from those presented in Tables I and II that increased cell age had no apparent effect upon the degree of inhibition encountered. In fact, the 42 and 48 day old cells were subject to about the greatest degree of inhibition found. The data

TABLE II

COMPARISON OF THE GALACTOSE REMOVAL RATE IN THE SHOCKED UNIT WITH THE RATE IMMEDIATELY PRIOR TO THE SHOCK

Fig. No.	Age days	Time after shock hours	Glucose present mg/l	Rate at time of shock mg/l/hr.	Rate after shock mg/l/hr.	Ratio of rate after to rate before
		0		118		
2	1	0.5	507		26	.220
		1.5	388		39	.330
		2.5	207		51	.432
		0		138		
3	8	0.5	478		34	.261
		1.5	292		52	.377
		2.5	105		60	.435
		0		94		
		0.5	507		48	.510
4	15	1.5	350		27	.287
		2.5	194		47	.500
		3.5	35		85	.905
		0		95		
5	21	0.5	468		14	.147
		1.5	295		40	.421
		2.5	116		72	.758
		0		144		
6	28	0.5	466		43	.298
		1.5	246		68	.472
		2.5	28		87	.604
		0		97		
7	35	0.5	476		37	.382
		1.5	302		42	.433
		2.5	127		62	.639
		0		123		
8	42	0.5	445		28	.228
		1.5	238		46	.374
		2.5	33		75	.610
		0		106		
		0.5	455		28	.264
9	48	1.5	338		25	.236
		2.5	219		36	.340
		3.5	99		54	.510

TABLE III

COMPARISON OF THE EXTENT OF GALACTOSE REMOVAL IN THE PRESENCE OF GLUCOSE FOR ALL EXPERIMENTS WITH THE HETEROGENEOUS POPULATION ACCLIMATED TO GALACTOSE

1	2	3	4	5	6	7	8	9	10	11
Fig. no.	Age days	Time when glucose = 100 mg/l in comb. unit hours	Gal. removed comb. unit in this time mg/l	Gal. removed control unit in this time mg/l	Time after shock when glucose = 100 mg/l in shock unit, hours	Gal. removed shock unit in this time mg/l	Gal. removal rate at time of shock mg/l/hr	Col.6 x col.8	Ratio of col.4:col.5	Ratio of Col.7:col.9
2	1	4.8	65	365	3.05	118	118	360	.178*	.328
3	8	2.6	127	294	2.55	120	138	352	.432	.341
4	15	3.1	136	254	3.1	127	94	292	.535	.435
5	21	2.72	79	260	2.6	90	95	247	.304	.364
6	28	2.57	134	304	2.17	125	144	312	.441	.401
7	35	2.85	106	245	2.65	119	97	257	.420	.463
8	42	2.28	65	246	2.18	84	123	268	.264	.313
9	48	2.8	65	241	3.5	110	106	371	.270	.297

*Not a measure of enzyme inhibition.

indicate absolutely no trend in the magnitude of inhibition observed, and this strongly suggests that the results were due to whatever population happened to be predominating at the time of the experiment. This conclusion is supported by the results obtained from observation of the unit and from the spread plates, which definitely showed that the population was a dynamic one and that it underwent many changes.

2. Sorbitol-Acclimated Cells

Observation of the color and settling characteristics of the heterogeneous population acclimated to sorbitol indicated that this unit also underwent several changes in predominance. The color of the mixed liquor was initially yellow. Some floc began to form after 6 days of operation, and the mixed liquor became green by the 9th day. By the time the unit was 14 days old, the settleability had become very good and the color had changed to grey. By the 24th day of operation, the mixed liquor exhibited a green-brown color. The sludge started to show signs of bulking on the 29th day, and by the 36th day settleability was virtually nonexistent; during this time the mixed liquor was green. The cells remained in a bulked condition during the remaining period of operation, but the mixed liquor did undergo two more color changes. By the 38th day the color had changed to grey, and it again changed to a green-grey by the 45th day.

The results which were obtained using the cells that were harvested for experimentation from a portion of the wasted mixed liquor at various times during the operation of the batch unit are presented in Figures 10-18. In the figures for the glucose controls there are curves which have been designated as apparent sorbitol COD. These curves represent periodate-reactive intermediates which were formed

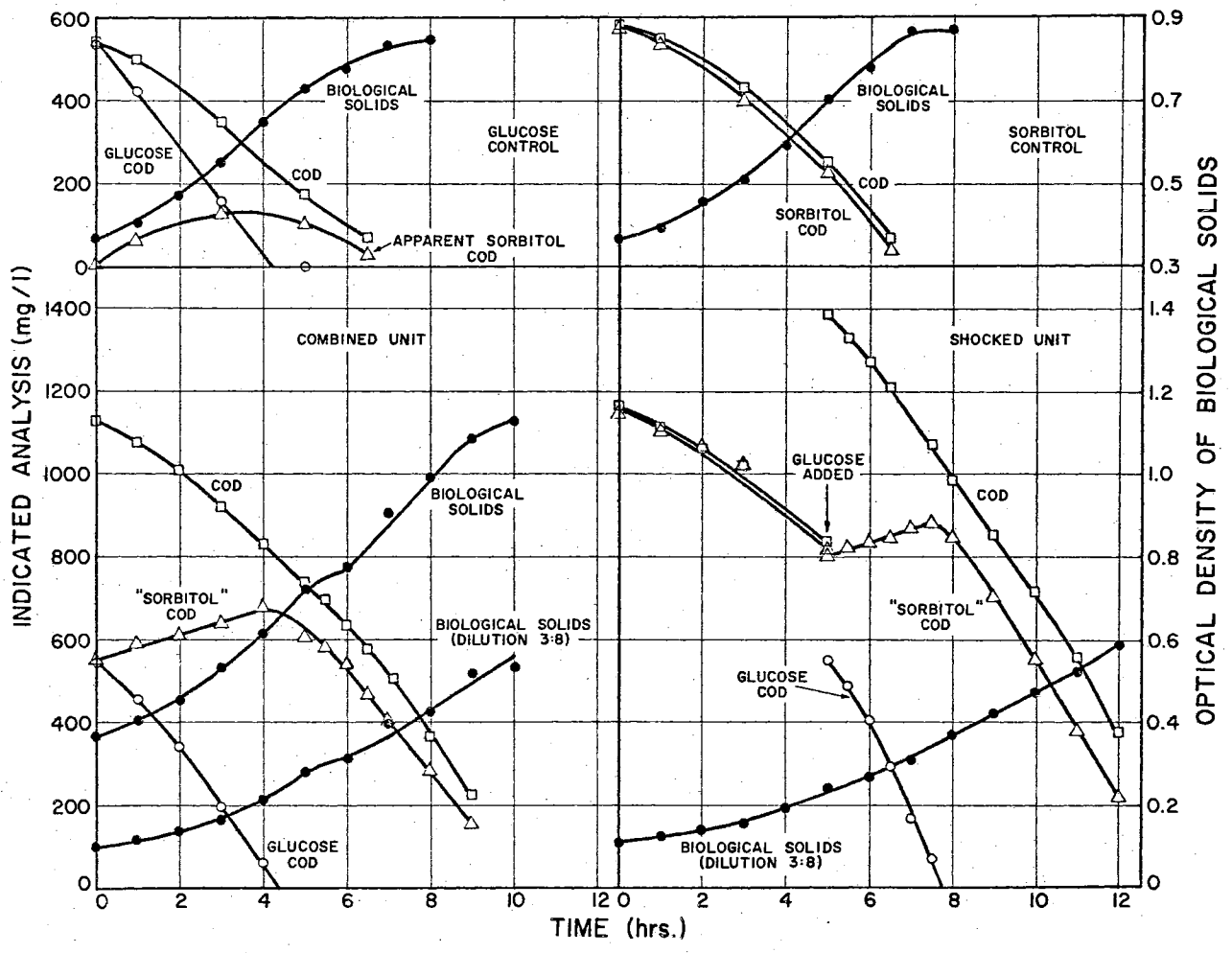


Figure 10. Metabolic response of a 2 day old culture of sorbitol-acclimated cells.

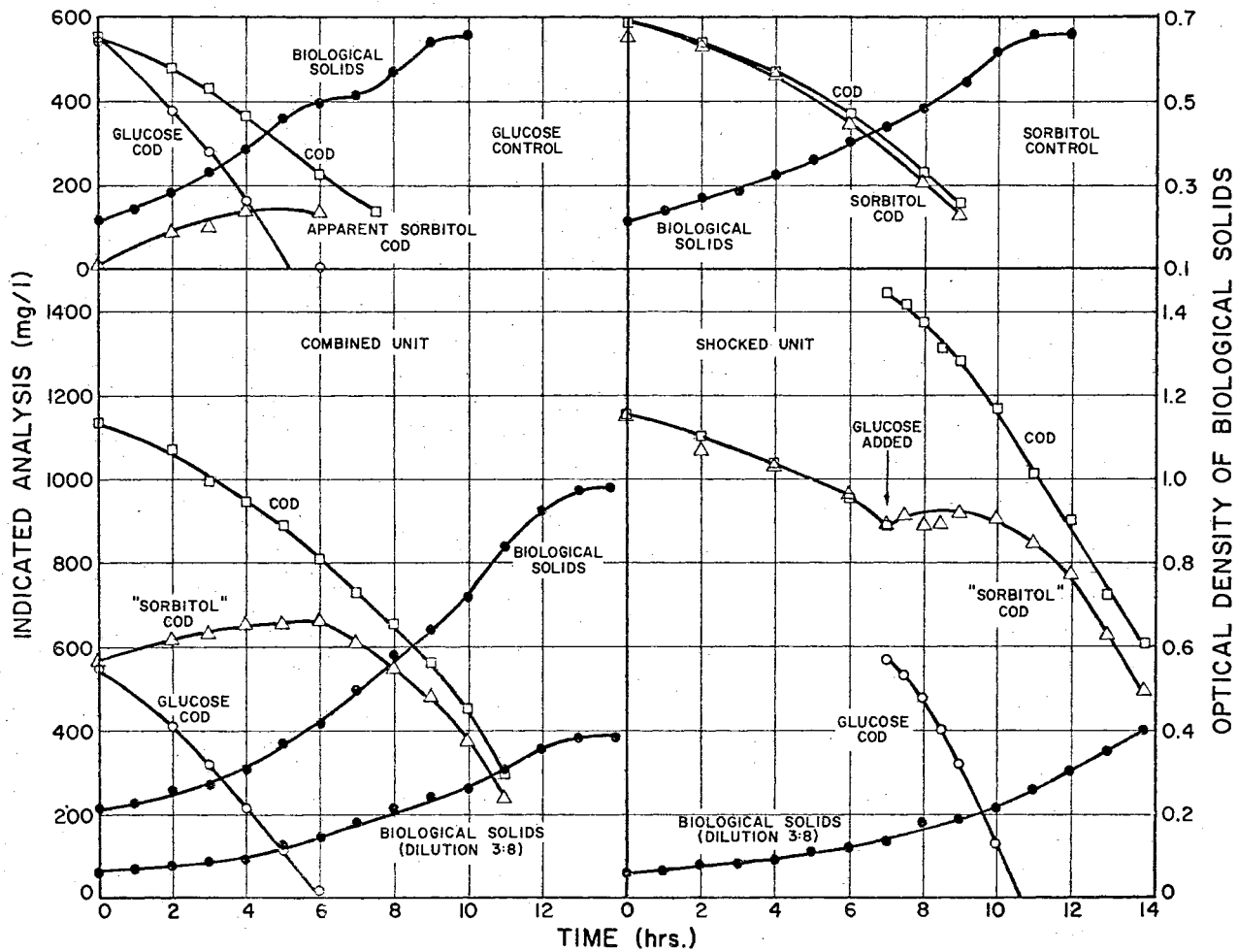


Figure 11. Metabolic response of a 6 day old culture of sorbitol-acclimated cells.

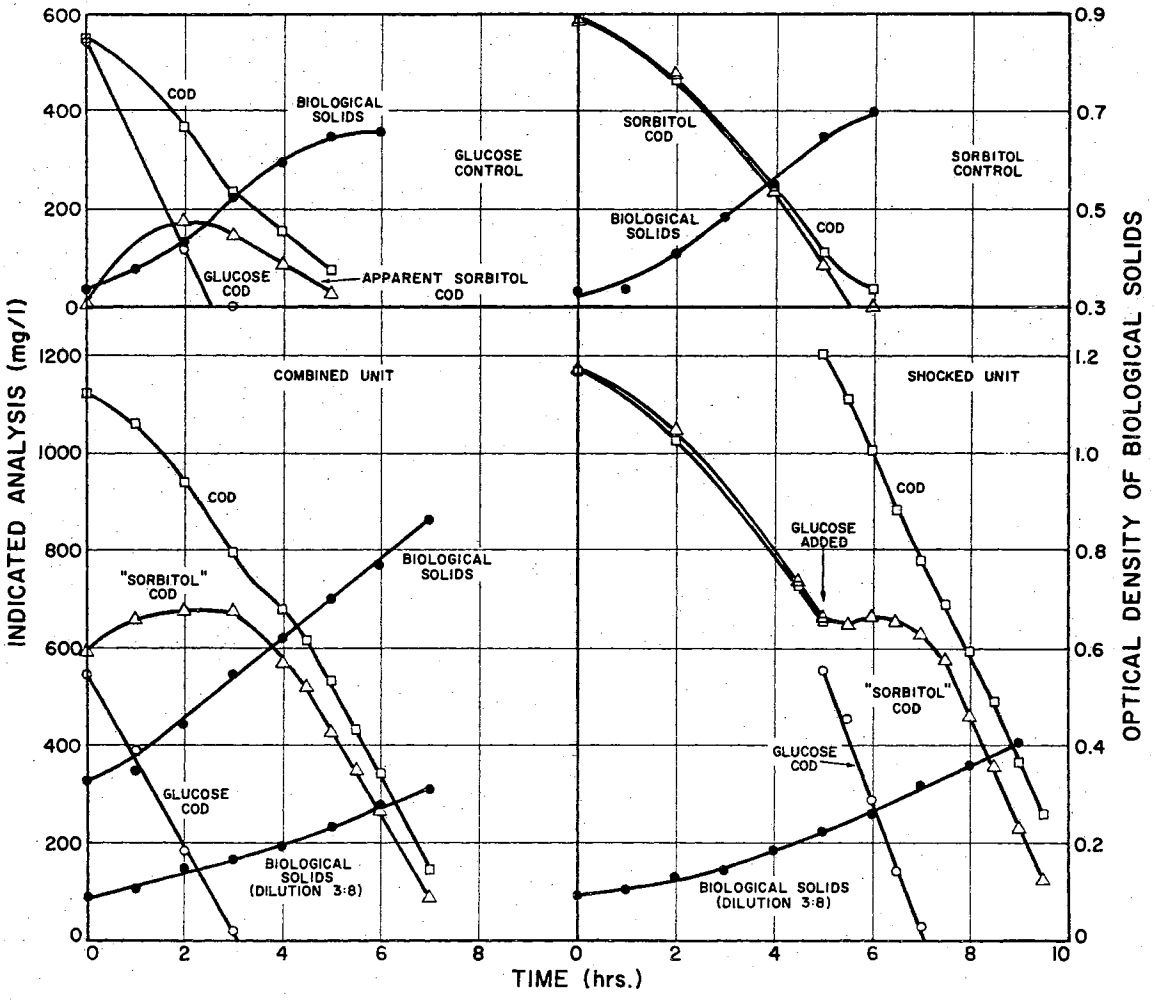


Figure 12. Metabolic response of a 10 day old culture of sorbitol-acclimated cells.

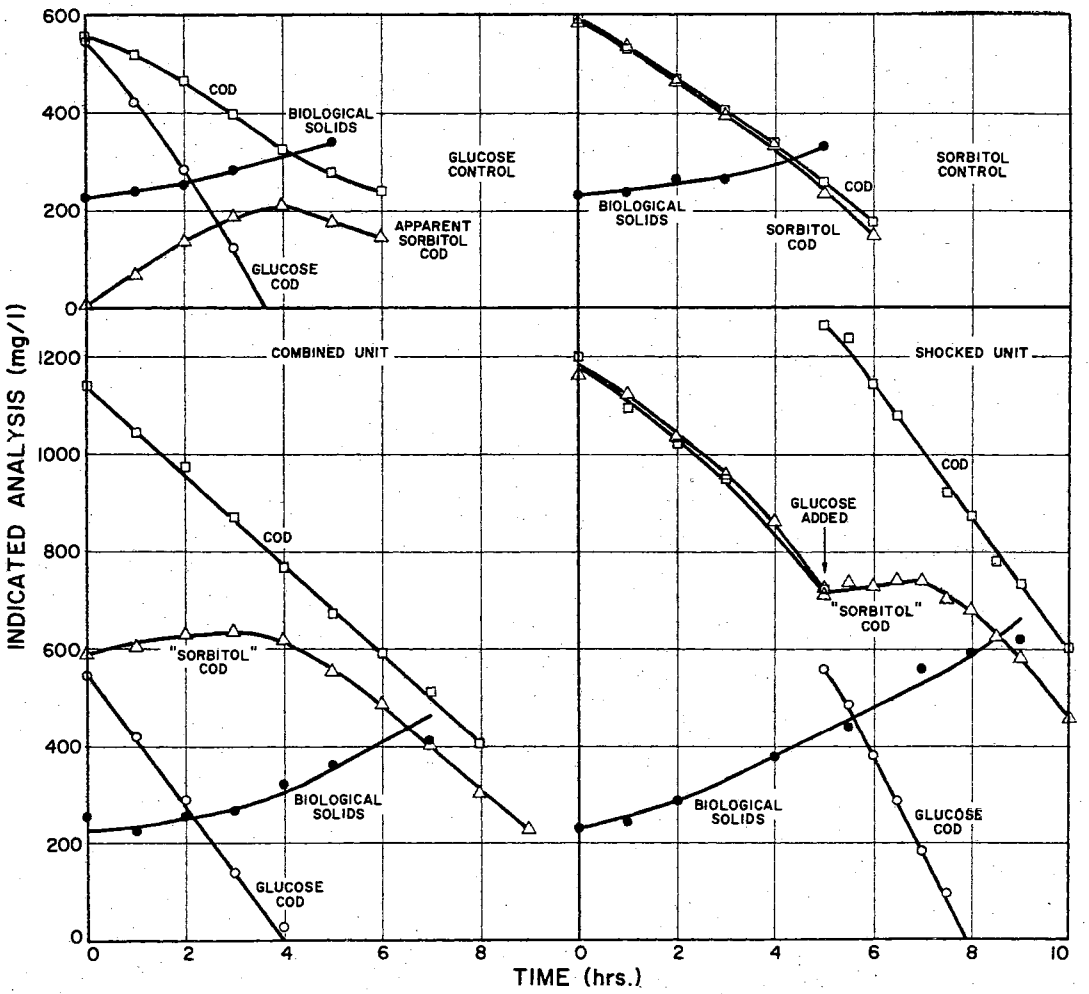


Figure 13. Metabolic response of a 14 day old culture of sorbitol-acclimated cells.

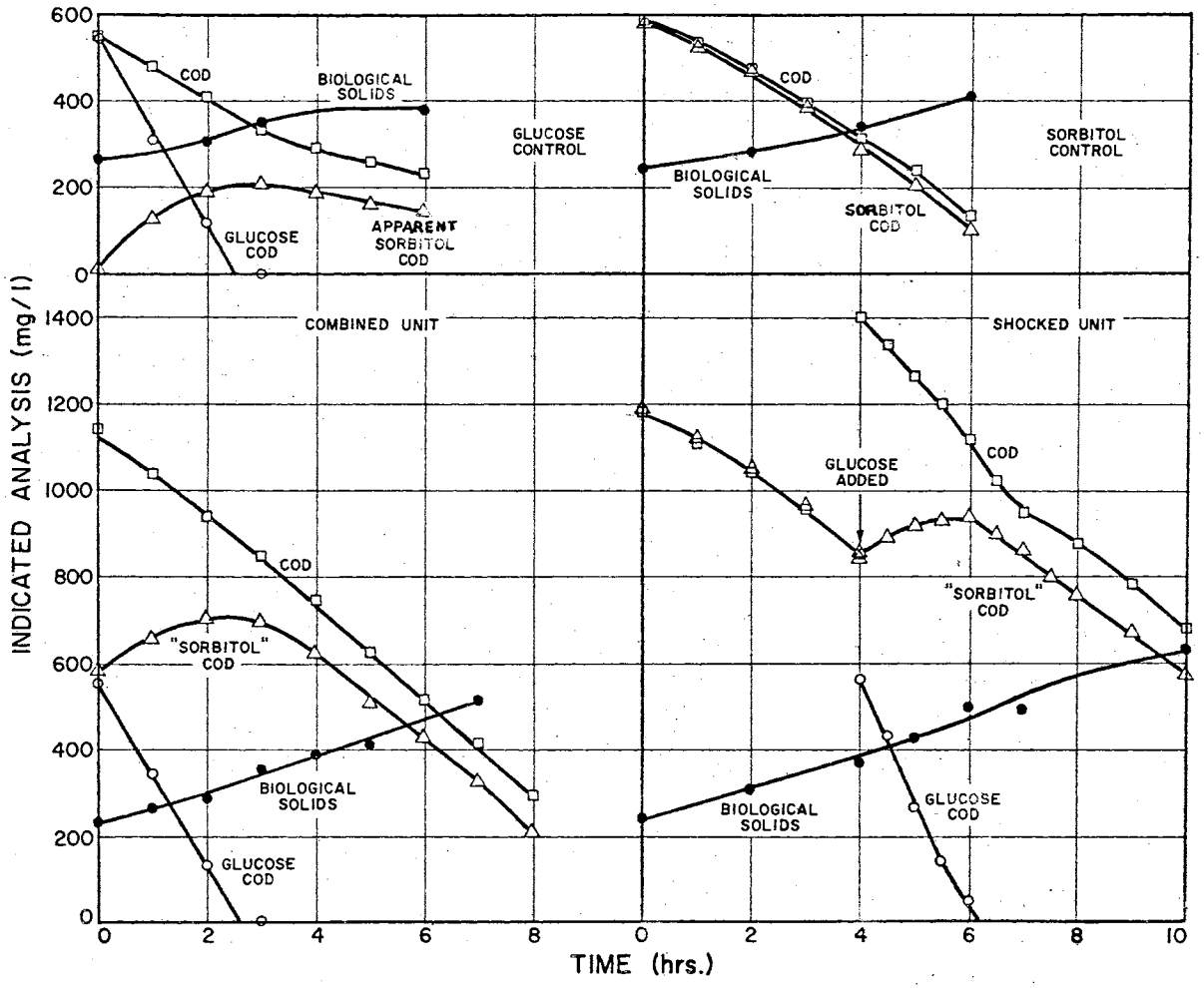


Figure 14. Metabolic response of a 19 day old culture of sorbitol-acclimated cells.

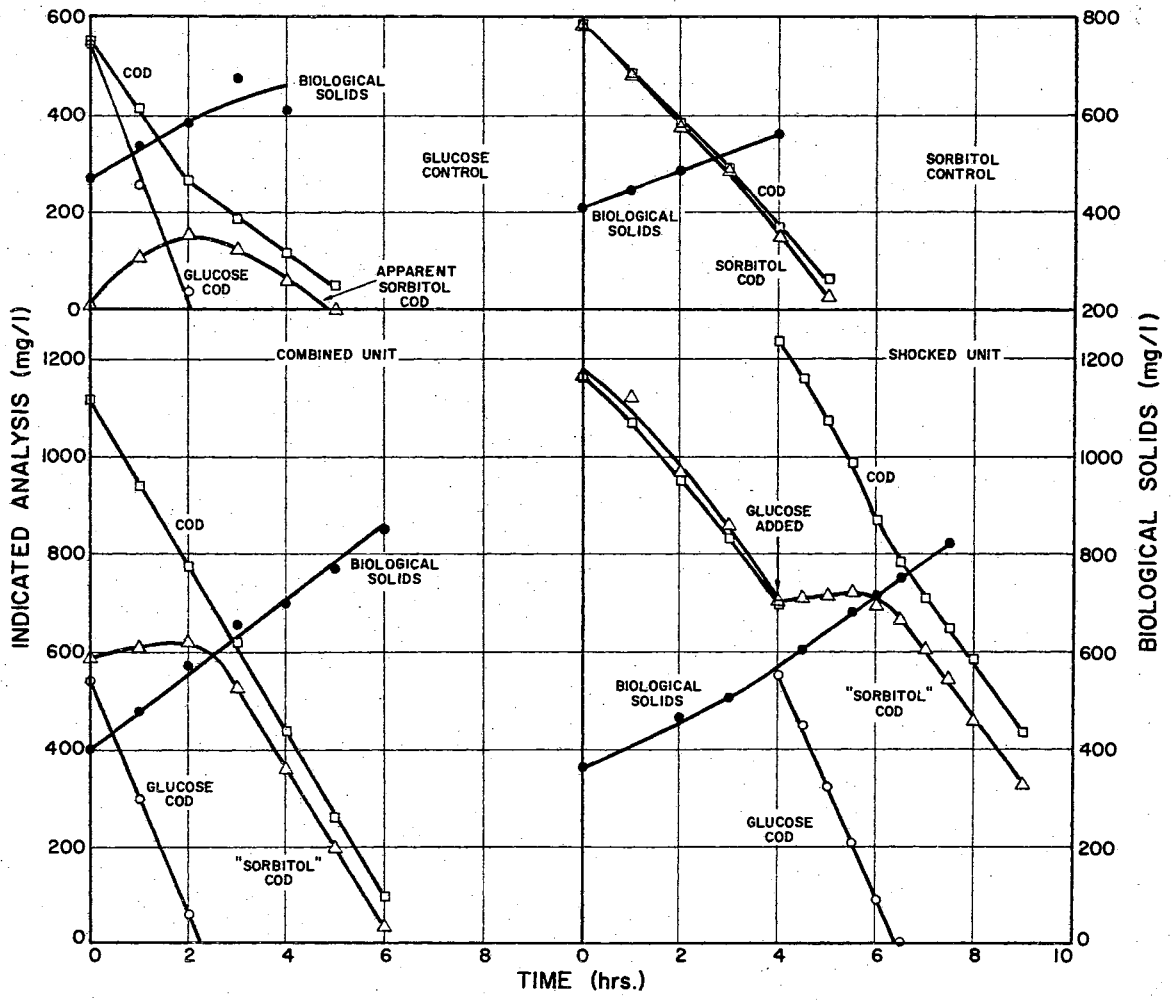


Figure 15. Metabolic response of a 24 day old culture of sorbitol-acclimated cells.

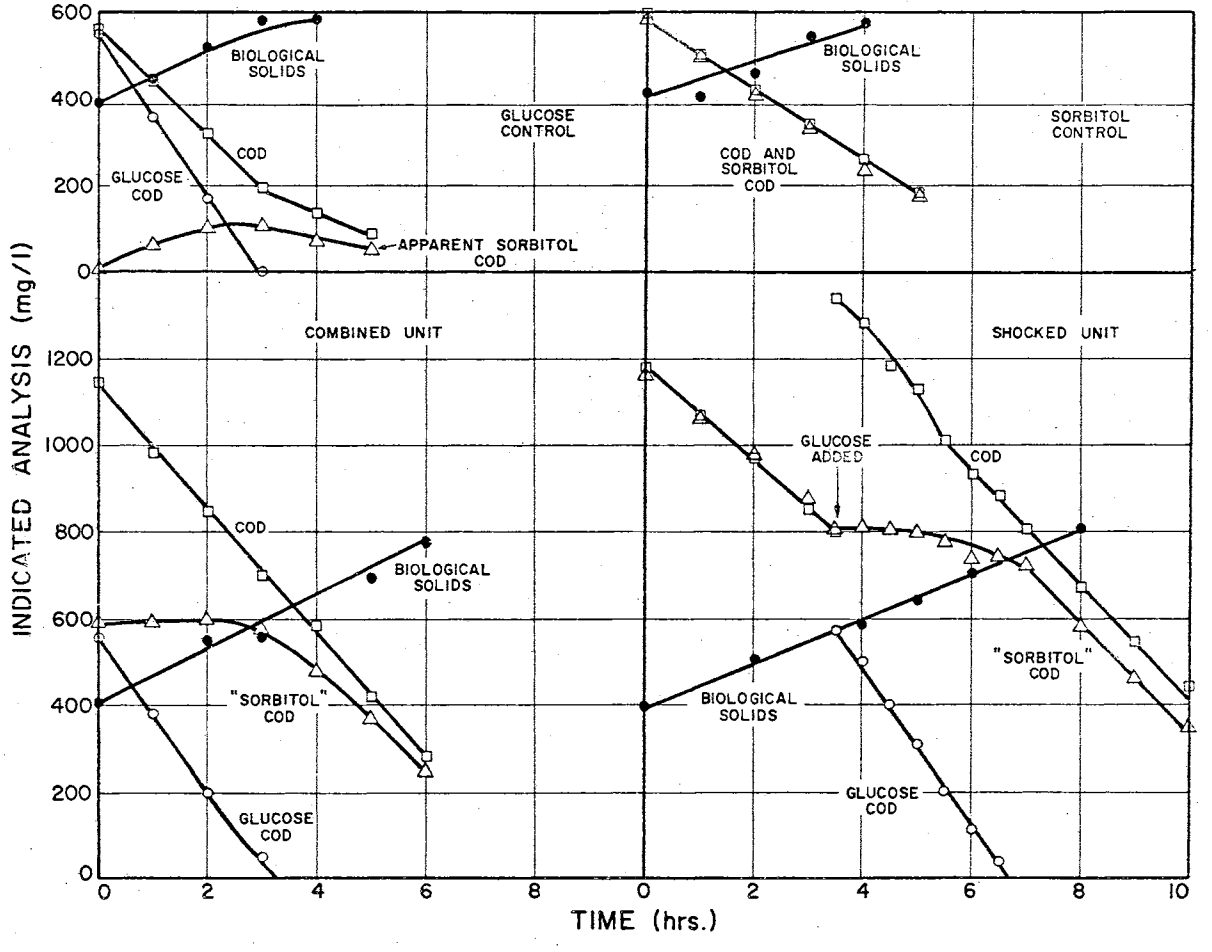


Figure 16. Metabolic response of a 29 day old culture of sorbitol-acclimated cells.

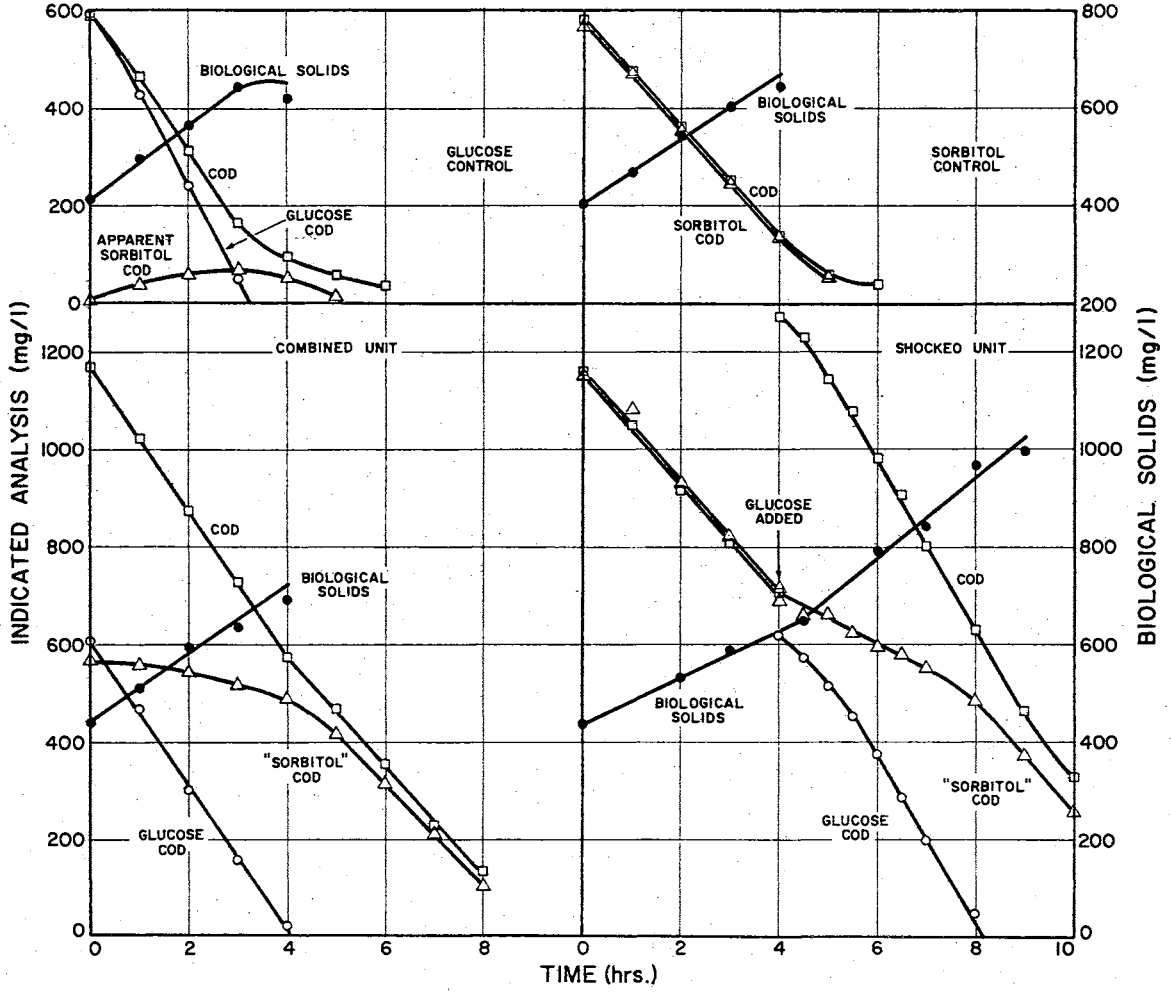


Figure 17. Metabolic response of a 36 day old culture of sorbitol-acclimated cells.

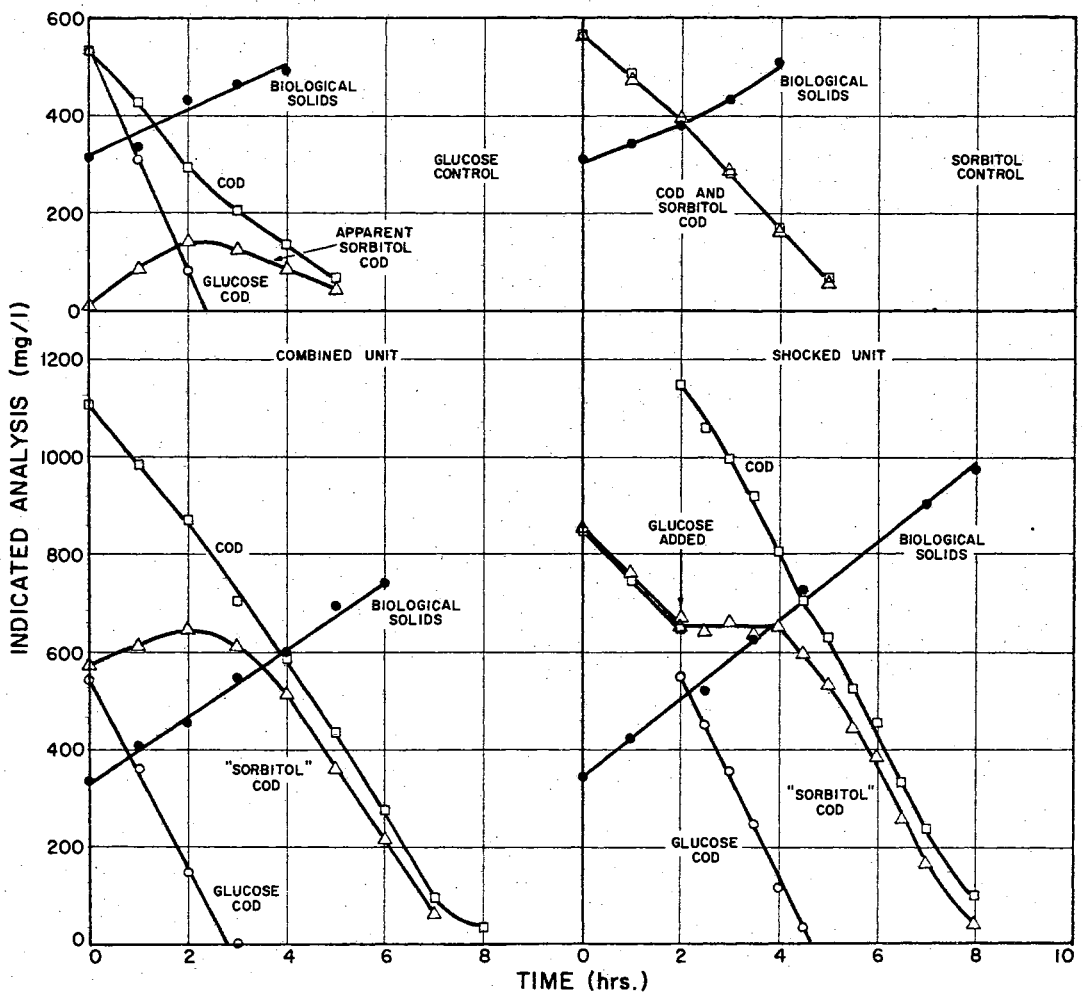


Figure 18. Metabolic response of a 45 day old culture of sorbitol-acclimated cells.

during the catabolism of glucose. They were designated as apparent sorbitol COD only because sorbitol was used as the standard when the periodate test was performed. This designation is not meant to imply that the periodate-reactive intermediates were actually sorbitol. It should be recalled that abundant quantities of these intermediates were also produced in the glucose control units when galactose-acclimated cells were studied, and, hence, prior growth of the cells on sorbitol was not directly responsible for the production of these intermediates. However, since a periodate analysis was also used to measure sorbitol concentrations, the results of that analysis presented in the combined units and in the shocked units after glucose addition include both sorbitol and those intermediates produced from glucose which react with periodate; therefore, these curves have been labeled "sorbitol" COD to indicate that they represent the sum of sorbitol and periodate-reactive glucose intermediates. A comparison of the COD and sorbitol COD curves in the sorbitol control units indicates that only very small quantities of intermediates were produced, and, in some cases, the curves were identical. In these units the designation sorbitol COD has been used. The sorbitol COD curves are assumed to represent the actual sorbitol concentrations because it seems unlikely that sorbitol could be converted to some periodate-reactive intermediate which would react at such a rate as to yield sorbitol concentrations nearly identical to the COD values at all times.

The fact that the observation of periodate-reactive intermediates produced from glucose was not the result of the introduction of some unknown factor which resulted from the modification of the sorbitol test given by Komolrit (42) is shown in Table IV. Several samples from the

TABLE IV

STATISTICAL COMPARISON OF THE RESULTS OBTAINED BY USING TWO DIFFERENT ANALYTICAL TECHNIQUES FOR MEASURING PERIODATE-REACTIVE COMPOUNDS

1	2	3	4	5	6
Fig. No.	Time hours	Sorbitol COD (Komolrit)	Sorbitol COD (Heidman)	Col. 3- col. 4	Difference squared
10	3	127.6	126	1.6	2.56
	5	103.4	101.6	1.8	3.24
	0	5.5	10.47	-4.97	24.70
12	2	176.9	174.2	2.7	7.29
	3	151.2	148	3.2	10.24
	0	2.93	5.4	-2.47	6.10
13	3	182.8	190.5	-7.7	59.29
	4	215.5	215	0.5	0.25
	5	186.1	177	9.1	82.81
	0	3.96	15.3	-11.34	128.59
	1	126.2	129	-2.8	7.84
	2	198	192	6	36
14	3	216	210	6	36
	4	196.5	188	8.5	72.25
	5	170.3	164.6	5.7	32.49
	6	151	144.8	6.2	38.44
15	2	147	151	-4	16
	3	127.7	121.2	6.5	42.25
	0	7.14	12.3	-5.16	26.62
18	2	139.5	143.2	-3.7	13.69
	3	128.9	126.5	2.4	5.76
	$\sum$	2763.73	2746.07	18.06	652.41

degrees of freedom = 20

Student's t = 0.682

$t_{.05}$ for 20 df = 2.086

glucose control units for 6 different experiments were analyzed exactly as described by the procedure given by Komolrit, and the results of that analysis are presented in column 3. The results which were obtained by the method for sorbitol analysis used throughout this study and previously described in the analytical techniques section in Chapter III are presented in column 4. A statistical analysis for paired observations (44) indicated that there was no significant difference in the results obtained.

An examination of Figures 10-18 reveals that in every experiment the rate of glucose removal at any given time in the glucose control was greater than the corresponding rate of sorbitol removal in the sorbitol control. There was never any lag in glucose removal in the control units, and thus it appears that the population at all times contained constitutive enzymes for the metabolism of glucose. The utilization of glucose was always accompanied by the release of metabolic intermediates and/or end products and the quantities of these compounds were frequently quite large. Because the "sorbitol" COD curves in the combined and the shocked units do not actually represent sorbitol, a simple examination of these curves by themselves is quite misleading. Therefore, a method to evaluate the contribution of the periodate-reactive intermediates produced from glucose in these units was employed. The assumptions which were made in determining these corrections were as follows:

a. Any given amount of glucose removal in the combined or in the shocked unit produced a quantity of periodate-reactive intermediates from glucose identical to that quantity produced for an equivalent amount of glucose removal in the glucose control unit.

b. No periodate-reactive intermediates were produced from sorbitol.

c. At any time up to the exhaustion of glucose in the combined or in the shocked units, the actual sorbitol concentrations could be determined by subtracting the periodate-reactive intermediates produced from glucose (calculated according to assumption a) from the "sorbitol" COD values. Once the glucose has been removed, there is no way to know if the "sorbitol" COD curve represents removal of glucose metabolites prior to sorbitol removal or sorbitol removal prior to removal of glucose metabolites or concurrent removal of all periodate-reactive compounds.

Although the results of the experiments with the galactose-acclimated cells were slightly different from those obtained with the sorbitol-acclimated cells since there was a small production of periodate-reactive intermediates from galactose, whereas none were produced from sorbitol, the systems are of sufficient similarity to show that the assumptions given above can be justified. The results presented in Table V were obtained from the experiments with the galactose-acclimated cells previously given in Figures 6, 7 and 8. The concentrations of glucose and galactose removed from the combined or shocked units at any given time are presented in columns 4 and 5, respectively. The data in columns 6 and 7 indicate the amount of periodate-reactive intermediates, expressed as sorbitol, which were produced in the control units for substrate removals equivalent to those observed in the combined or shocked units. For example, the results from the combined unit of Figure 6 indicate that after 2 hours, 389 mg/l of glucose and 90 mg/l of galactose had been removed from this

TABLE V

COMPARISON OF THE QUANTITIES OF PERIODATE-REACTIVE INTERMEDIATES PRODUCED IN THE VARIOUS UNITS BY THE GALACTOSE-ACCLIMATED POPULATION

1	2	3	4	5	6	7	8	9
Fig. No.	Unit	Time hours	Glucose COD removed	Galactose COD removed	"Sorbitol" COD from glucose control	"Sorbitol" COD from galactose control	Sum of control COD's	"Sorbitol" COD in stated unit
		0	0	0	5	2	7	5
		1	207	36	98	10	108	109
	Combined	2	389	90	143	18	161	178
		3	513	166	175	29	204	216
6		3.5	570	200	184	30	214	226
		3	0	438	5	40	45	61
		3.5	105	460	60	40	100	112
	Shocked	4	212	482	100	40	140	157
		4.5	321	511	128	38	166	193
		5	429	546	157	35	192	220
		0	0	0	6	6	12	11
		1	160	26	88	11	99	100
	Combined	2	321	58	142	16	158	167
		3	481	113	181	21	202	229
		3.5	563	145	192	23	215	242
7		3	0	294	6	31	37	52
		3.5	87	310	56	32	88	91
		4	173	327	93	32	125	131
	Shocked	4.5	260	348	122	33	155	169
		5	347	373	149	32	181	208
		5.5	434	399	170	32	202	237
		6	520	434	188	32	220	260
		6.2	560	449	192	32	224	263
		0	0	0	4	2	6	8
		1	196	18	91	6	97	97
	Combined	2	392	52	139	11	150	156
		2.8	548	95	160	13	173	188
8		3	0	364	4	21	25	37
		3.5	102	375	58	20	78	78
		4	207	391	94	20	114	118
	Shocked	4.5	311	414	122	19	141	158
		5	415	442	142	16	158	182
		5.5	519	476	157	14	171	199
		5.65	550	486	160	12	172	200

unit. For equivalent amounts of glucose and galactose removal in the control units, 143 mg/l of periodate-reactive intermediates expressed as sorbitol were produced in the glucose control, and 18 mg/l of periodate-reactive intermediates were produced in the galactose control. If the previously given assumptions were true (modified to include the periodate-reactive intermediates produced from galactose) one would expect to find 161 mg/l (column 8) of periodate-reactive intermediates in the combined unit. The amount found by direct measurement was 178 mg/l. A comparison of the values presented in column 8 with the corresponding values in column 9, clearly indicates that while the assumptions concerning the equivalent production of intermediates did not give exact results, a good approximation of the actual values in the combined and shocked units could be made. It should be noted that it was necessary to include the periodate-reactive intermediates produced from galactose in the calculations; whereas one of the assumptions given for the calculation of sorbitol concentrations was that these intermediates were not produced from sorbitol. However, since the largest quantities of periodate-reactive intermediates were produced from glucose, it would seem that the results definitely substantiate the assumption that the actual sorbitol concentrations (within a magnitude of error no greater than that shown in Table V) in the combined and shocked units can be calculated by using the assumptions previously given.

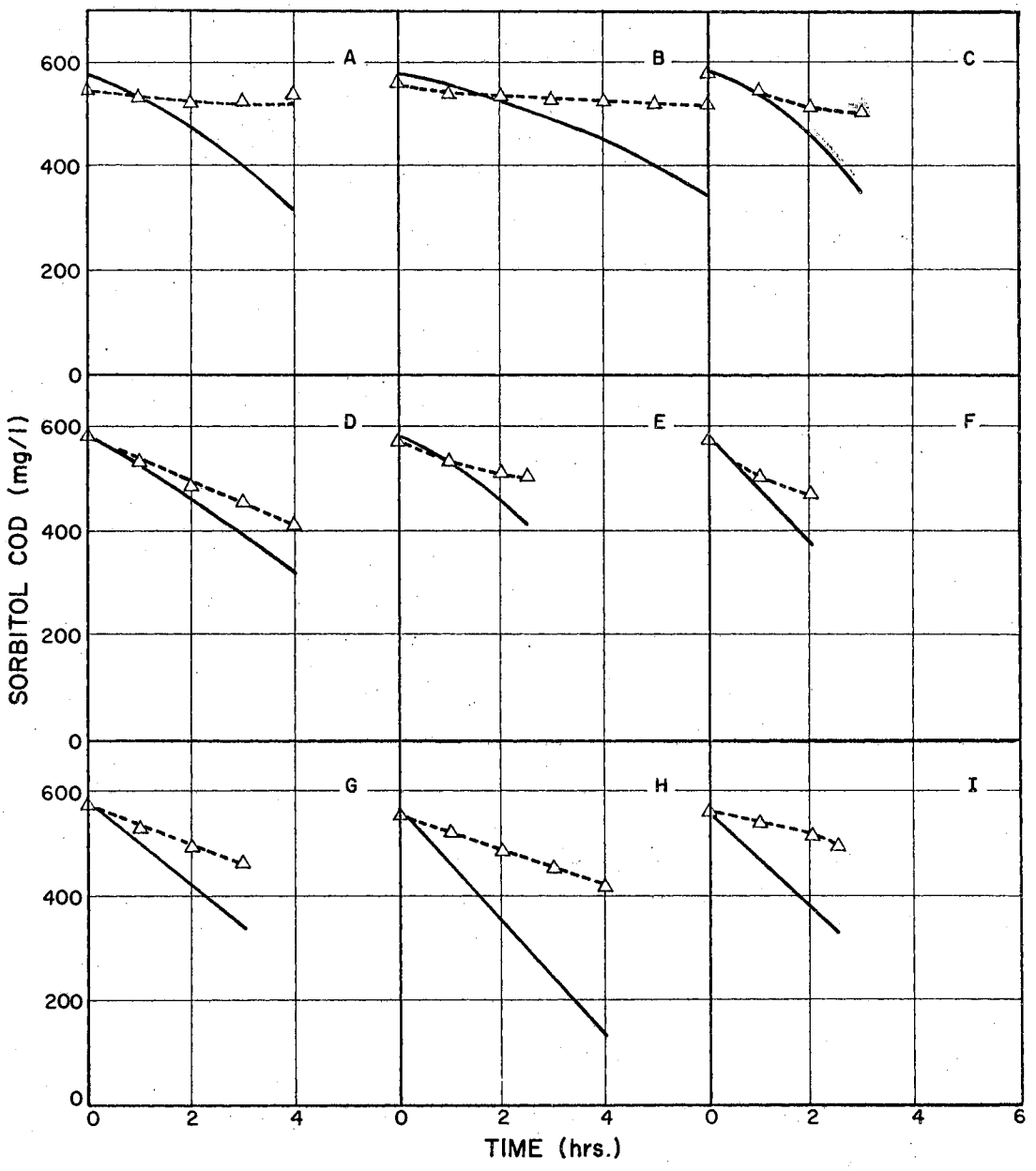
The calculated sorbitol removals in the presence of glucose for all of the combined units shown in Figures 10-18 are given in Figure 19. An examination of Figures 19A - 19E reveals that sorbitol removal occurred at a continually increasing rate in the control units, and

Figure 19. Comparison of sorbitol removals in the control units with the calculated values of sorbitol removal in the combined units.

The solid lines represent the sorbitol removal curves for each of the control units and they were reproduced from the corresponding figures.

The dashed lines represent the sorbitol removal curves in the combined units, and they were determined in accordance with the assumptions previously described.

A. From Figure 10
B. From Figure 11
C. From Figure 12
D. From Figure 13
E. From Figure 14
F. From Figure 15
G. From Figure 16
H. From Figure 17
I. From Figure 18


A
B
C
D
E
F
G
H
I
600
400
200
0
SORBITOL COD (mg/l)
0
2
4
6
TIME (hrs.)

hence the divergence of the sorbitol removal curves could have resulted from both repression of enzyme synthesis and inhibition of enzyme function. Since the sorbitol removal curves for the control units presented in Figures 19F-19I were all linear, it can be assumed that there was also no synthesis of the sorbitol enzymes in the combined units during this time, and, therefore, the difference in the removal curves can be attributed to inhibition of preformed enzymes (catabolite inhibition and/or inhibition of sorbitol transport into the cell. In any event, glucose was found to retard the removal of sorbitol in all systems.

The effect of the addition of glucose on sorbitol removal for all of the shocked systems presented in Figures 10-18 is shown in Figure 20. The dashed lines indicate what the response in the units would have been if the addition of glucose had caused complete repression of any further synthesis of sorbitol degrading enzymes but had not inhibited the activity of the preformed enzymes. It can be seen that in every case the addition of glucose resulted in the inhibition of the preformed sorbitol enzyme system. Whether the variations in the sorbitol removal patterns in the presence of glucose (linear, concave upward, or concave downward) are significant or whether they resulted from experimental error because of the indirect method of calculation used, is not known. Hence definite conclusions beyond that of the existence of partial inhibition at all times cannot be drawn.

The data presented in Table VI provide a means of comparing the results of all of the experiments presented in Figures 10-18. The values for sorbitol presented in columns 4 and 7 were obtained from the calculated values in Figures 19 and 20, respectively. Columns 4 and 5 refer to the time in column 3, and column 7 refers to the time in

Figure 20. Calculated sorbitol removals in the shocked units.

The dashed lines represent the rates of sorbitol removal at the time the shock was applied.

The sorbitol removal curves shown from 2 hours prior to the shock to the time of the shock have been reproduced from the corresponding figures.

The sorbitol removal curves shown after the addition of the shock have been calculated in accordance with the assumptions previously described.

A. From Figure 10
B. From Figure 11
C. From Figure 12
D. From Figure 13
E. From Figure 14
F. From Figure 15
G. From Figure 16
H. From Figure 17
I. From Figure 18

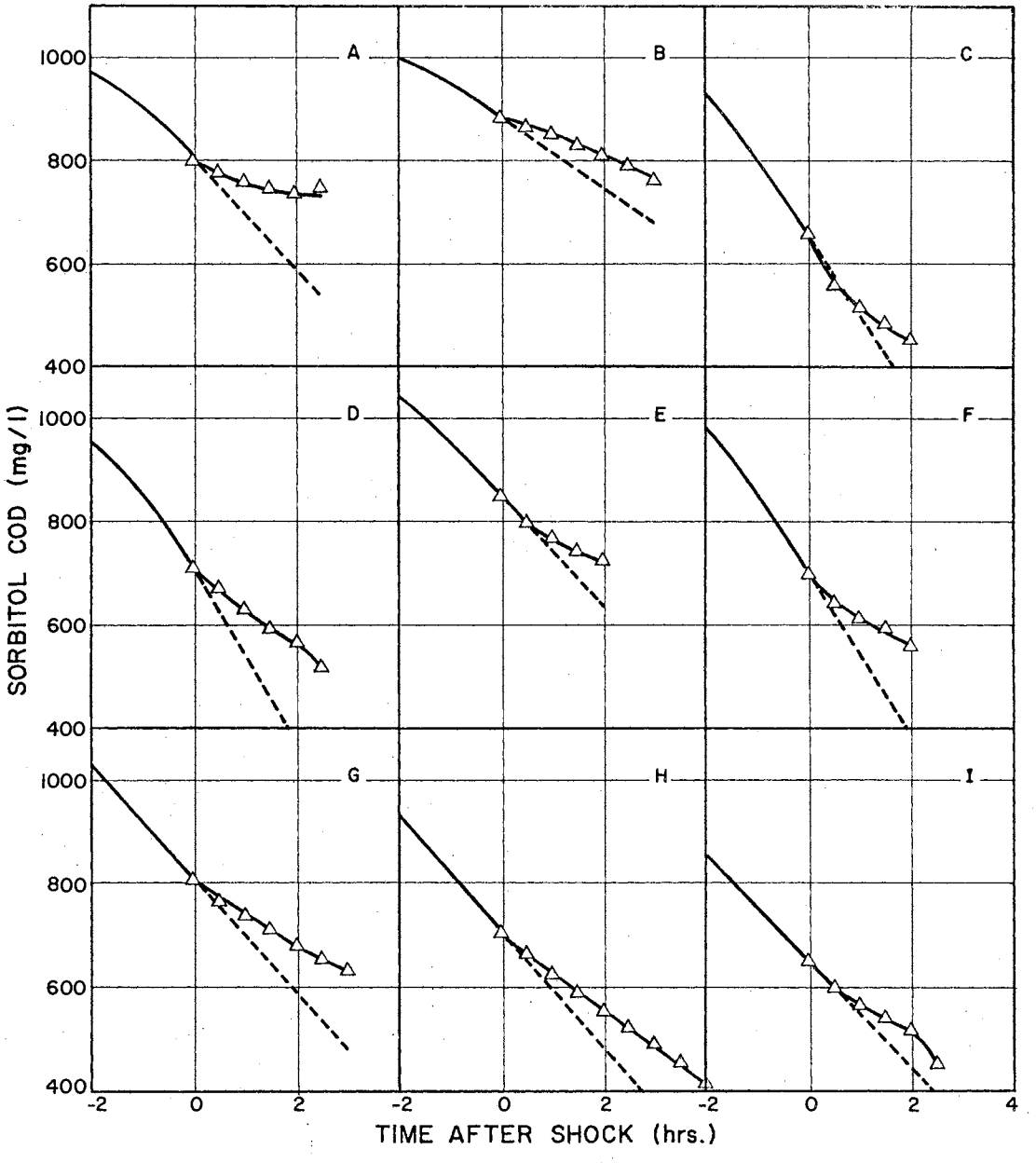

A
B
C
D
E
F
G
H
I
1000
800
600
400
-2
0
2
4
SORBITOL COD (mg/l)
TIME AFTER SHOCK (hrs.)

TABLE VI

COMPARISON OF THE AMOUNTS OF CALCULATED SORBITOL REMOVAL IN THE PRESENCE OF GLUCOSE FOR ALL EXPERIMENTS WITH THE HETEROGENEOUS POPULATION ACCLIMATED TO SORBITOL

1	2	3	4	5	6	7	8	9	10	11
Fig. No.	Age days	Time when glucose = 100 mg/l in comb. unit hours	Sorbitol removed comb. unit in this time mg/l	Sorbitol removed cont. unit in this time mg/l	Time after shock when glucose = 100 mg/l in shock unit hours	Sorbitol removed shock unit in this time mg/l	Sorbitol rate at time of shock mg/l/hr	Col.6 x col.8	Ratio of col.4:col.5	Ratio of col.7:col.9
10	2	3.7	28	236	2.28	65	107	244	.119	.266
11	6	5.13	40	186	3.13	126	68	213	.215	.592
12	10	2.55	75	183	1.72	188	155	267	.410	.704
13	14	3.28	143	210	2.4	179	167	400	.681	.448
14	19	2.16	66	139	1.7	114	103	175	.475	.651
15	24	1.83	103	190	1.98	138	157	311	.542	.444
16	29	2.58	99	206	2.65	161	110	292	.480	.551
17	36	3.43	116	375	3.6	267	113	407	.309	.657
18	45	2.28	55	210	2.2	152	102	224	.262	.679

column 6. The values presented in column 11 provide a means of determining the extent of inhibition of the preformed sorbitol enzymes (assuming complete repression) caused by the rapid addition of glucose. As previously explained, the ratios in column 10 are not all measures of enzyme inhibition alone. Because of the involved method of calculating the sorbitol concentrations in the combined and the shocked units, the ratios presented in columns 10 and 11 should certainly not be considered as exact values. Therefore, it would be inappropriate to attempt to make specific comparisons among units on the basis of these ratios. However, it is apparent that glucose or glucose catabolites always inhibited the preformed sorbitol enzymes, and the extent of this inhibition appears to be unaffected by the time of operation of the batch unit. Although the values in column 11 did increase during the period from 2 to 10 days, the values obtained for the remaining time of operation were entirely random. It could perhaps be argued that the changes in the extent of inhibition which occurred during the course of the first three experiments were attributable to an increase in cell age. However, when the results of the entire 45 day period of operation are considered together, the particular response observed would seem to be due to whatever organisms happened to be predominating at the time of each experiment.

B. Ageing of Pure Cultures

1. Blue Organism

The results which were obtained from the batch operation of a unit containing a pure culture of Blue organism are presented in Figures 21-25. The cells for each experiment were obtained from that portion of

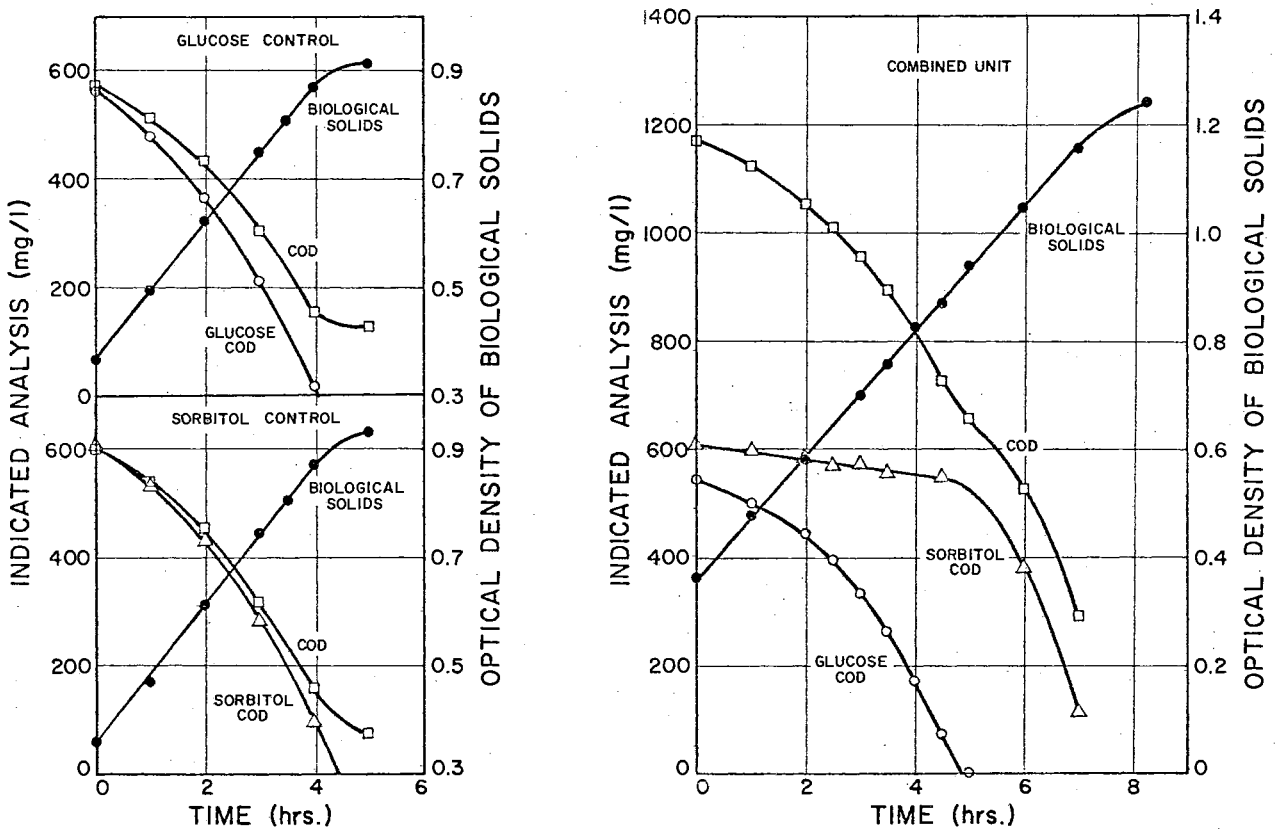


Figure 21. Metabolic response of a 2 day old culture of Blue organism acclimated to sorbitol.

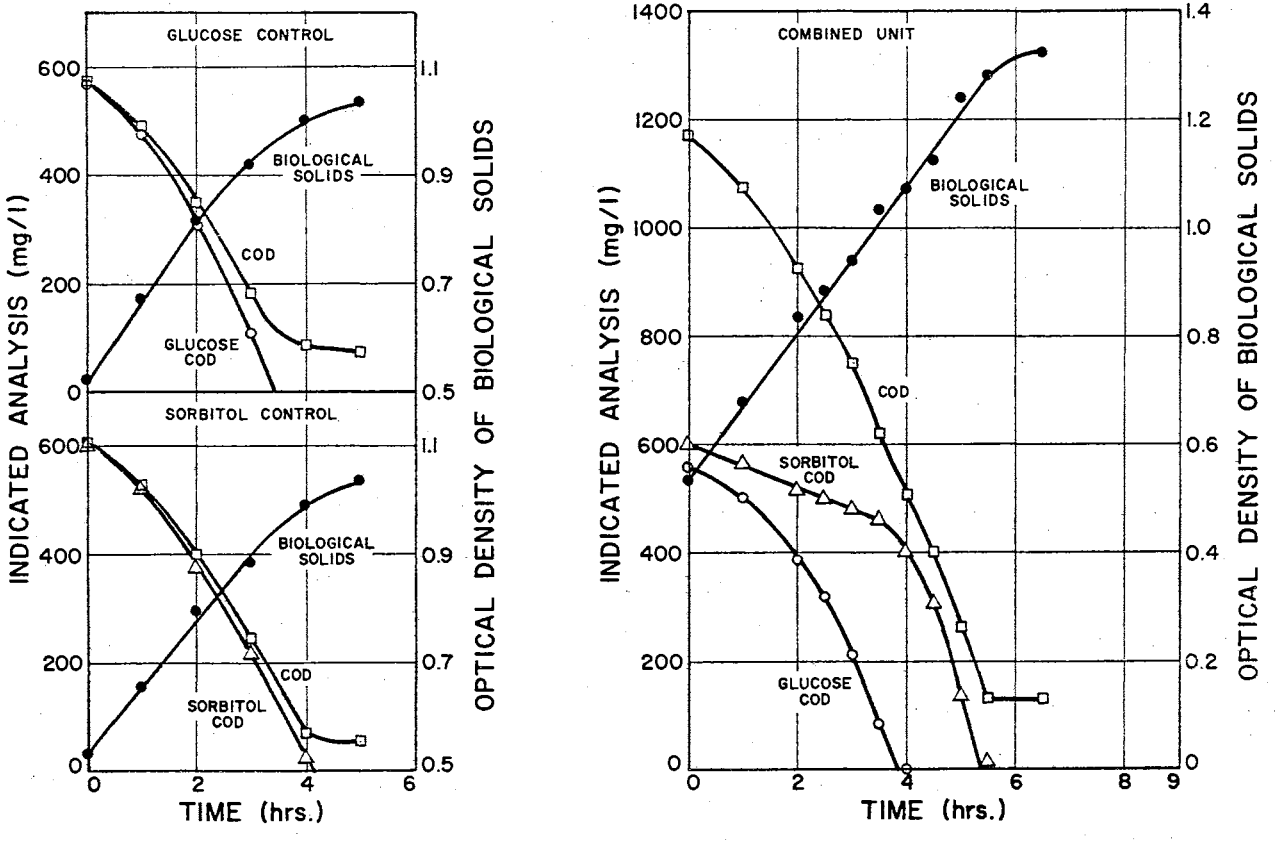


Figure 22. Metabolic response of a 6 day old culture of Blue organism acclimated to sorbitol.

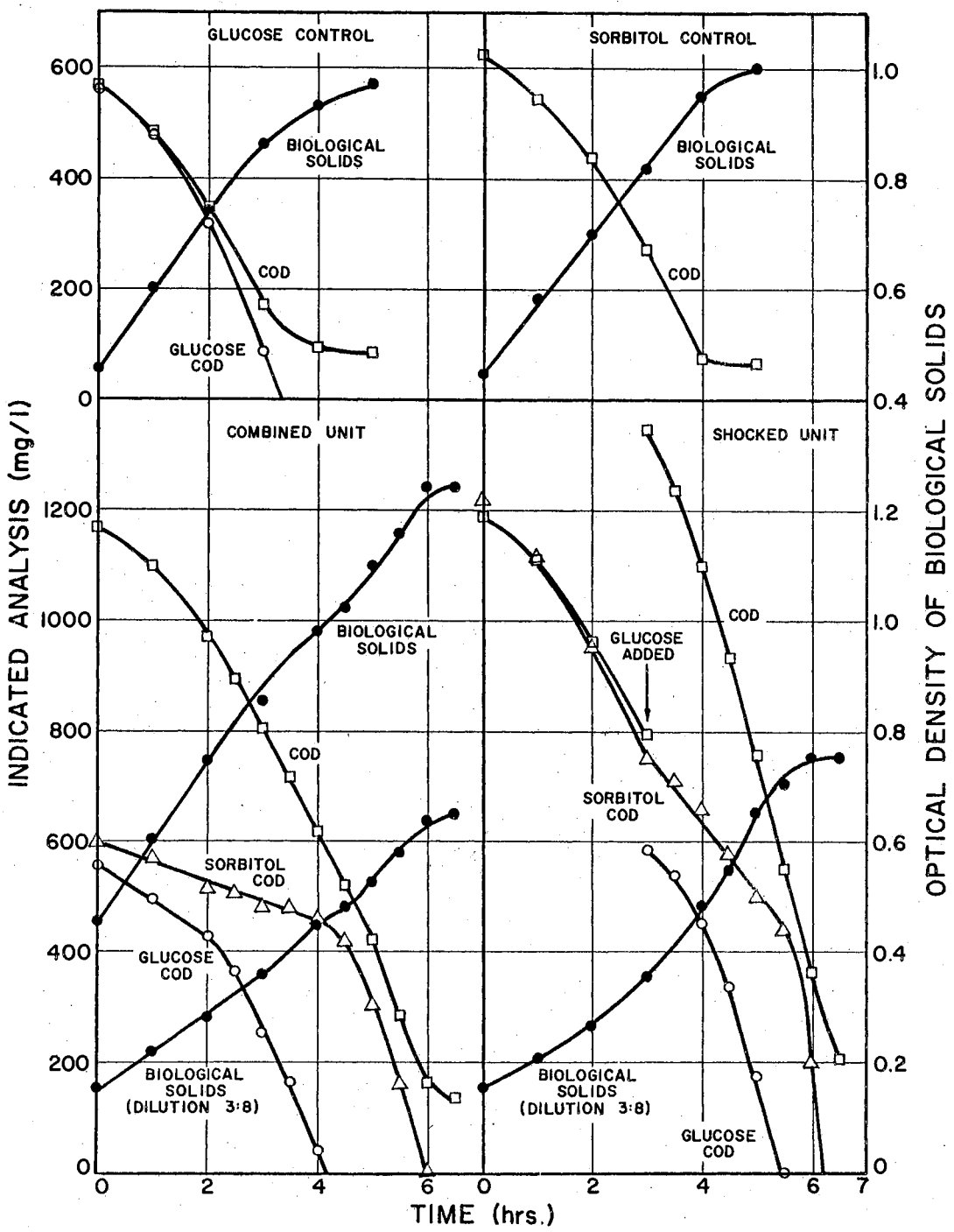


Figure 23. Metabolic response of a 12 day old culture of Blue organism acclimated to sorbitol.

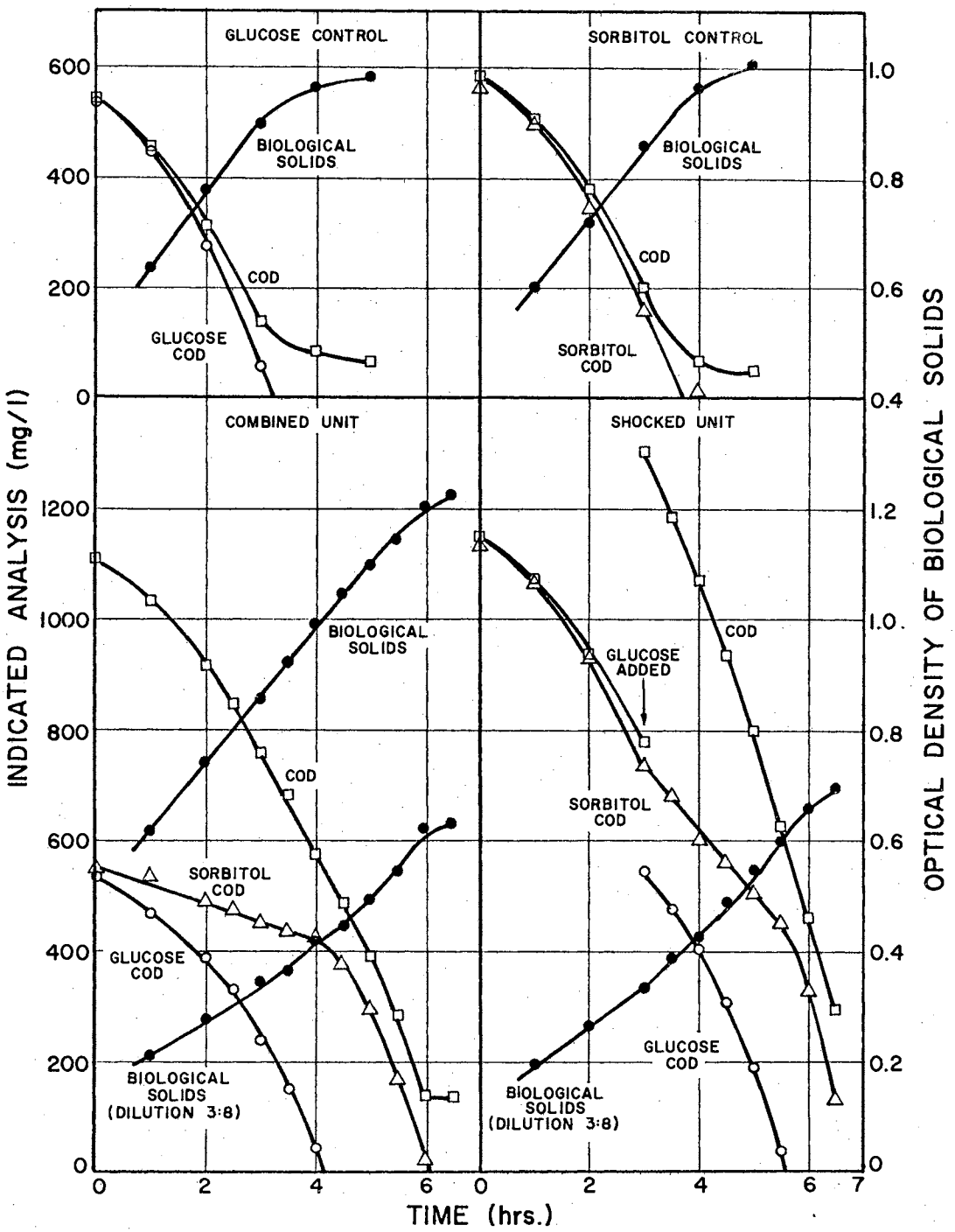


Figure 24. Metabolic response of a 16 day old culture of Blue organism acclimated to sorbitol.

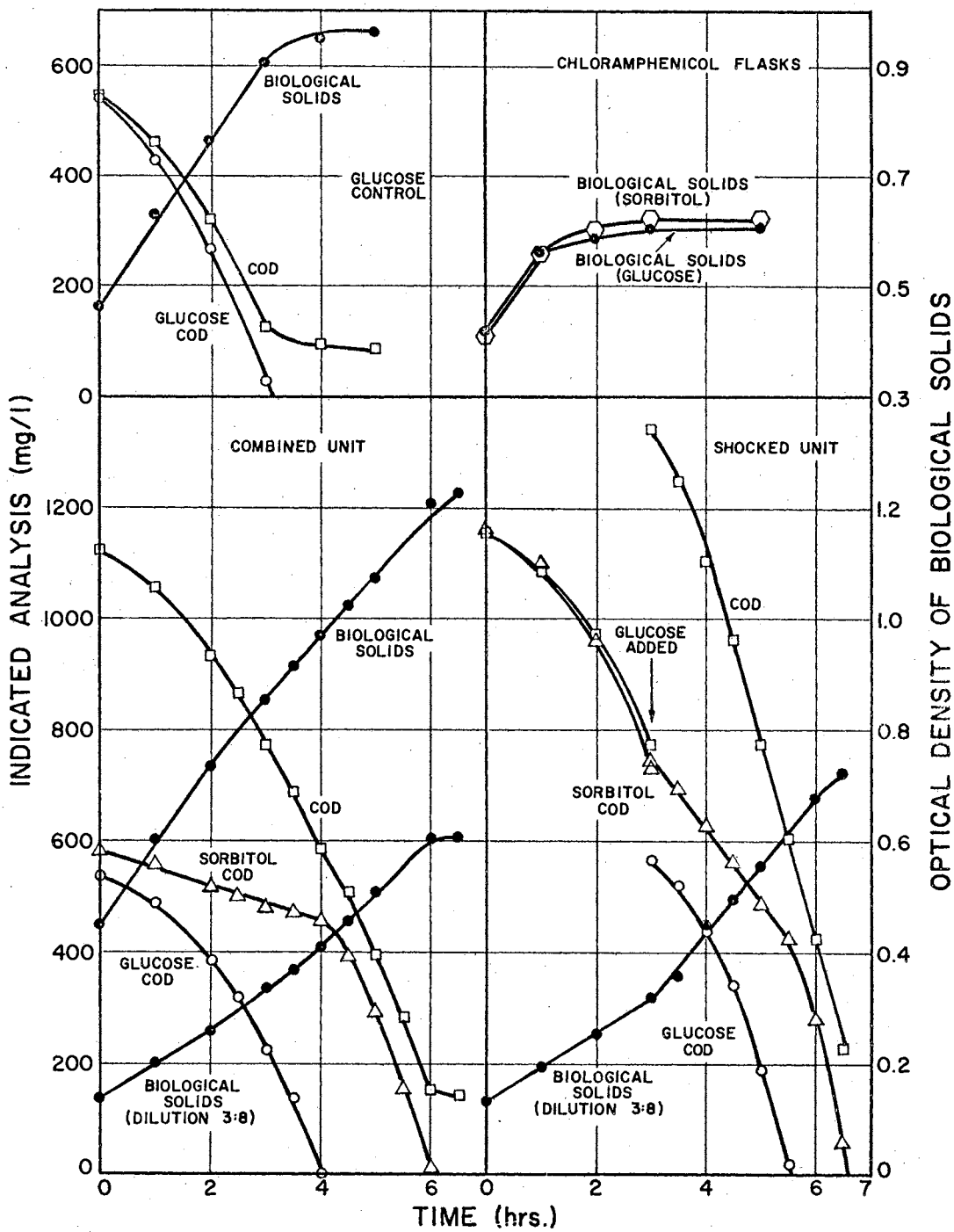


Figure 25. Metabolic response of a 26 day old culture of Blue organism acclimated to sorbitol.

the mixed liquor which was removed prior to feeding, and they remained completely dispersed throughout the period of operation of the unit. A shocked unit was not studied in the experiments shown in Figures 21 and 22. At the time this series of experiments was performed, the author was unaware of the problems which can arise when periodate-reactive intermediates are produced from glucose, and, hence, an analysis for periodate-reactive intermediates in the glucose control units was not made. Later experimentation with a young cell culture of this organism revealed that no periodate-reactive intermediates were produced from glucose. Since the total quantity of intermediates produced by the older cells was slightly less than was obtained from the young cells, there is no reason to believe that periodate-reactive intermediates were produced. As will be shown later, when increased ageing leads to the production of periodate-reactive intermediates there is a corresponding increase in the production of total intermediates.

Instead of a sorbitol control unit, Figure 25 shows the results which were obtained from 2 flasks which contained chloramphenicol in addition to the substrates. Prior to the addition of substrate, the cells were aerated for 30 minutes in the standard medium (minus the carbon source) to which 100 mg/l of chloramphenicol had been added. Glucose was then added to one flask and sorbitol to the other to yield final concentrations of 500 mg/l of each compound. Only optical density was monitored. From these results it can be discerned that the cells contained the enzyme systems necessary for the removal of glucose and sorbitol prior to exposure to either of these compounds.

An examination of the sorbitol removal curves in the combined units shown in Figures 21-25 reveals that in every case sorbitol

removal proceeded linearly in the presence of glucose. Variations in the initial biological solids concentrations resulted in variations in the initial "enzymatic capabilities" to remove sorbitol, and, hence, the actual removal rate varied somewhat from experiment to experiment. That the cells possessed a functioning preformed enzyme system for sorbitol removal at the start of the experiments can be seen from the results obtained from the chloramphenicol unit shown in Figure 25. Therefore, sorbitol removal was not dependent upon synthesis of sorbitol enzymes, and the constant sorbitol removal rates in the presence of glucose indicate that the synthesis of the sorbitol enzymes probably was completely repressed. It is readily apparent that increased ageing of the cells did not result in a relief of the repression mechanism.

An examination of the shocked systems shown in Figures 23-25 reveals that sorbitol removal in the presence of glucose was again essentially linear. A comparison of the sorbitol removal rates immediately prior to the shock with the corresponding rates after the shock indicates that the preformed sorbitol enzyme system was inhibited 36%, 43%, and 46% (assuming complete repression) in the shocked units represented in Figures 23-25, respectively. Since the sorbitol removal rate was changing quite rapidly prior to the addition of glucose, it was very difficult to obtain accurate estimates of the rate at the time the shock load was administered, and the variations in the degree of inhibition appear to be attributable more to experimental error than to any difference in the performance of the biological systems. Since the rates of sorbitol removal in the presence of glucose were essentially linear, it would appear that the previous conclusion that further synthesis of sorbitol enzymes in the presence of glucose was blocked is

substantiated. It would also appear that glucose did not inhibit the transport of sorbitol into the cell because there was no increase in the sorbitol removal rate as the glucose concentration decreased. This conclusion is also supported by the results obtained from the combined units.

To verify that the degree of inhibition encountered was not a result of the age of the cells (since the unit had been in operation for 12 days before inhibition was first accurately measured by including a shocked unit in the experimental protocol) a 1 ml portion of cells was withdrawn from the batch unit on the 24th day of operation and transferred aseptically to a flask containing 50 ml of standard medium. After 24 hours, 1 ml from the flask was transferred to fresh medium and this procedure was repeated daily. After a total of 4 transfers to fresh medium had been made, 12 flasks were inoculated on the 5th transfer, and an experiment was conducted with the young cells on the following day. The results are given in Figure 26. The degree of inhibition in the shocked unit was determined to be 44%, and once again the patterns of substrate removals in the combined and shocked units were the same as had been observed in the previous experiments.

On the basis of the substrate removal responses which were obtained with the Blue organism, it is apparent that the extent of enzyme repression and the extent of enzyme inhibition manifested by an organism need not be related. In all cases, glucose appeared to cause 100% repression of sorbitol enzymes, but the degree of inhibition encountered was only about 42% (average value). It is also readily apparent that increased ageing of the unit had absolutely no effect upon the degree of repression or inhibition encountered.

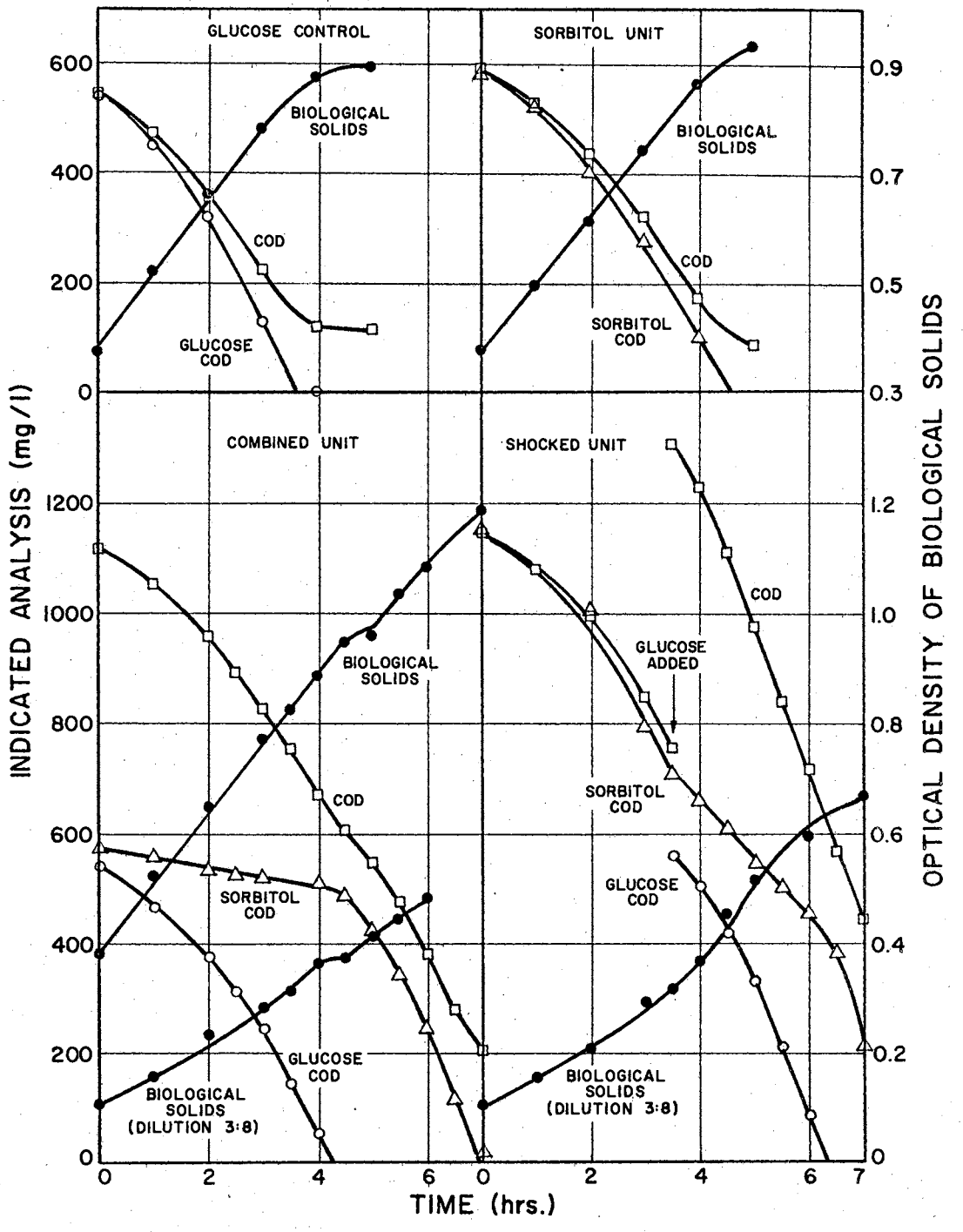


Figure 26. Metabolic response of a young cell culture of Blue organism grown from an initial inoculum of 24 day old cells.

2. Escherichia coli, Strain K-12

Additional experiments with a batch-operated culture of E. coli, strain K-12, were also performed. The organisms were grown on the standard medium, and they were fed once every 24 hours. The daily operating procedures (reactor volume, feed concentrations, etc.) were the same as those used in the previous experiments. The temperature was maintained at 35° C. After the unit had initially "grown up," it was defined as being 1 day old, and each succeeding 24-hour period was defined as increasing the age by 1 day.

The results obtained from an experiment with 2 day old cells are presented in Figure 27. Glucose utilization did not result in the production of any periodate-reactive intermediates, and, therefore, there was no interference with any of the sorbitol determinations. COD removal and biological solids production in the combined unit were diphasic, and the production of the enzymes necessary to degrade sorbitol appears to have been completely repressed in the presence of glucose. The linearity of sorbitol removal in the shocked unit during the period of glucose removal also suggests that further synthesis of sorbitol degrading enzymes was completely repressed. Furthermore, the linear removal indicates that glucose did not interfere with sorbitol transport into the cell. The addition of glucose to the shocked unit inhibited the preformed sorbitol enzymes by 77%.

The batch culture lysed some time between the 8th and 9th day of operation. At the time the culture would normally have been fed for the 9th time, the biological solids level had become extremely low and the "mixed liquor" was practically transparent. A sample was withdrawn and passed through a 0.45 μ membrane filter. When 1 ml of the

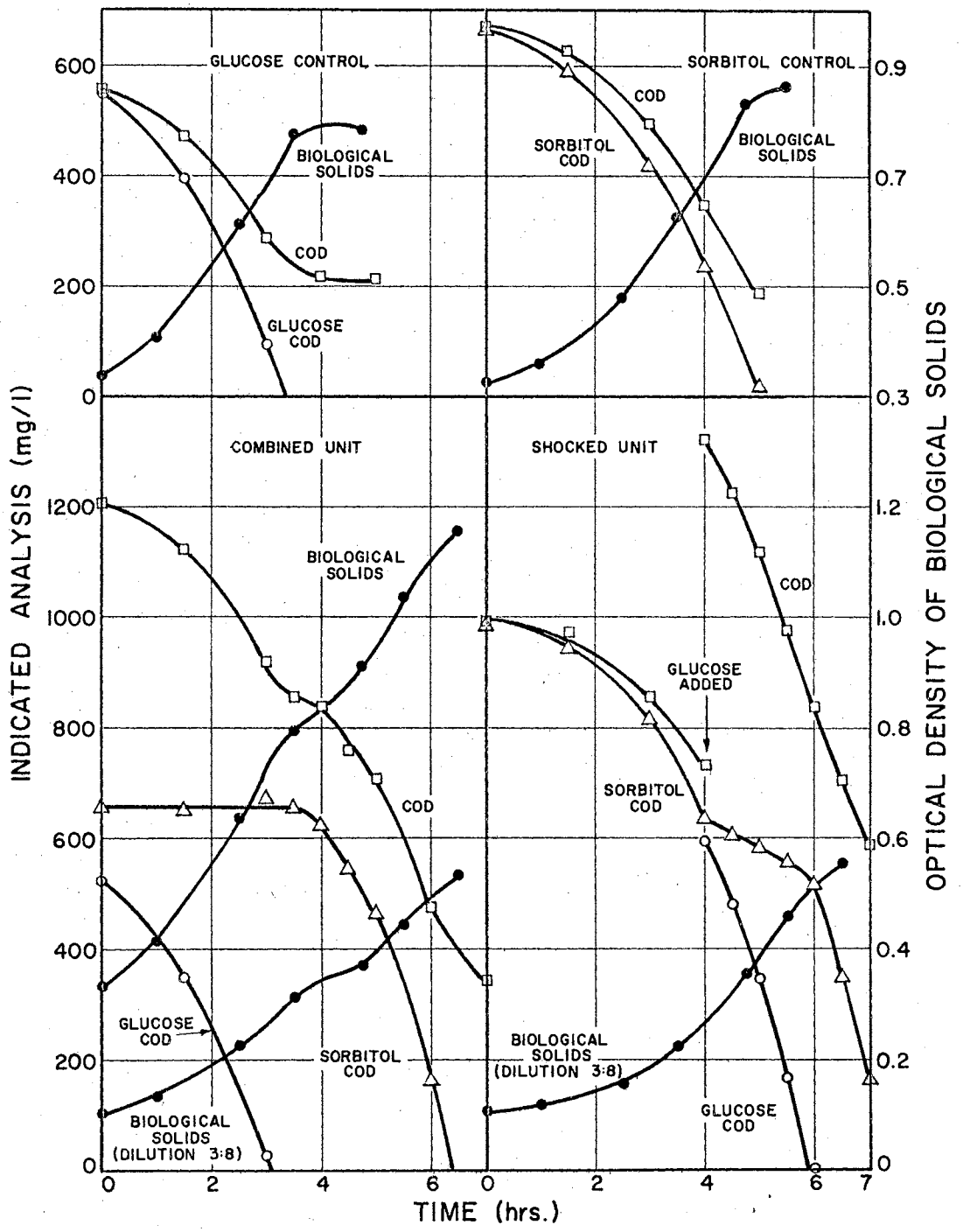


Figure 27. Metabolic response of a 2 day old culture of E. coli acclimated to sorbitol.

filtrate was added to a flask containing an actively growing culture of E. coli, lysis again occurred within 8 hours.

The batch unit and feed reservoirs were emptied and all components of the reactor were sterilized. Operation of the batch unit was reinitiated with an inoculum of E. coli, strain K-12, which had been grown from the same slant as the first inoculum. The unit was then operated exactly as previously described for this organism.

The results of an experiment with cells taken from an 8 day old unit are presented in Figure 28. It can be seen that a very small amount of periodate-reactive intermediates was produced from glucose. Therefore, the curves in the combined and shocked units have been labeled "sorbitol" COD because they include this small quantity of intermediates in addition to sorbitol. Once again it appears that the presence of glucose caused a complete repression of further synthesis of the enzymes necessary to degrade sorbitol. When a correction for the periodate-reactive intermediates produced from glucose was applied to the shocked unit, it was found that glucose addition had inhibited the preformed sorbitol enzyme system by 79%. Therefore, the results of increasing the age from 2 days to 8 days were negligible.

Approximately 3 hours after the batch unit had been fed for the 10th time, an excessive amount of foaming began to occur. Once again the unit underwent lysis, and the viable count dropped from 1.78×10^9 to 4×10^5 within 9 hours. When a sample of membrane filtrate which had been obtained from the unit was added to a flask containing an actively growing culture of E. coli, the cells lysed within several hours. No further attempts to age a pure culture of E. coli by this method of batch operation were undertaken.

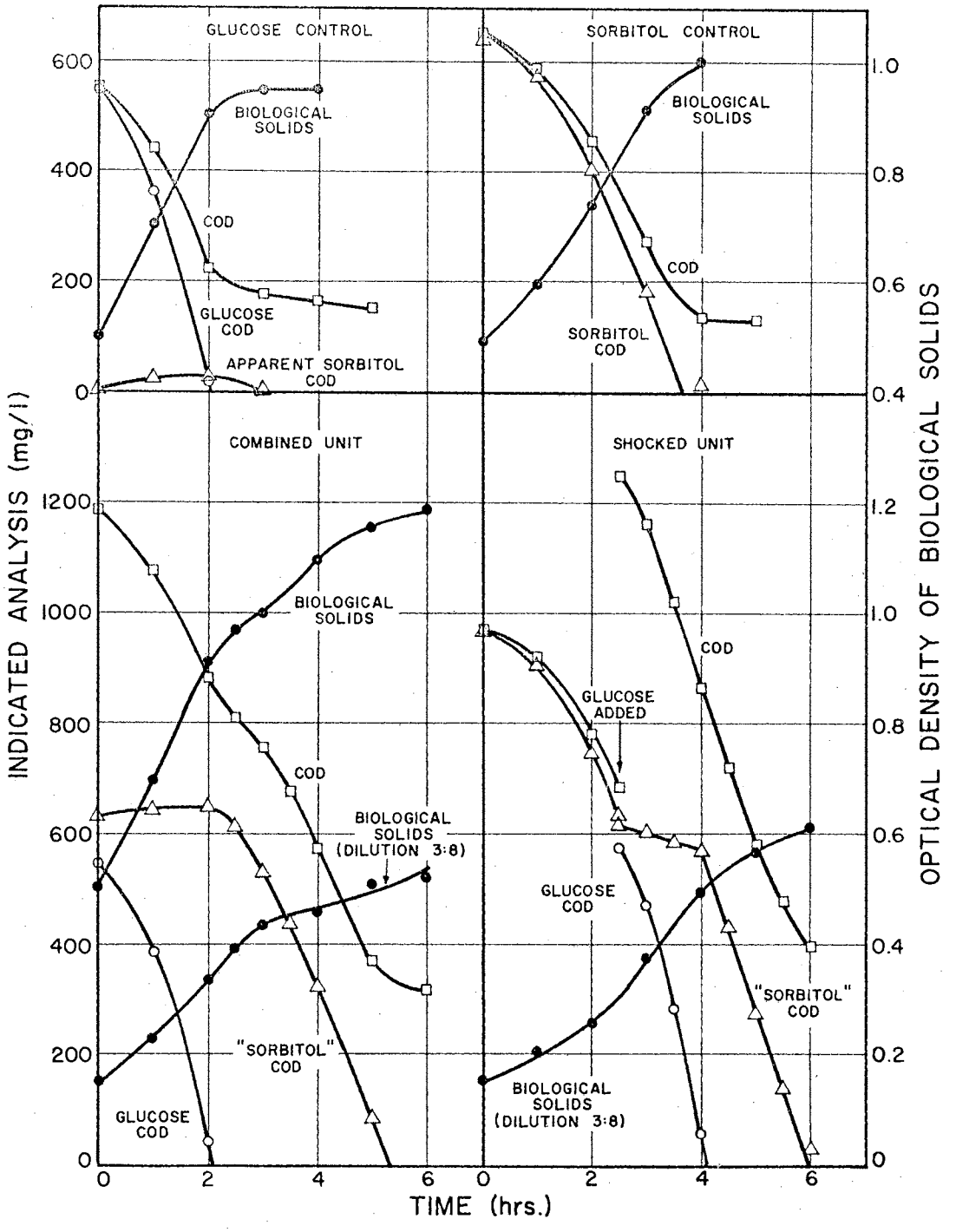


Figure 28. Metabolic response of an 8 day old culture of E. coli acclimated to sorbitol.

From the results presented thus far, it is apparent that increased ageing of a population by extended batch operation has not resulted in any apparent differences in the degrees of repression or inhibition observed. However, after a certain period of time a batch operated unit reaches equilibrium with respect to biological solids concentration, and although the age may be defined as increasing with an increase in the time of operation of the unit, the physiological age of the cells can be expected to reach an equilibrium point. For example, after a batch unit had become flocculent, the mode of operation utilized in this study resulted in a wasting of 1/3 of the cells each time the unit was fed. Therefore, the average retention time for a cell became constant, and increasing lengths of batch operation did not result in a corresponding increase in physiological age. To determine if an increase in cell age could result in changes in the extent of repression or inhibition encountered, it was felt that additional methods of ageing should be conducted in such a manner that the physiological cell age would be directly related to age expressed as a function of time. Consequently a reactor was prepared as follows: sorbitol, 7,500 mg/l; 1 M phosphate buffer (pH 7.0), 50 ml/l; all of the salts, except $(NH_4)_2SO_4$, at a concentration 2.5 times that given in the standard medium; $(NH_4)_2SO_4$ at a concentration 0.25 times that in the standard medium; and tap water, 100 ml/l. The resulting COD:N ratio was slightly greater than 160:1. The reactor was inoculated with 5 ml of actively growing E. coli, strain K-12, and was then aerated for 8.75 days. The temperature was maintained at 35^{o} C during this time. When the cells were harvested for experimentation the pH was 6.85. An analysis of the filtrate revealed that 5,800 mg/l of periodate-reactive

material expressed as sorbitol were present. In all probability, a significant portion of this material was sorbitol.

The results of the experiment (in double strength standard medium in which the buffer concentration had been increased to 50 ml/l) in which these cells were utilized are presented in Figure 29. The apparent sorbitol COD in the glucose control unit at time zero was the result of "carry over" of periodate-reactive material with the cell inoculum. Examination of the glucose control unit reveals that glucose removal was accompanied by the release of large quantities of metabolic intermediates and/or end products, and a significant portion of this material reacted with periodate. This is in marked contrast to the results obtained with actively growing young cells of this organism (Figure 27). The lag in sorbitol removal in the control system was greater than the time required for glucose removal in the combined system. Therefore, the results presented for the combined unit do not provide proof that glucose repressed the synthesis of sorbitol enzymes. It should be noted that once glucose was removed from the combined unit, the rate of "sorbitol" COD removal was significantly greater than the rate of sorbitol removal in the control. It is readily apparent that this method of ageing resulted in complete loss of the initial ability of the cells to utilize sorbitol.

3. Escherichia coli, Strain 45

Because of the lysis which was encountered with E. coli, strain K-12, subsequent studies were undertaken with a different strain. To ascertain the performance of a young cell culture of this strain, an experiment was performed with cells which had been grown by daily transfers (for 3 days) of a 1 ml inoculum to 50 ml of fresh sorbitol

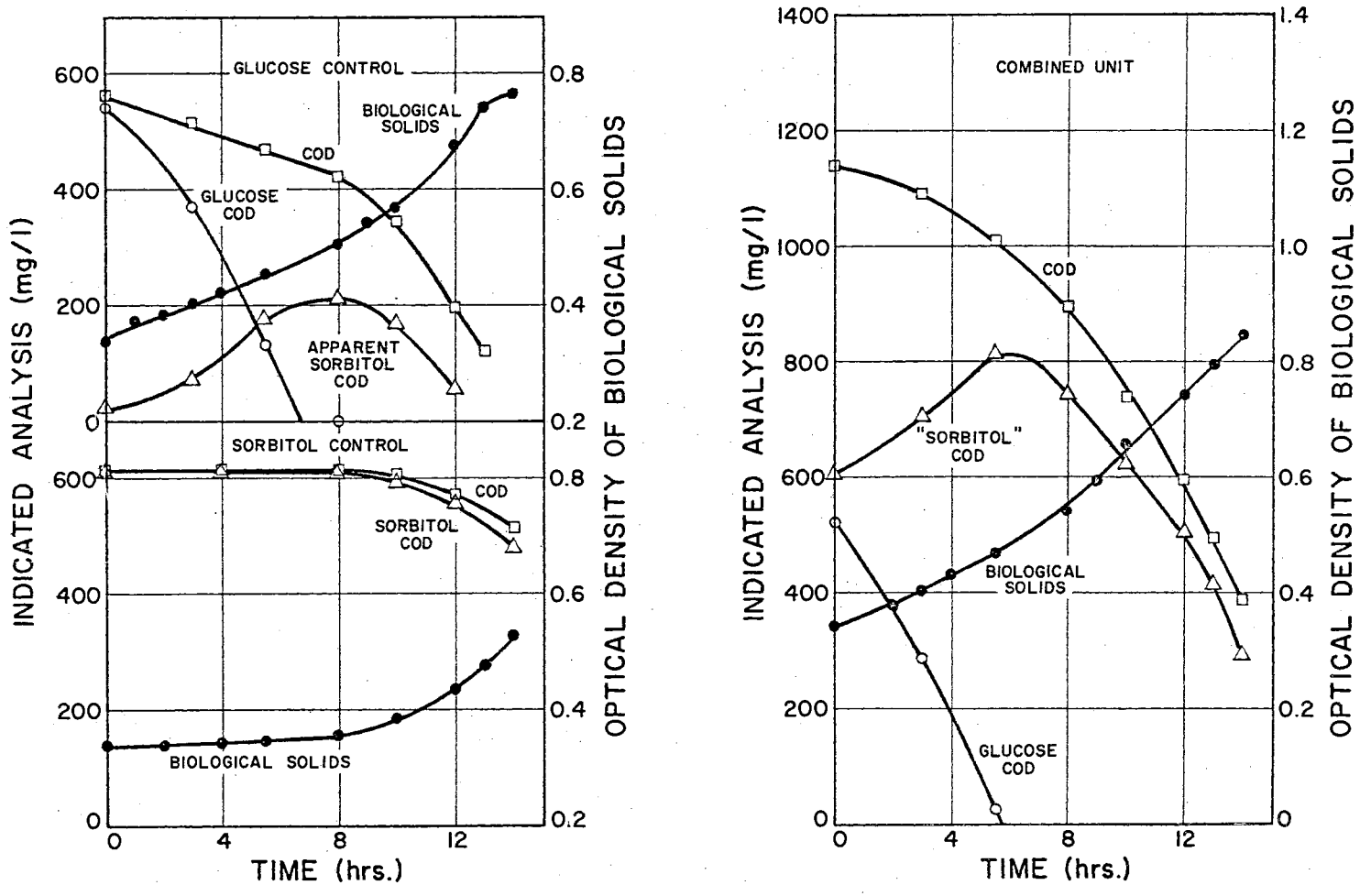


Figure 29. Metabolic response of a culture of E. coli which had been aged under nitrogen limiting conditions.

medium. Eighteen hours prior to the experiment, a portion of the cells was used to inoculate 1 liter of standard medium and this was aerated at 35° C. The cells were then harvested and used for experimentation. The results are presented in Figure 30. Small quantities of periodate-reactive intermediates were produced from glucose, and, therefore, the designation "sorbitol" COD has again been employed in the combined and shocked units. From the results presented for the combined unit, it would appear that the synthesis of the sorbitol enzymes was completely repressed in the presence of glucose, and the preformed enzymes in the shocked unit appear to have been inhibited by 83% when the glucose was added. Therefore, the response of a young cell culture of this organism was essentially the same as that observed with E. coli, strain K-12.

Various methods of ageing this organism were investigated. A reactor containing 1.5 l of standard medium (buffer increased to 30 ml/l) was inoculated with 25 ml of an actively growing culture of E. coli. Temperature was maintained at 35° C, and the culture was aerated for 4.5 days. The cells were then harvested for experimentation. The results are presented in Figure 31. Although the cells had been grown in sorbitol, the length of the endogenation period resulted in a complete loss in the initial ability of the cells to utilize this compound. In marked contrast, the cells rapidly removed the glucose. Because the cells had lost all or part of the enzymes necessary to utilize sorbitol, the results observed for the combined unit do not provide proof of repression of enzyme synthesis. It should be noted that all of the "sorbitol" COD in the combined unit was removed in slightly over 6 hours. During this same time period, the sorbitol COD

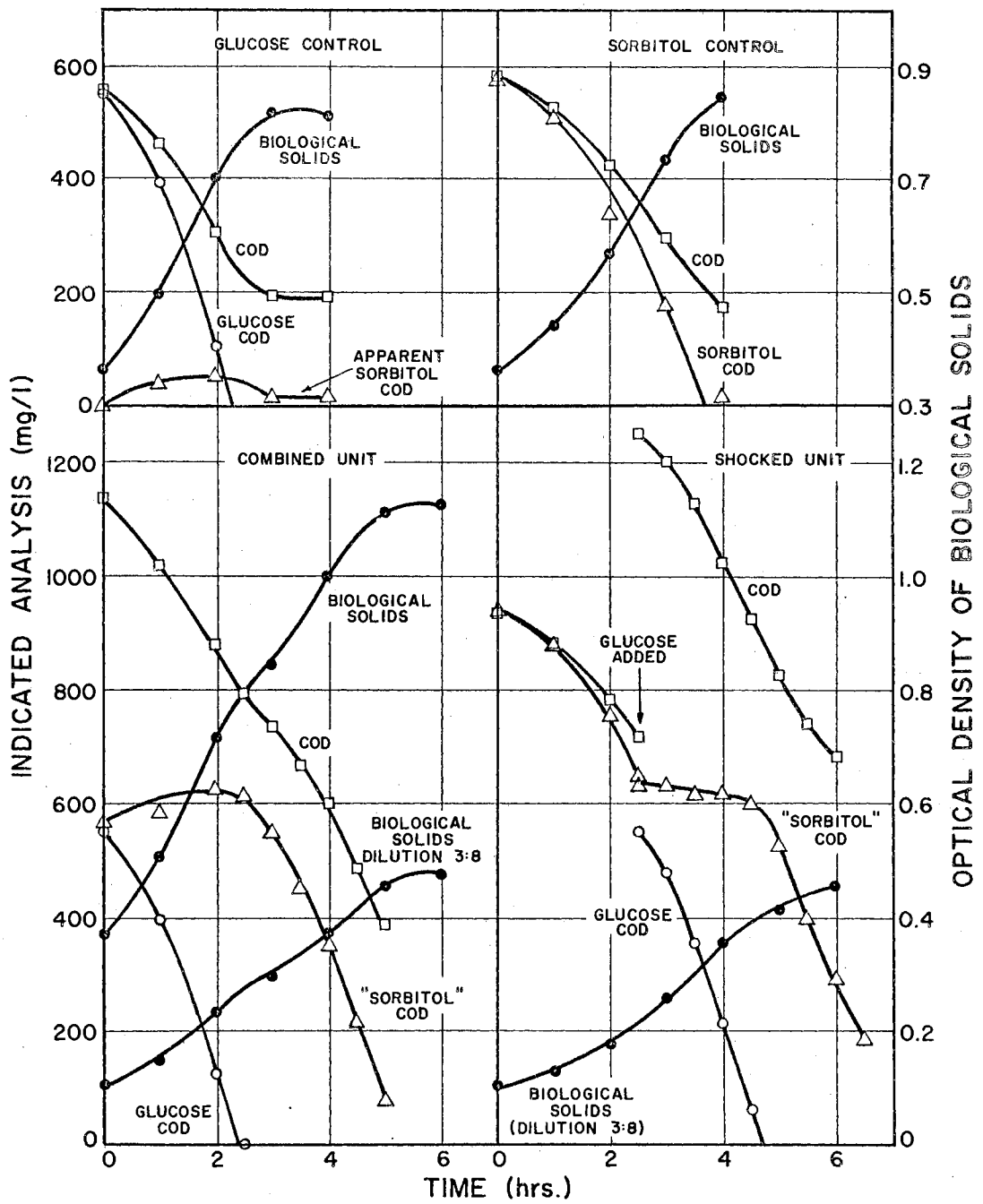


Figure 30. Metabolic response of a young cell culture of E. coli 45 acclimated to sorbitol.

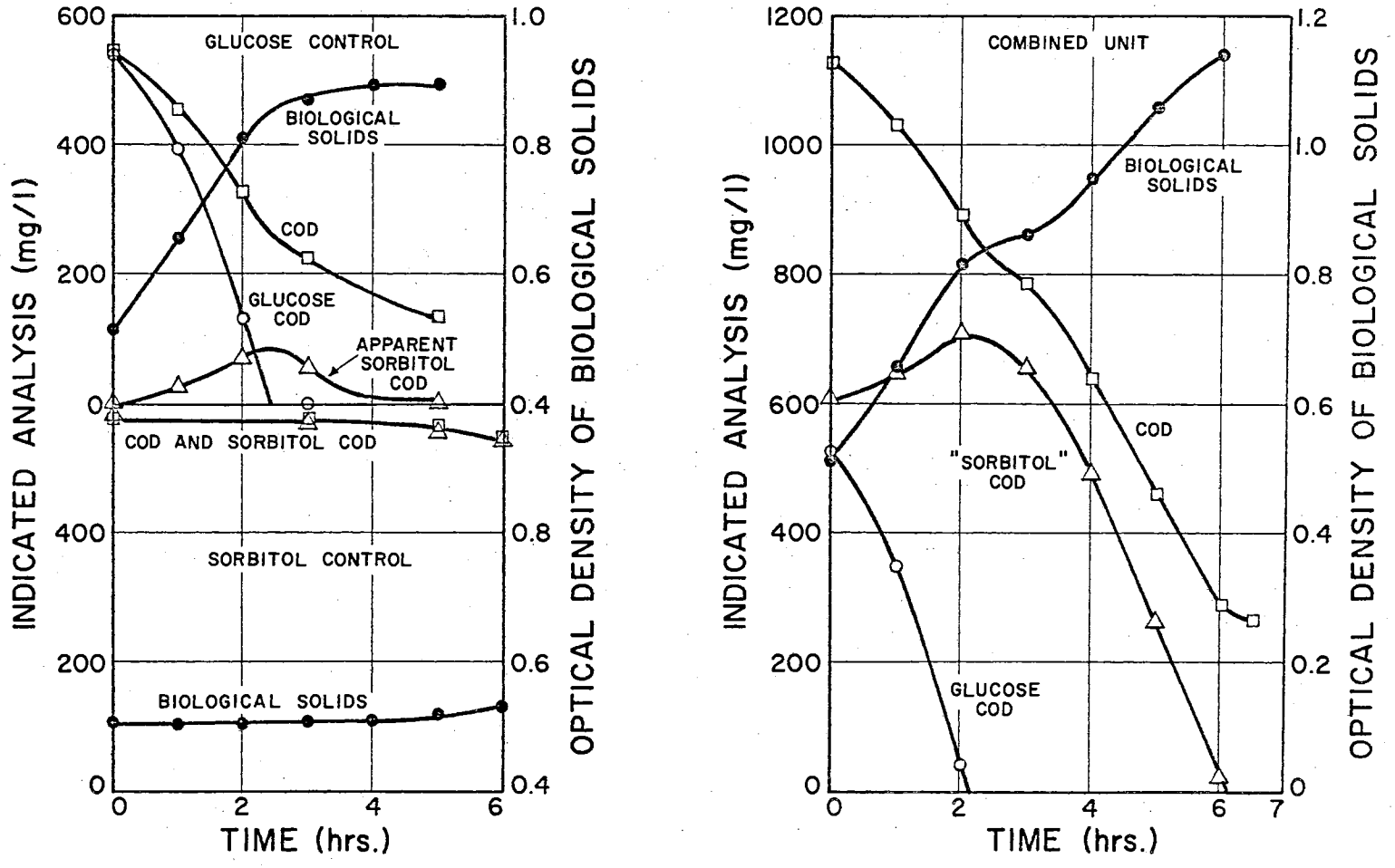


Figure 31. Metabolic response of a culture of E. coli 45 which had been aged by endogenous metabolism.

in the control unit was only reduced by 28 mg/l.

In an effort to prevent the loss of the ability of the cells to utilize sorbitol, the following method of ageing was employed. A reactor containing 2,000 mg/l sorbitol, 1.5 times the concentration of salts in the standard medium (thus providing sufficient inorganic constituents for an additional 1,000 mg/l of sorbitol), and 2 times the normal concentration of buffer was inoculated with E. coli, strain 45. After the unit had "grown up" it was fed 50 mg/l/day of sorbitol. No cells were wasted. Evaporation of the medium caused by aeration enabled the feed to be added (plus distilled water) so that the fluid volume of the reactor was returned to its initial volume at each feeding. Feeding occurred once every 24 hours, and the temperature was maintained at 35^{o} C. When the unit was to have been fed for the 7th time, the cells were harvested for experimentation. The pH of the reactor at this time was 6.75.

The results obtained from the experiment with these cells are presented in Figure 32. It is readily apparent that the cells had an extremely low complement of the enzymes necessary to remove sorbitol. In the time required for the removal of 550 mg/l of glucose, only 15 mg/l of sorbitol were removed in the control. Hence, once again, the results obtained from the combined unit are not proof that glucose repressed the formation of sorbitol enzymes. The production of periodate-reactive intermediates totally obscured any slight reduction of sorbitol which may have occurred in the presence of glucose. The "sorbitol" COD was removed from the combined unit prior to any significant amount of sorbitol removal in the sorbitol control.

A second unit (1 day younger) identical to the one just described

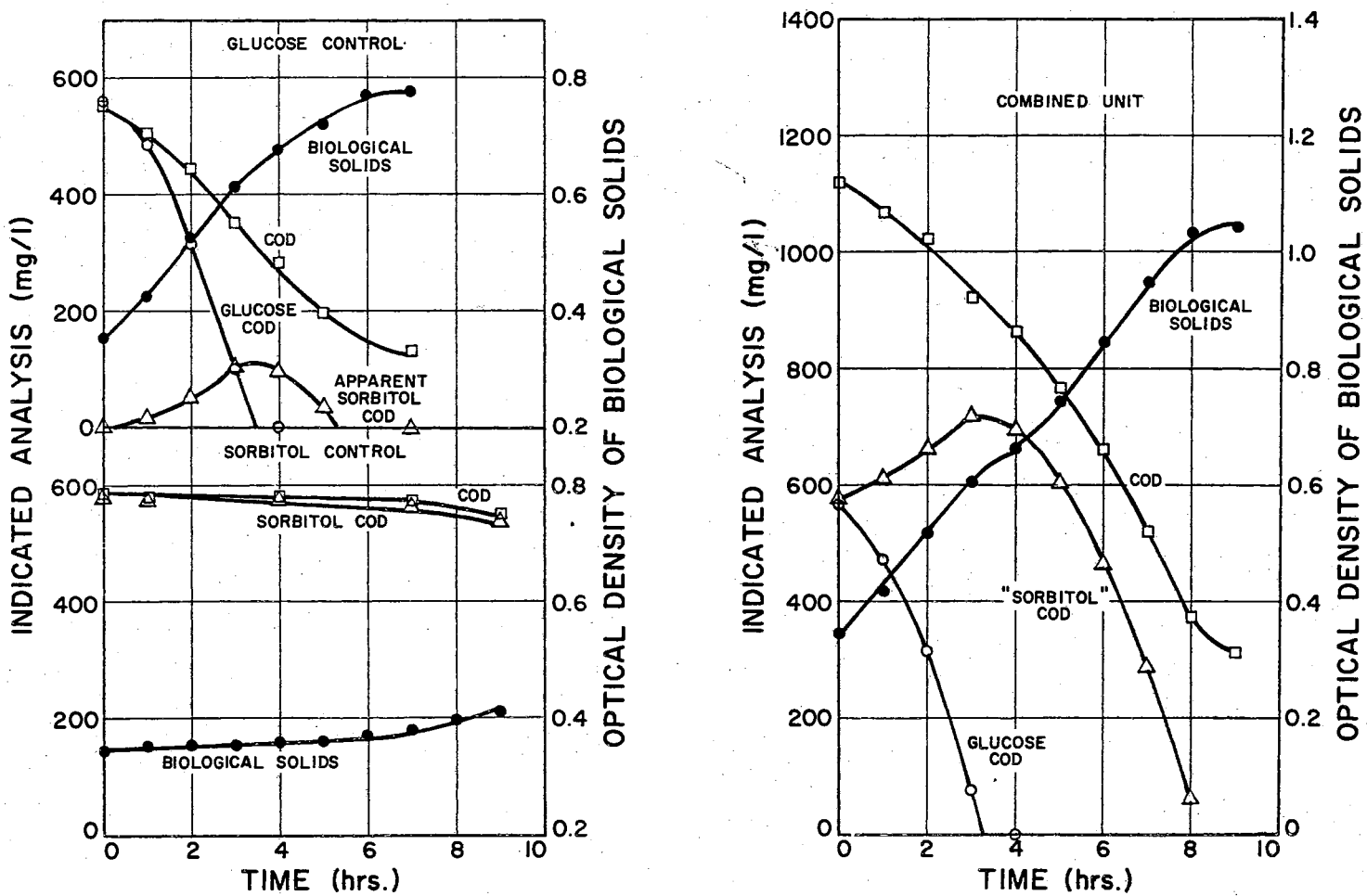


Figure 32. Metabolic response of a culture of E. coli 45 which had been aged by the addition of 50 mg/l/day of sorbitol.

had been operated concurrently with the other one. Since it was apparent that the cells had essentially lost their ability (relative to glucose) to remove sorbitol, the feeding procedure was modified in this unit on the 7th day. At this time, the physiological condition of the cells was the same as that of the cells harvested for the previous experiment. Concentrated sorbitol was added to yield a final concentration of 700 mg/l. The unit was then aerated for an additional 24 hours, and on the 8th day a portion of the cells were harvested. The reactor pH was 6.7.

Figure 33 shows the results obtained with these cells. A large enough component of sorbitol enzymes had been resynthesized so that a measurable rate of sorbitol removal in the sorbitol control unit was observed from the beginning of the experiment. The addition of glucose to the shocked unit caused a rise in the "sorbitol" COD, and when the correction for periodate-reactive intermediates from glucose was applied, the response still appeared to be 100% inhibition of the preformed enzymes. Therefore, the failure of sorbitol to be removed from the combined unit in the presence of glucose cannot be unequivocally attributed to the repression of sorbitol enzymes. Thus when the aged culture had been given enough sorbitol so that the initial sorbitol removal rate in the control unit was such that it could be measured, the response was one of total inhibition of preformed enzymes and probably total repression of enzyme synthesis. Once again, increased cell age was found to provide no relief from the extent of inhibition associated with the young cell culture. In fact, the inhibition appears to be somewhat greater than was found previously (83%). It is possible, however, that this was the result of the error inherent in applying the

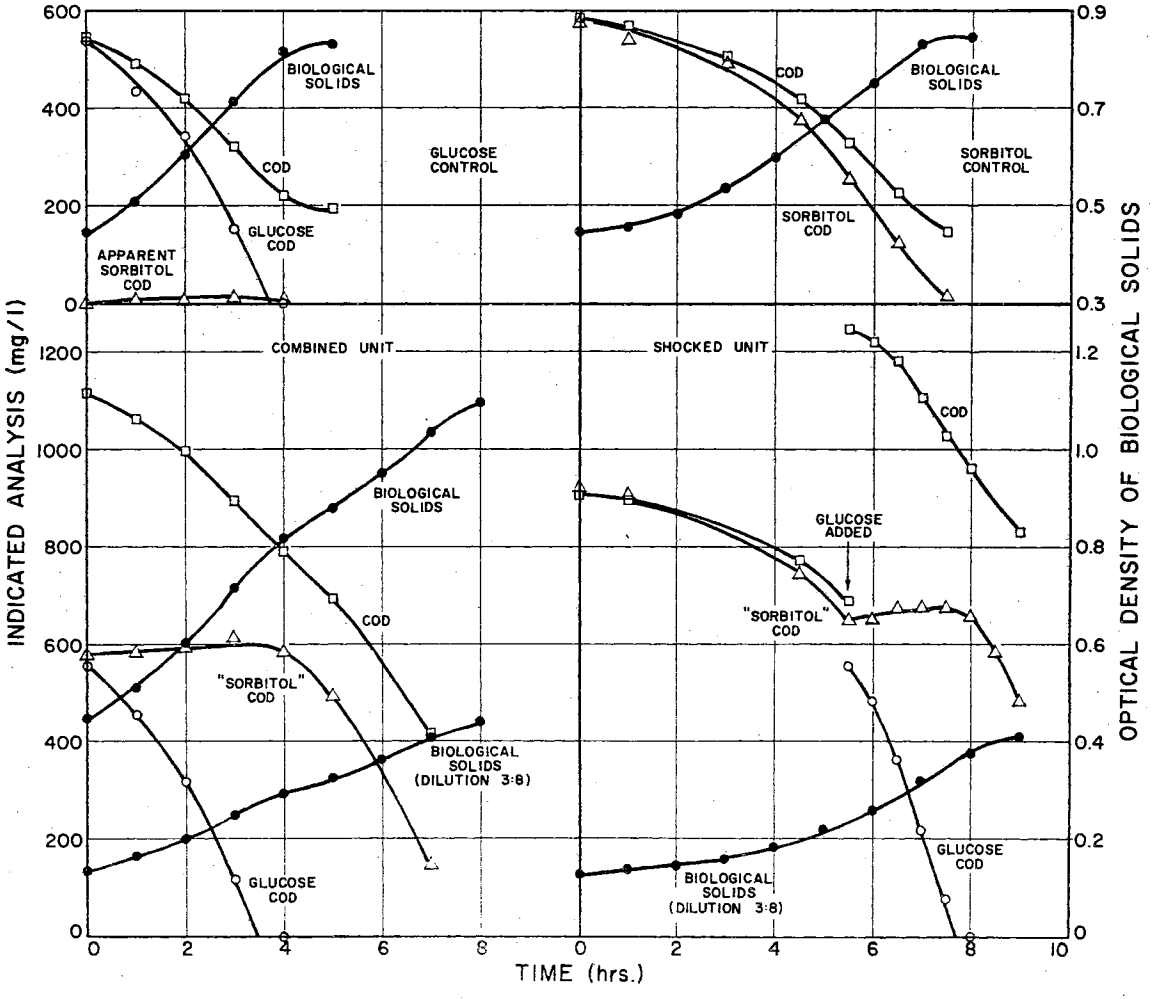


Figure 33. Metabolic response of an aged culture of E. coli 45 which had received 700 mg/l of sorbitol 24 hours before experimentation.

correction for the periodate-reactive intermediates produced from glucose.

C. Substrate Removal with Young Cell Cultures

1. Escherichia coli, Strain K-12

An additional experiment with E. coli, strain K-12, was performed to ascertain whether a reduction in the glucose removal rate caused by decreasing the temperature relative to that used in previous experiments with this organism (35^{o} C) would result in a relief of the repression or inhibition which was observed when this compound was combined with sorbitol. A young cell culture was grown by daily transfer of a small inoculum of cells to fresh sorbitol medium. The temperature was maintained at 25^{o} C. The results obtained using the cells harvested for experimentation are shown in Figure 34. No periodate-reactive intermediates were produced from glucose, and, therefore, the sorbitol COD curves are true measurements of the sorbitol concentrations. A comparison of the initial biological solids concentration in the glucose control with that found in the experiment with E. coli given in Figure 27 (92 mg/l), shows that the initial biological solids concentration was double that previously utilized; however, a greater time was required for glucose removal. The substrate removal patterns in the combined unit indicate that the synthesis of sorbitol enzymes was completely repressed in the presence of glucose. The results obtained from the shocked unit show that the preformed enzymes were inhibited by about 81%. The level of inhibition present in the shocked unit in Figure 27 was 77%. Therefore the decrease in the glucose removal rate, which resulted from a lower temperature, did not alter

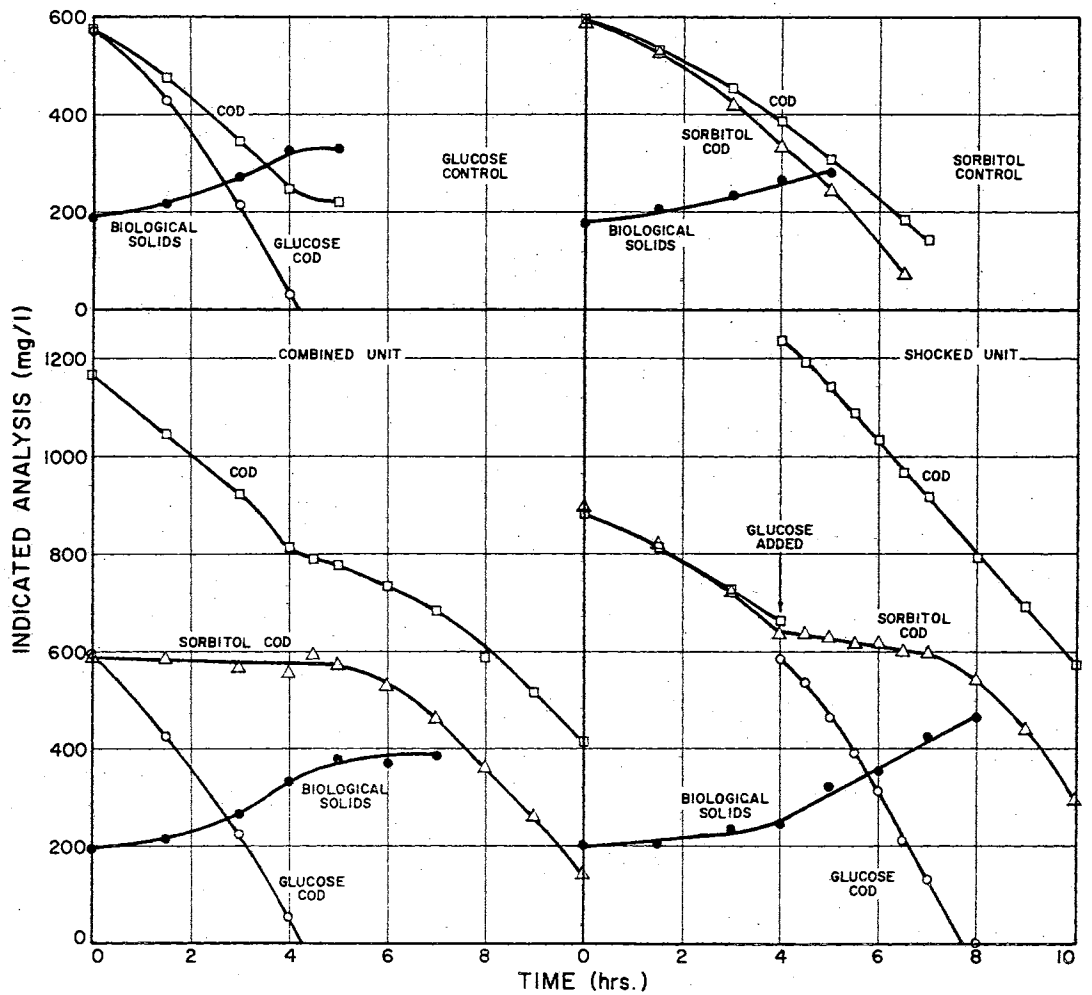


Figure 34. Metabolic response of a young cell culture of E. coli acclimated to sorbitol.

the extent of repression or inhibition previously observed.

2. Serratia marcescens

A culture of Serratia marcescens was grown by daily transfer of a 1 ml inoculum to 50 ml of fresh sorbitol minimal medium. After a total of 4 transfers, 1 liter of double strength standard medium was inoculated, and after 24 hours a portion of the cells was harvested for experimentation. The temperature was maintained at 25° C. The results of the experiment are presented in Figure 35. The rate of glucose removal in the glucose control at any time was significantly greater than the rate of sorbitol removal in the sorbitol control for the corresponding time. Examination of the results for the glucose control unit reveals that large quantities of periodate-reactive intermediates were produced, and the apparent sorbitol COD reached a maximum of 158 mg/l. The rise in "sorbitol" COD in the combined unit during the course of glucose removal was 140 mg/l. Thus it would appear that glucose completely repressed the formation of the sorbitol enzymes. At the point of glucose removal in the shocked unit, the "sorbitol" COD had risen by 65 mg/l over that concentration observed when the glucose was first added. Application of the assumptions previously outlined for calculating the interference from periodate-reactive intermediates reveals that the true sorbitol removal in the shocked unit in the presence of glucose was 75 mg/l. This corresponds to a linear removal rate of 37.5 mg/l/hour. The rate immediately prior to the shock was 155 mg/l/hour. Hence, the degree of inhibition of the preformed sorbitol enzymes, when averaged over the entire time period of glucose removal, was 76%.

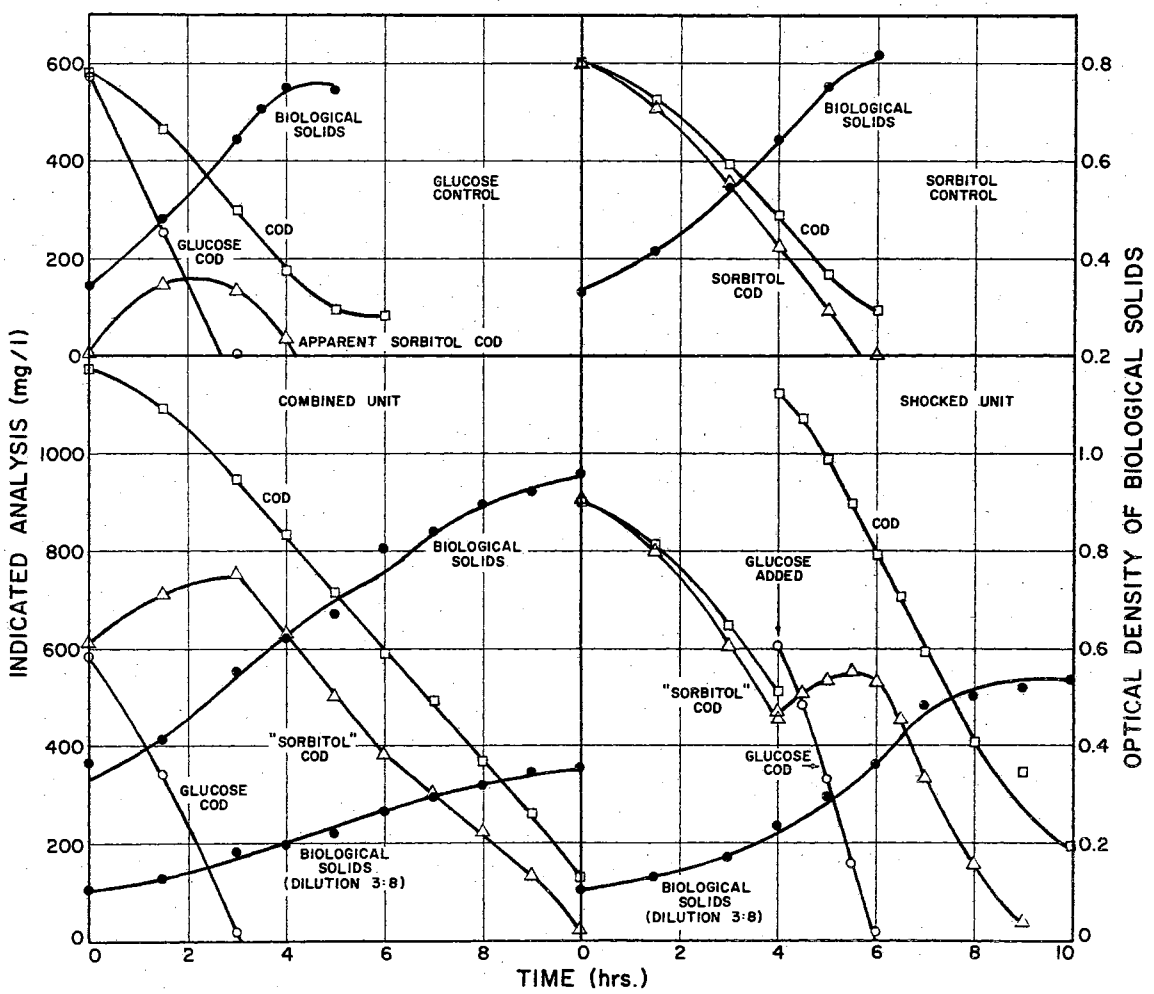


Figure 35. Metabolic response of a young cell culture of Serratia marcescens acclimated to sorbitol.

3. Escherichia intermedia

A culture of Escherichia intermedia (tentative identification) was grown at 30° C by daily transfer of a 1 ml inoculum to 50 ml of standard medium. After 3 transfers, the cells were used to inoculate 1 liter of standard medium, and a portion of these cells were then harvested for experimentation 20 hours later. The results obtained from the experiment with these cells are presented in Figure 36. No periodate-reactive intermediates were produced from glucose, and, hence, all sorbitol COD curves are true measurements of sorbitol. An examination of the results obtained from the combined unit reveals that the repression of sorbitol enzymes in the presence of glucose may have been less than complete because it appears as if sorbitol removal occurred at a continually increasing rate. However, a straight line could be drawn through the data points given at 1, 2, 2.5, 3, and 3.5 hours with a linear correlation coefficient of -0.986. Thus if the initial data point were in error, a straight line would adequately represent the removal of sorbitol in the presence of glucose. An examination of the shocked unit reveals that the sorbitol removal rate in the presence of glucose was 64% of the rate immediately prior to glucose addition.

To ascertain whether the repression of the sorbitol enzymes was less than complete, an additional experiment with young cells acclimated to sorbitol was performed. The glucose concentrations in the combined and shocked units were increased to extend the period of sorbitol removal in the presence of glucose. The results of this experiment are presented in Figure 37. In the combined unit, sorbitol removal in the presence of glucose was nearly linear. Thus it would appear that the repression of the sorbitol enzymes was complete or very

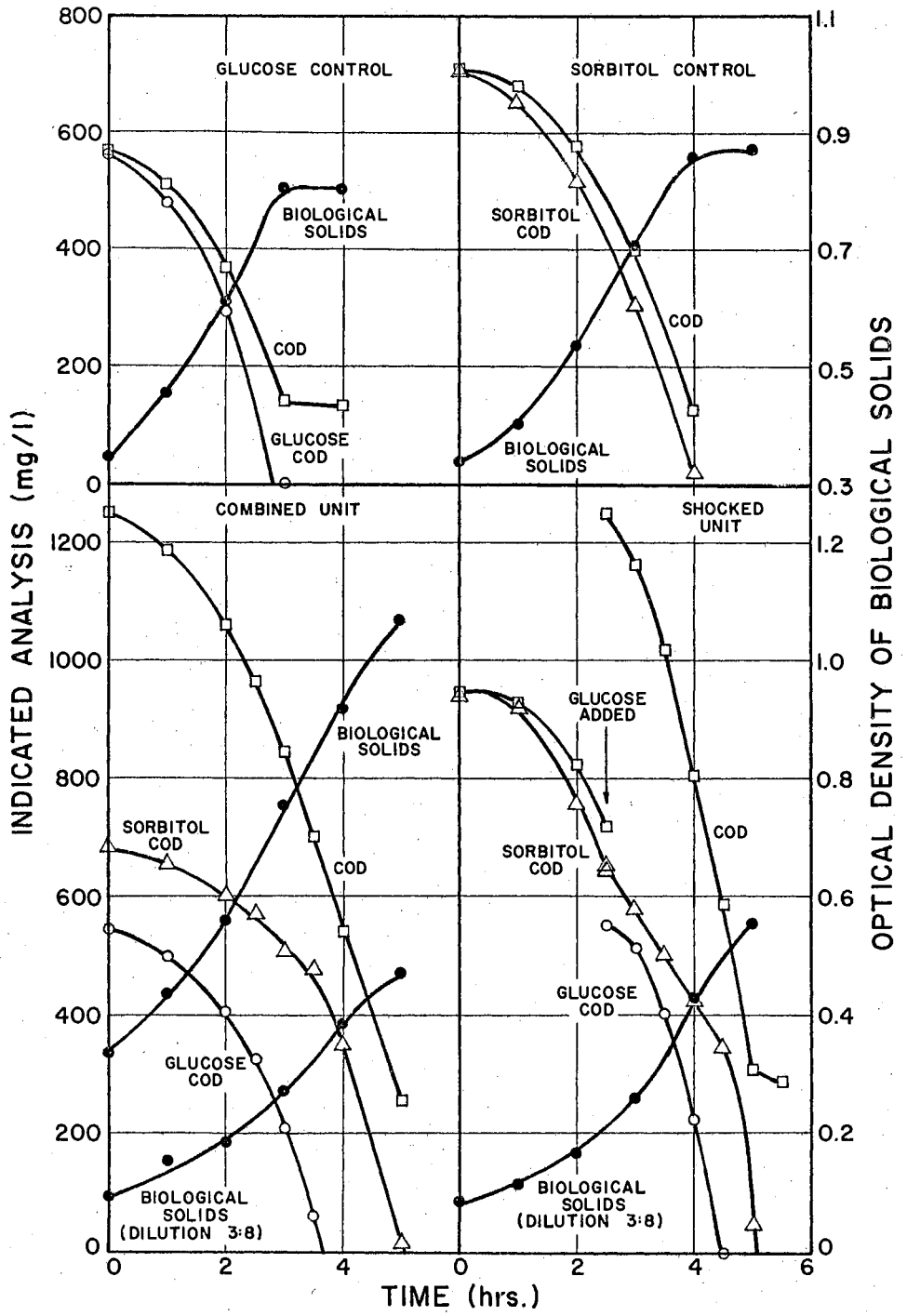


Figure 36. Metabolic response of a young cell culture of Escherichia intermedia acclimated to sorbitol. (Normal glucose concentrations.)

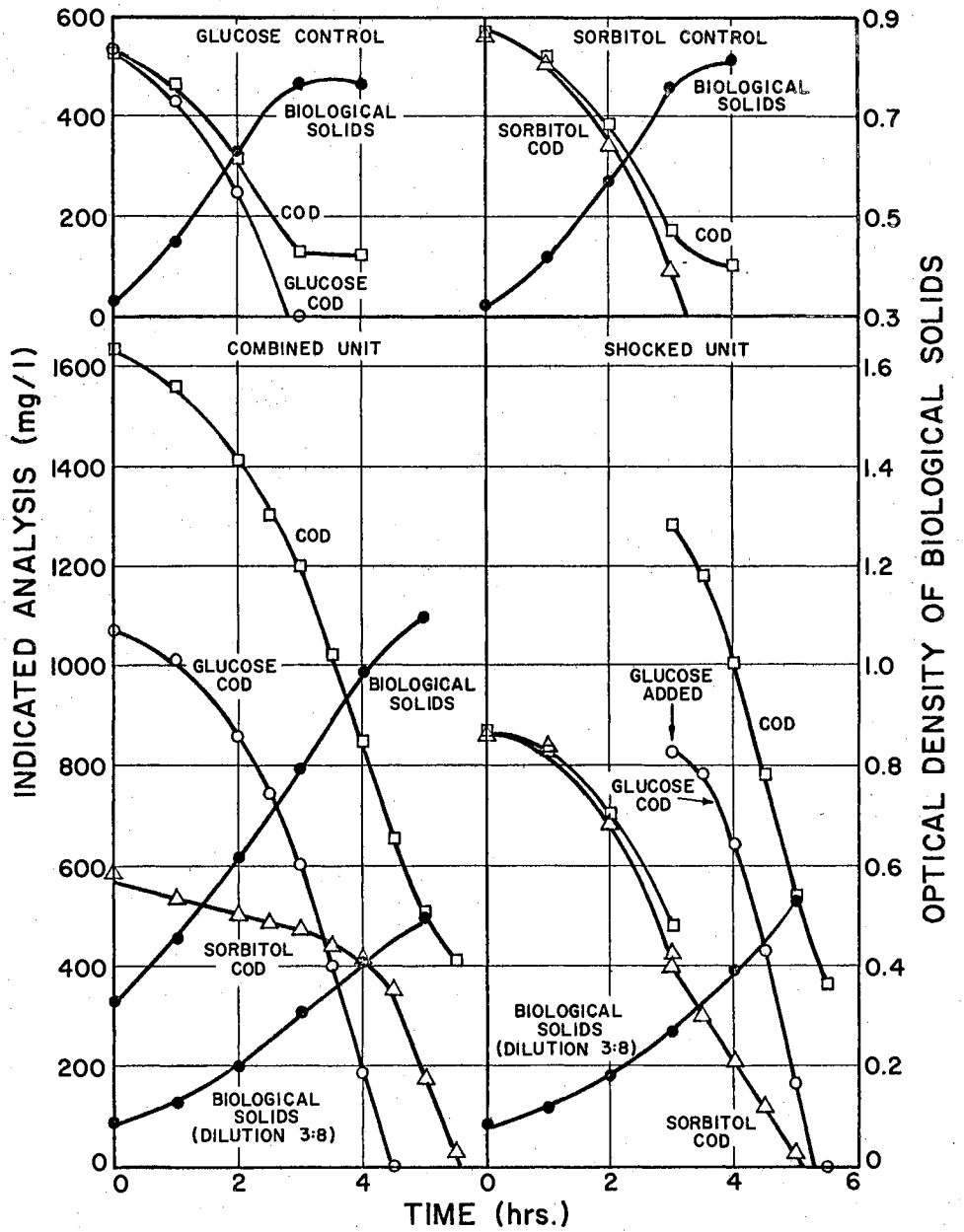


Figure 37. Metabolic response of a young cell culture of Escherichia intermedia acclimated to sorbitol. (High glucose concentrations.)

nearly complete. The reduction in the sorbitol removal rate in the shocked unit caused by glucose addition was 40%. The linearity of sorbitol removal in the presence of glucose in the shocked units of this and the preceding experiment suggests that glucose did not interfere with the transport of sorbitol into the cells.

CHAPTER V

DISCUSSION

A. Substrate Removal in Relation to Control Mechanisms

As stated in the introduction, the classification of substrate removal patterns as sequential or concurrent without consideration of the metabolic control mechanisms which dictated those patterns can lead to completely erroneous conclusions concerning biological behavior. However, it is equally important to understand that substrate removal patterns do not constitute absolute proof of the expression of a particular control mechanism. For any pattern of substrate removals observed, several interpretations of the control mechanisms which were operative are possible. Consider, for example, the results obtained from the combined unit which were presented in Figure 25. The constant sorbitol removal rate in the presence of glucose was attributed to the functioning of the preformed sorbitol enzymes (inhibited by a constant amount) and to the complete repression of further sorbitol enzyme synthesis. However, other mechanisms could have been operative. It is possible (but extremely unlikely) that there was no repression, but that increasing catabolite inhibition was caused by the continually increasing rate of glucose utilization. If the increasing percentage of inhibition was "balanced" by increased enzyme synthesis, linear substrate removal could have resulted. Alternatively, the linearity could have resulted from a combination of complete repression, varying

catabolite inhibition, and varying transport interference. At the beginning of the experiment, glucose could have caused a rather significant interference with sorbitol transport, but the catabolite inhibition of preformed enzymes could have been rather low. As increasing quantities of glucose were removed, the increased flow of glucose catabolites may have increased the extent of catabolite inhibition, but this effect on sorbitol utilization could have been negated by increased sorbitol transport which resulted from the continually decreasing external concentration of glucose. Finally, it is conceivable that the extent of repression, catabolite inhibition, and transport interference could all have varied in such a manner as to yield the linear substrate removal pattern observed.

When the results obtained from the shocked unit presented in Figure 25 are considered separately and not in relation to the combined unit (for purposes of illustration) it is again apparent that several possible control mechanisms could have interacted to produce the results obtained. The simplest and most logical interpretation of the mode of sorbitol removal in the presence of glucose is that further synthesis of sorbitol enzymes was completely repressed and that the preformed sorbitol enzymes were inhibited by a constant degree as the result of catabolite inhibition. Consequently, glucose could not have measurably interfered with sorbitol transport. However, as in the case of the combined unit, there could have been no repression but continually increasing inhibition, or complete repression and a "balancing" of catabolite inhibition with transport interference, or a rather involved interaction among the mechanisms of incomplete repression, increasing catabolite inhibition, and decreasing transport interference.

Therefore, the statement in Chapter IV that the preformed sorbitol enzyme system was inhibited by 46% should be viewed as one which accurately describes the nature of sorbitol removal in the presence of glucose in relation to the most logical and probable control mechanisms and not one which is unequivocally correct in terms of the basic control mechanisms which could have been operative.

When the results obtained from the combined and shocked units of Figure 25 are considered in conjunction, the most logical explanation, by far, is that further synthesis of sorbitol enzymes was completely repressed in the presence of glucose and that the inhibition resulted from a constant degree of catabolite inhibition. Although it is possible that catabolite inhibition and transport interference could have "balanced" in each case to yield linear removals, the probability of this occurrence would seem to be very low. The combination of no enzyme repression and continuously increasing inhibition could have produced the substrate removal patterns observed, but if this were the case, the cells would have wasted energy and amino acids synthesizing enzymes which would have been inhibited at a continually increasing rate; therefore, this is a "self-defeating" combination of control mechanisms and as such would appear to have had little chance of having developed in the evolutionary process. Finally, the possibility of the complex balancing of incomplete repression, increasing catabolite inhibition, and decreasing transport interference cannot be eliminated, but the probability that all of these mechanisms could have "balanced" in both the shocked and combined units appears very remote.

The preceding discussion illustrates that an explanation of substrate removal patterns in terms of control mechanisms, while perhaps

not absolutely correct in terms of biochemical mechanisms, can be entirely adequate from an engineering viewpoint. Therefore, the fact that one cannot (from experiments of the type conducted here) determine with absolute certainty the exact control mechanisms which were operative, should not discourage practical utilization of these mechanisms in explaining the observed behavior of a biological system. For example, the explanation that the presence of glucose completely represses further synthesis of sorbitol degrading enzymes and inhibits the preformed enzymes by a constant percentage enables the engineer to calculate the amount of sorbitol removal that can be expected to occur at any time. It is for this reason that all of the results presented in Chapter IV were discussed in terms of enzyme repression and inhibition, for it is only if one uses descriptions that can subsequently be transformed into meaningful mathematical relationships that the results can be of any tangible value for the prediction of substrate removal rates.

B. Heterogeneous Galactose-Acclimated Cells

The experiments with the heterogeneous population acclimated to galactose were primarily measurements of inhibition of preformed enzymes. In every case, except for the results in Figure 2, the rates of galactose removal in the control units were essentially constant. Therefore, there was no apparent synthesis of the enzymes necessary for galactose removal. In every experiment, the glucose was removed from the combined unit within the time period required for galactose removal in the control, and, therefore, it would appear that the results obtained in the combined units of Figures 3-9 should be interpreted

entirely from the viewpoint of inhibition of preformed enzymes. In all of the shocked units of Figures 3-9, the rate of galactose removal up to the point of glucose addition was constant. Therefore, if there was no new synthesis of the galactose enzymes during the time period of glucose removal (either because there was no new synthesis occurring anyway or because of complete repression) the galactose removal curves after glucose addition can be used to calculate the true inhibition of the preformed enzymes. If there was some new synthesis of the galactose enzymes occurring during the course of glucose removal, then the degrees of inhibition would be underestimated when the removal rates after the addition of the shock are compared to their respective removal rates prior to the addition of glucose.

As indicated previously, numerous investigators (8, 10, 11, 13) have reported that glucose inhibits galactose transport in E. coli. Therefore, in any study of the inhibition of galactose removal, one must recognize that this is a possible cause. It has also been shown (11) that glucose catabolites only partially repress the enzymes of the gal operon. This also could be an important factor in some of the shocked units where the galactose removal rate after glucose had been removed increased to a rate greater than that observed prior to the shock, for it is possible that galactose enzyme synthesis may have been occurring during glucose removal. Finally, Stumm-Zollinger (2) observed linear removal of galactose in the presence of glucose when a high initial solids inoculum was used. This would indicate that the inhibition which she observed was due almost entirely to catabolite inhibition, and consequently the effect caused by transport interference was small or entirely absent.

When one considers all of the possible mechanisms by which the presence of glucose can influence the mode of galactose removal, it becomes apparent that some caution must be exercised when interpreting the results of an experiment in which these compounds were present. The results presented in Figure 5 provide a good example of a case in which all of the mechanisms just discussed were probably operative to some extent. The rate of galactose removal in the shocked unit prior to the addition of glucose was 95 mg/l/hour. The ratios of the rates after the shock to the rate before the shock continually increased and reached 0.905 (glucose = 32 mg/l) after 6 hours of experimentation. Since the ratio of the rates was 0.147 when the glucose concentration was 468 mg/l, one might conclude that the inhibition observed was primarily the result of transport interference, and that catabolite inhibition had little if any effect. However at 8.5 hours, the rate of galactose removal was 150 mg/l/hour. Therefore, it is entirely possible that there was some synthesis of galactose-degrading enzymes during the course of glucose removal. If this were the case, then catabolite inhibition would have been exerted to a greater extent than was attributed to it on the basis of the observation of the ratios of the rates. Indeed, the results obtained from the combined unit indicate that this was the case. As previously indicated, the linearity of substrate removal in the galactose control unit implies that there was no synthesis of galactose enzymes in the combined unit during the time period of glucose removal. At 3.5 hours, when the glucose concentration was nearly zero, the ratio of the rate of galactose removal in the combined unit to the rate in the control unit was 0.594. Therefore, the results from the combined unit clearly indicate that

catabolite inhibition played an important role in determining the amount of inhibition of galactose removal observed. This indicates that the percentages of inhibition, which can be calculated from the ratios presented in the last column of Table II, may be regarded as the minimum estimate of the true extent of the inhibition of the preformed galactose enzymes. Only if no galactose-degrading enzymes were synthesized during the course of glucose removal do these ratios provide the means of calculating the actual inhibition of the preformed galactose enzyme system at any time.

Now that the complexity of trying to determine which metabolic control mechanisms were operative in the glucose-galactose experiments by examination of the substrate removal curves has been amply demonstrated, it should be apparent what the values in columns 10 and 11 in Table III actually do or do not represent. One would expect that the values in these two columns would be equal only if: a) glucose caused a complete repression of the synthesis of the galactose enzymes, b) there was no interference with the transport of galactose into the cell, and c) that there was no variation in the degree of catabolite inhibition during the course of glucose removal. The values in column 10 are probably true estimates of the extent of inhibition of preformed enzymes which resulted from a combination of catabolite inhibition and transport interference, but this is only true of the values in column 11 if there was no further synthesis of the galactose enzymes during the course of glucose removal.

In view of the results which suggest the dual operation of two distinct inhibition mechanisms, i.e., catabolite inhibition and transport interference, it would seem that an additional criticism of the

work of Stumm-Zollinger (4) may be warranted. When she performed her experiment with the young cell population acclimated to galactose, the initial substrate concentrations were approximately 200 mg/l each of glucose and galactose, and it was observed that glucose inhibited galactose utilization. However, when the experiment with the reconstituted population was performed, the initial substrate concentrations were only 10 mg/l of each compound, and she concluded (wrongly) that there was no inhibition. Interference with galactose transport could have played an important role when the initial substrate concentrations were 200 mg/l, but it should be recalled that Horecker et al. (8) have shown that 10 mg/l of glucose had virtually no effect on galactose uptake with E. coli. Therefore, the experiments were so designed that transport interference could have contributed to the inhibition in the first experiment but probably not in the second. It is apparent that the results of both of these experiments could have been essentially duplicated with a pure culture where the predominant inhibition mechanism was the interference by glucose with the uptake of galactose. This illustrates another important consideration necessary for studies of inhibition of preformed enzymes; where transport interference plays an important role, variations in the ratios and quantities of the substrate concentrations present at the start of an experiment will provide varying degrees of inhibition. Therefore, substrate concentrations and their respective ratios must be considered whenever a comparison between systems within an experiment or between systems in different experiments are made.

The results of the experiments with the galactose-acclimated cells show that the designation of cells as young or old (in accordance with

the length of the batch operation period) is irrelevant to the degree of inhibition to be expected. Glucose was found to inhibit galactose removal even though the age of the culture was varied from 1 to 48 days during the period of experimentation, and the extent of inhibition which was observed at any particular time was entirely unpredictable. The variations in the extent of inhibition were found instead to be correlated to the frequent variations in the species of organisms present.

C. Heterogeneous Sorbitol-Acclimated Cells

Since periodate-reactive intermediates were produced from glucose in every experiment with the heterogeneous sorbitol-acclimated population, it was necessary to employ several assumptions when the amount of interference was calculated in the combined and shocked units. Although the results presented in Table V indicate that the assumptions which were made provided a means of obtaining good estimates of the interference in each of these units, exact values were not obtained. Therefore, it would be unrealistic to attempt to make a precise evaluation of the sorbitol removal curves presented in Figures 19 and 20 on the basis of the probable mechanisms which produced the various shaped removal curves, for much of the variation in the sorbitol removal curves may have been more the result of experimental error than the result of any differences in the control mechanisms which were operative at the time of each experiment.

There is another method by which the amount of sorbitol removal in the combined and shocked units may be estimated. If the assumption that any given amount of glucose removal in the combined or in the shocked unit produced a quantity of periodate-reactive intermediates

from glucose identical to that quantity produced for an equivalent amount of glucose removal in the glucose control unit is true, then it should also be true that any given amount of glucose removal in the combined or in the shocked unit produced a reduction in the total COD identical to that reduction for an equivalent amount of glucose removal in the glucose control unit. Therefore, at any time during the course of glucose removal in the combined or shocked unit, any reduction in total COD in excess of that calculated from the glucose control unit may be attributed to a reduction in COD caused by sorbitol removal. If the sorbitol control unit indicates that the reduction in COD and sorbitol COD was the same, i.e., no intermediates, then the COD removal in the combined or shocked unit in excess of that attributable to glucose removal may be assumed equal to the sorbitol COD removed.

Before proceeding further, it is advisable to examine the assumptions just given and see what is actually implied in their use. First, even though the assumptions concerning the production of equivalent amounts of periodate-reactive intermediates for equivalent amounts of glucose removal may have been true, it is not absolutely necessary that there were equivalent reductions in COD values. However, if the amounts of sorbitol removal calculated using the assumptions concerning COD values are widely divergent from those removals obtained using the assumptions concerning the equivalent production of periodate-reactive intermediates, there is no reason to believe that either set of assumptions was true. On the other hand, if the values for sorbitol removal calculated according to the COD assumptions are the "same" as those obtained using the periodate-reactive assumptions, this is additional evidence to support (but not prove) the validity of the assumptions

which were made to calculate the amount of periodate-reactive intermediates produced from glucose in the combined and shocked units. Secondly, it would be absurd to expect to obtain identical answers by these two methods; the introduction of a separate method of calculation (COD method) introduces an additional source of experimental error, and when this is coupled with the error inherent in "reading" the values from the plotted curves, it is readily apparent that the two methods of calculation can be expected to yield only approximately the same answers. Consider the results presented in Table VII for the combined unit of Figure 14 (discussed in the following paragraph). If the values in columns 4 and 5 are 99% accurate (± 1% error), the value in column 6 is correct within approximately ± 20. If the values in columns 7 and 8 are 99% accurate, the value in column 9 is correct within approximately ± 10. Therefore, the value in column 10 (column 6 -column 9) can be expected to be correct within about ± 30. Since there was also some error inherent in obtaining the value presented in column 11, the values in columns 10 and 11 should be considered the "same." It was felt that differences of 40 or less between the values in columns 10 and 11 should not be considered significant because of the error inherent in the method used to obtain them.

The results obtained by applying the assumptions concerning the equivalent reductions in COD to each of the combined units which were presented in Figures 10-18 are presented in Table VII. The values given in column 10 represent the reduction in total COD in each of the combined units which can be attributed to sorbitol removal. An examination of all of the sorbitol controls presented in Figures 10-18 reveals that intermediate production was very small and in most cases

TABLE VII

COMPARISON OF THE SORBITOL REMOVALS IN THE COMBINED UNITS OBTAINED BY UTILIZING TWO DIFFERENT METHODS OF CALCULATION FOR ALL OF THE EXPERIMENTS WITH THE HETEROGENEOUS SORBITOL-ACCLIMATED POPULATION

1	2	3	4	5	6	7	8	9	10	11
Fig. No.	Age days	Glucose removed at gluc. = 100 mg/l comb. unit	Initial COD comb. unit	COD at gluc. = 100 mg/l comb. unit	COD removed comb. unit	Initial COD glucose control	COD in gluc. control for removal given in col. 3	COD removed glucose control	Col.6-col.9	Sorbitol COD removed from Table VI column 4
10	2	443	1129	866	263	543	307	236	27	28
11	6	443	1132	877	255	552	332	220	35	40
12	10	448	1123	863	260	550	358	192	68	75
13	14	446	1138	840	298	557	393	164	134	143
14	19	452	1125	940	185	550	398	152	33	66
15	24	436	1112	801	311	552	308	244	67	103
16	29	458	1140	760	380	559	266	293	87	99
17	36	503	1167	657	510	598	202	396	114	116
18	45	443	1108	827	281	534	296	238	43	55

nonexistent. Therefore, the values in column 10 essentially represent true sorbitol removals, and any attempt to convert the COD removals to equivalent sorbitol removals by utilizing the results in the sorbitol control units would introduce more error than benefit. The sorbitol removals were calculated in such a manner so that the results could be directly compared to those presented in column 4 of Table VI (reproduced in column 11). In all cases the values are quite close, and the differences between them could easily have resulted entirely from experimental error rather than as a result of any inherent differences emanating from the use of two separate sets of assumptions. Therefore, the validity (allowing for small errors) of the assumptions concerning the periodate-reactive intermediates used in calculating the sorbitol removals in the combined units has been further substantiated.

When the assumptions concerning the equivalent reductions in COD values were applied to each of the shocked units presented in Figures 10-18, the results given in Table VIII were obtained. Again the method of calculation was such that the COD reductions given in column 10 could be directly compared with the values given in column 7, Table VI, and reproduced here in column 11. A comparison of the values presented in columns 10 and 11 reveals that only the differences obtained from the experiments in Figures 11 and 17 appear large enough to perhaps be attributable to something beyond the normal variations induced as the result of experimental error. An examination of Figure 11 indicates that the glucose was not added as a shock load until 7 hours after the experiment was initiated, and its concentration did not reach 100 mg/l until after 10 hours. Therefore, the differences in this case might be attributable to the occurrence of a sufficient change in the metabolic

TABLE VIII

COMPARISON OF THE SORBITOL REMOVALS IN THE SHOCKED UNITS OBTAINED BY UTILIZING TWO DIFFERENT METHODS OF CALCULATION FOR ALL OF THE EXPERIMENTS WITH THE HETEROGENEOUS SORBITOL ACCLIMATED POPULATION

1	2	3	4	5	6	7	8	9	10	11
Fig. No.	Age days	Glucose removed at glucose = 100 mg/l shock unit	COD after shock	COD at glucose = 100 mg/l shock unit	COD removed shock unit	Initial COD glucose control	COD in gluc. control for removal given in col.3	COD removed glucose control	Col.6-col.9	Sorbitol COD removed from Table VI col.7
10	2	449	1380	1097	283	543	304	239	44	65
11	6	470	1444	1145	299	552	321	231	68	126
12	10	455	1202	839	363	550	354	196	167	188
13	14	454	1261	948	313	557	389	168	145	179
14	19	460	1401	1160	241	550	401	149	92	114
15	24	450	1233	874	359	552	308	244	115	138
16	29	480	1342	920	422	559	258	301	121	161
17	36	518	1271	698	573	598	187	411	162	267
18	45	451	1145	763	382	534	294	240	142	152

patterns of the cells in the shocked unit during the 7 hour period so that intermediate production from glucose varied somewhat from that response observed in the glucose control. The largest difference in values appears in the results obtained from Figure 17. The majority of this discrepancy was probably the result of an error in the initial COD value obtained immediately after the shock. The addition of 618 mg/l glucose COD produced an increase of only 565 mg/l of total COD. Thus it appears that the COD value immediately after the shock was in error by 53 mg/l. If this were the case, the difference in the values in columns 10 and 11 would amount to only 52 mg/l. Therefore, with the possible exceptions of the shocked units in Figures 11 and 17, the assumptions concerning the equivalency of periodate-reactive intermediate production were again supported. Even in Figures 11 and 17, the results did not differ sufficiently to completely invalidate the assumptions utilized in obtaining them. Although there was some decrease in the extent of enzyme inhibition encountered in the experiments presented in Figures 11-13, the extent of inhibition present after this time was entirely random. When the degrees of inhibition obtained up to the time of bulking are considered together (Figures 11-16) there is no noticeable trend in the results. Thus although there was a rather significant change in the characteristics of the microbial population after the first 28 days of batch operation (resulting in the establishment of a predominating population having the characteristics of fungi) there still is little evidence to indicate that increased ageing can produce a decrease in the extent of inhibition associated with young cell cultures. It would seem that the results were due, instead, to whatever organisms happened to be

predominating at the time of each experiment. The results presented in Figure 19A-E suggest that in all cases the synthesis of sorbitol-degrading enzymes in the presence of glucose was completely or very nearly completely repressed, and, hence, cell age appeared to provide no decrease in the extent of repression. Gaudy et al. (22, 24) also found that there was no relief of the repression mechanism in old or intermediate age sorbitol-acclimated systems. Therefore, it appears that complete or very nearly complete repression of sorbitol enzyme synthesis in the presence of glucose is a very general phenomenon. This response was also observed with all of the pure cultures examined in this study. Although substrate removal in young cell populations has, in general, been found to be strongly inhibited in the presence of glucose as a result of enzyme inhibition, this has not always been observed. For example, Gaudy et al. (27) found that in one case the preformed sorbitol enzymes in young cells were only inhibited by about 42% (assuming complete repression) in the presence of glucose. Dulcitol removal with young dulcitol-acclimated cells (29) and in some cases glycerol removal with young glycerol-acclimated cells (31) have also been shown to be only partially inhibited in the presence of glucose. This provides additional proof that cell age is not responsible for the degrees of inhibition observed. Instead, it would seem that the development of young cell heterogeneous populations tends to favor a selection of biological species where enzyme inhibition may be more pronounced, and for this reason significant degrees of inhibition have frequently been observed with such cell populations. When one considers that the degree of inhibition of the preformed sorbitol enzymes observed with each of the pure cultures was strictly a function of the

organism studied, it is evident how the varying degrees of enzyme inhibition observed with the heterogeneous sorbitol-acclimated population can be directly related to predominance changes.

D. Pure Culture Systems

1. Blue Organism

It could be argued that the numerous predominance changes which occurred during the operation of the batch unit with heterogeneous populations acclimated to sorbitol resulted in the presence of a rather young cell population at all times. However, the results obtained with the pure culture of Blue organism clearly indicate that increased cell age caused by extended batch operation did not result in a change in the degrees of enzyme repression or inhibition observed. Although this unit never became flocculent and hence 2/3 of the cells present were wasted at each feeding, it was fed only once every 48 hours, and, therefore, the average retention time for the cells should have been essentially the same as for the heterogeneous populations which did flocculate but were fed once every 24 hours. As explained at the beginning of this chapter, the substrate removal patterns can be explained on the basis of several control mechanisms. However, when the results are examined on the basis of the most logical and most probable mechanisms, it can be concluded that sorbitol enzyme synthesis was always completely repressed while glucose was present and that the degree of inhibition of preformed sorbitol enzymes was constant. Transport interference appeared to play no part in the inhibition mechanism. The results from this series of experiments offer conclusive proof that increased cell age attained by extended batch operation

did not result in any change in the metabolic control mechanisms which were operative.

2. Escherichia coli

When increased ageing as a result of batch operation was studied with E. coli, strain K-12, it was again found that increased cell age did not induce any changes in the extent of repression or inhibition encountered. The problem of lysis of this culture made it impossible to extend the length of the study beyond the experiment with an 8 day old culture. Hence, the limited time span makes it impossible to conclude that prolonged batch ageing could not affect the degree of repression or inhibition. However from the results discussed thus far, there is no reason to believe that increased ageing would have produced any changes. Since compressed air was utilized to aerate the batch unit and since there was no way to remove any bacteriophage which could have been present, the apparent reason for the lysis was the introduction of an E. coli phage into the unit. It will be recalled that when filtrate from the lysed unit, which had been passed through 0.45 μ membrane filters, was used to inoculate young cell cultures of the organism, they also underwent lysis. Since the actual cause for the lysis was in no way related to the objectives of the research reported herein, no attempt was made to determine if phage or some other factor was responsible.

The results which were obtained from the culture of E. coli, strain K-12, which had been grown in nitrogen-limited sorbitol minimal medium (COD:N slightly greater than 160:1) and then allowed to "endogenate" in this medium, indicated that the cells had completely lost the enzymes necessary to metabolize sorbitol when they were

examined 8.75 days after the medium had been inoculated. In contrast, the cells still had a marked ability to remove glucose. This observation indicates that the glucose-degrading enzymes were much more resistant to degradation than were the sorbitol enzymes. Although the "sorbitol" COD remaining in the batch unit at the time the cells were harvested was in excess of 5,800 mg/l, it is conceivable that the sorbitol had been converted to some periodate-reactive intermediate and, therefore, that the cells had not always been aged in the presence of an inducer (sorbitol). However, the method of ageing utilized by Tsay (36) in which 10,000 mg/l of sorbitol were added daily to E. coli cells in nitrogen-limited medium, insured that the inducer was present, and she also found that increased age resulted in a loss in the ability of the cells to utilize sorbitol. Therefore, even by ageing the cells in the presence of an inducer, it was not possible to prevent the loss of all or part of the enzymes necessary for sorbitol utilization.

Similar results were obtained from the experiment with E. coli, strain 45, in which the cells had been grown in sorbitol and then allowed to "endogenate." When the cells were harvested, it was found that glucose removal was quite rapid, but the cells had virtually no initial ability to metabolize sorbitol. This again indicated that all or part of the sorbitol enzyme system was subjected to degradation during the endogenous period but that the glucose enzymes were much more resistant. However, as in the experiment with the nitrogen-limited cells, the results obtained from the combined unit did not offer proof that glucose catabolites were able to repress the synthesis of the enzymes necessary to degrade sorbitol because the glucose was removed from the combined units prior to any sorbitol removal in the

sorbitol control unit.

Even in the experiment (Figure 32) with the cells which had received 50 mg/l/day of sorbitol during the ageing process, the rate of sorbitol removal was so low compared to the rate of glucose removal that it was not possible to determine whether there was any sorbitol removal in the combined unit during the course of glucose removal. Thus even though there was excess nitrogen present in the medium throughout the ageing process, the small quantity of inducer (sorbitol) added daily was insufficient to maintain a sorbitol enzyme system of sufficient quantity so that its "metabolic capability" could be measured. Once again, it was observed that the glucose enzyme system was much more resistant to degradation during ageing than was all or part of the sorbitol enzyme system. McGrew and Malette (45) reported that 50 mg/l/day of glucose was just sufficient to provide for the maintenance requirements of 375 mg/l of E. coli. Clifton (46) reported that between 24 and 48 mg/l/day of glucose was required to meet the daily maintenance requirements of 100 mg/l of E. coli. Since the cell population was initially grown on 2,000 mg/l of sorbitol prior to the addition of the 50 mg/l/day of feed, it would appear that even the maintenance requirements of the cells were not being fulfilled, and this provides a possible explanation of the reduction of the sorbitol enzymes to such a low level.

When the aged cells were provided with sufficient sorbitol to return the sorbitol enzyme system to a level at which sorbitol removal could be measured (Figure 33), it was again observed that synthesis of the sorbitol enzymes was probably completely repressed in the presence of glucose. The preformed sorbitol enzymes appeared to have been

completely inhibited. Studies by Tsay (36) with this organism indicated that interference with sorbitol transport into the cell was not an important mechanism, and, hence, the inhibition evidently resulted from catabolite inhibition. Since the sorbitol enzymes with a young cell culture of this organism were only inhibited by 83%, it is possible that increased age may have resulted in a slight increase in the degree of inhibition present. However, since there was some production of periodate-reactive intermediates from glucose in this experiment, the apparent complete inhibition may have been entirely the result of unequal intermediate production in the glucose control and the shocked unit for equivalent amounts of glucose removal. In any event, it is certainly obvious that the aged cells did not undergo a loss in the extent of enzyme inhibition encountered in the presence of glucose.

3. Escherichia intermedia

In the two experiments with the young cell cultures of Escherichia intermedia, the addition of glucose as an instantaneous shock was found to inhibit sorbitol removal by an average of 38%. Since the sorbitol removal rate was constant in the presence of glucose, it appears that the inhibition of substrate removal was the result of catabolite inhibition. This conclusion is further substantiated by the fact that although the shocked unit in Figure 36 received 552 mg/l glucose COD when the sorbitol COD concentration was 650 mg/l, whereas, the shocked unit in Figure 37 received 850 mg/l glucose COD when the sorbitol COD concentration was 400 mg/l, this large difference in the respective concentrations of these compounds immediately after addition of the glucose shock did not result in any significant difference

in the degrees of inhibition encountered. If glucose had been able to interfere with sorbitol transport, there should have been a much greater degree of inhibition manifested in Figure 37 than in Figure 36.

When the extent of enzyme inhibition observed with the young cell cultures of Escherichia intermedia (average value 38%) is compared with that found with a young cell culture of E. coli, strain 45 (83%), it is evident that two organisms as closely related as these are still subject to widely varying degrees of inhibition. Therefore, it is not possible to predict the degree of inhibition to be expected from a particular organism by knowing the response of a related organism. When one considers the dynamic state of a heterogeneous population, it is readily understandable how various degrees of inhibition can be observed at various times.

E. Engineering Controls

Briefly summarizing the results obtained from all of the glucose-sorbitol systems, it can be discerned that the glucose caused complete or very nearly complete repression of enzyme synthesis at all times (where repression could be measured) and that the degree of enzyme inhibition encountered appeared to be related only to the predominating species of organisms present. There was no indication that repression or inhibition was proportional to the age of the system. Therefore, one may be tempted to conclude that since cell age played no role in the results obtained, it is not possible to employ engineering control of this parameter to reduce the repression or inhibition which may be encountered with certain substrate combinations. However, the results must be analyzed in relation to the type of treatment process which they represent. When this is done, it is apparent that engineering controls can indeed be employed provided that intelligent design

procedures are used in selecting the treatment process.

Digressing momentarily, it will be recalled that Chian and Mateles (34) observed that glucose and butyrate could be removed concurrently in a completely mixed continuous flow reactor operated at a dilution rate of 0.35 hr^{-1}, but higher dilution rates left increasing quantities of butyrate in the effluent. Komolrit and Gaudy (47) found that sorbitol-fed continuous flow reactors operated at a 4 hour detention time could withstand glucose shock loads provided that the magnitude of the shock was not too great. In one experiment, when the feed was changed from 1,000 mg/l sorbitol to 1,000 mg/l sorbitol plus 1,000 mg/l glucose, there was only a slight rise in the effluent COD and no change in the effluent sorbitol concentration. When the detention time was changed to 2 hours and the experiment repeated, the sorbitol level remained at slightly over 1,000 mg/l for almost 12 hours after the shock was applied. At a detention time of 16 hours, the system was capable of receiving more severe shock loads with no disruption of sorbitol metabolism. The dependence of multicomponent substrate removal on detention time has also been demonstrated with pure cultures. It has been shown (34) that when fructose and glucose were the growth limiting nutrients, pure cultures of E. coli or Pseudomonas fluorescens consumed both sugars at low dilution rates, but the fructose was attacked slightly or not at all at high dilution rates. Harte and Webb (48) have shown that Klebsiella aerogenes removed glucose and maltose at low flow rates, but at high flow rates only glucose was removed. Since it has been demonstrated that the degree of repression of an enzyme subject to catabolite repression is dependent on the rate of metabolism of the repressing substrate (49), it seems reasonable that decreasing dilution

rates allow for concurrent substrate removal and provide the system with the ability to withstand shock loads of limited magnitude.

In all of the experiments reported in this work, the rate of glucose removal was not subject to any engineering control, i.e., the glucose was removed as rapidly as was possible for the combination of organisms and conditions present. Thus this entire series of experiments represents what would occur in an activated sludge unit which was designed so that "plug flow" occurred, and it is readily apparent that this type of design could lead to severe problems when certain substrate combinations are encountered. Hence where substrate interactions may arise, one should consider selecting a mode of operation in which the rate of metabolism of a compound known to produce repression can be placed under engineering control. Completely mixed continuous flow activated sludge systems (carbon-limited) in which the rate of metabolism of a repressing substrate can be controlled by selecting an appropriate dilution rate would appear to offer much greater possibilities of attaining suitable engineering control.

CHAPTER VI

CONCLUSIONS

1. An analysis of the results obtained from the heterogeneous populations, which were aged by prolonged batch operation, gave no indication that enzyme repression or inhibition was proportional to the age of the system. Instead, the extent of repression or inhibition appeared to be entirely random. Also, the studies with pure culture systems indicated that increased age could not relieve the enzyme inhibition or repression found with young cells.

2. When E. coli was aged by methods other than the standard batch operation, it was frequently found that part or all of the enzymes necessary for sorbitol degradation were essentially lost. In all cases, the cells still maintained the ability to utilize glucose. Therefore, all or part of the sorbitol enzymes were more easily degraded during the ageing process than were the glucose enzymes.

3. The repression of the enzymes necessary for sorbitol utilization by glucose catabolites appeared to be a very general phenomenon, and, in all cases, the repression was essentially complete.

4. Inhibition of the preformed sorbitol enzymes did not appear to result from interference with sorbitol transport by glucose.

5. The degree of enzyme inhibition encountered varied with each of the pure cultures studied, and there was no way to predict the degree of inhibition expected with an organism on the basis of the

results obtained from a related organism. This variation helps explain why the degrees of inhibition encountered in heterogeneous populations can vary widely.

6. Preinduction of the enzymes necessary for sorbitol metabolism appeared to offer no protection against repression.

7. In all cases, the utilization of galactose by the heterogeneous populations was inhibited in the presence of glucose. This inhibition appeared to be the result of both transport interference and catabolite inhibition.

CHAPTER VII

SUGGESTIONS FOR FUTURE WORK

From an applied engineering point of view, there would seem to be little which would be gained by extending this study. Although it may be possible that increased cell age could relieve the repression or inhibition normally associated with young cells, this would certainly appear to be the "exception" and not the general case. Consequently, the design of a treatment plant to insure the presence of old cells is meaningless from the viewpoint of eliminating repression or inhibition mechanisms. It would appear, therefore, that any future work would be better directed to studies with continuous flow reactors where the rate of metabolism of a repressing compound can be controlled by the choice of an appropriate dilution rate.

From a more theoretical point of view, the following would be of interest:

1. Other substrate combinations could be investigated to determine if an increase in cell age would result in a change in the extent of repression or inhibition associated with young cell cultures.

2. Additional pure cultures of organisms should be studied to determine if the complete repression of sorbitol enzymes by glucose catabolites is as general an occurrence as it appeared to be in this study.

3. The loss of all or part of an enzyme system necessary for the

utilization of a compound to which a cell has been acclimated as a result of increased age is of significance. This aspect should be further investigated with both heterogeneous and pure culture systems.

SELECTED BIBLIOGRAPHY

1. Gaudy, A. F. Jr., "Studies on Induction and Repression in Activated Sludge Systems." Applied Microbiol. 10, 264-271 (1962).

2. Stumm-Zollinger, E., "Effects of Inhibition and Repression on the Utilization of Substrates by Heterogeneous Bacterial Communities." Applied Microbiol., 14, 654-664 (1966).

3. Prakasam, T. B. S., and Dondero, N. C., "Aerobic Heterotrophic Bacterial Populations of Sewage and Activated Sludge, III. Adaptation In a Synthetic Waste." Applied Microbiol., 15, 1128-1137 (1967).

4. Stumm-Zollinger, E., "Substrate Utilization in Heterogeneous Bacterial Communities." J. Water Pollut. Control Fed., Research Suppl., 40, R213-R229 (1968).

5. Grady, C. P. L. Jr., "Metabolic Control Mechanisms Operative in Natural Microbial Populations Selected by Their Ability to Degrade Lysine." Doctoral Thesis, Oklahoma State University, Stillwater, Okla., (1969).

6. Doudoroff, M., Hassid, W. Z., Putman, E. W., Potter, A. L., and Lederberg, J., "Direct Utilization of Maltose by Escherichia coli." J. Biol. Chem., 179, 921-934 (1949).

7. Cohen, G. N., and Monod, J., "Bacterial Permeases." Bacteriol. Rev. 21, 169-194 (1957).

8. Horecker, B. L., Thomas, J., and Monod, J., "Galactose Transport in Escherichia coli, I. General Properties as Studied in a Galactokinaseless Mutant." J. Biol. Chem., 235, 1580-1585 (1960).

9. Horecker, B. L., Thomas, J., and Monod, J., "Galactose Transport in Escherichia coli, II. Characteristics of the Exit Process." J. Biol. Chem. 235, 1586-1590 (1960).

10. Rogers, D., and Yu, S. H., "Substrate Specificity of a Glucose Permease of Escherichia coli." J. Bacteriol. 84, 877-881 (1962).

11. Adhya, S., and Echols, H., "Glucose Effect and the Galactose Enzymes of Escherichia coli: Correlation Between Glucose Inhibition of Induction and Inducer Transport." J. Bacteriol. 92, 601-608 (1966).

12. Anraku, Y., "The Reduction and Restoration of Galactose Transport in Osmotically Shocked Cells of Escherichia coli." J. Biol. Chem. 242, 793-800 (1967).

13. Anraku, Y., "Transport of Sugars and Amino Acids in Bacteria, I. Purification and Specificity of the Galactose- and Leucine-Binding Proteins." J. Biol. Chem. 243, 3116-3122 (1968).

14. Lin, E. C. C., Koch, J. P., Chused, T. M., and Jorgensen, S. E., "Utilization of L-α-Glycerophosphate by Escherichia coli Without Hydrolysis." Proc. Natl. Acad. Sci. 48, 2145-2150 (1962).

15. Hayashi, S., and Lin, E. C. C., "Capture of Glycerol by Cells of Escherichia coli." Biochim. Biophys. Acta 94, 479-487 (1965).

16. Sanno, Y., Wilson, T. H., and Lin, E. C. C., "Control of Permeation to Glycerol in Cells of Escherichia coli." Biochem. Biophys. Res. Comm. 32, 344-349 (1968).

17. Zwaig, N., and Lin, E. C. C., "Feedback Inhibition of Glycerol Kinase, a Catabolic Enzyme in Escherichia coli." Science, 153, 755-757 (1966).

18. Cohn, M., and Horibata, K., "Inhibition by Glucose of the Induced Synthesis of the β-Galactosidase-Enzyme System of Escherichia coli. Analysis of Maintenance." J. Bacteriol., 78, 601-612 (1959).

19. Mandelstam, J., "The Repression of Constitutive β-Galactosidase in Escherichia coli by Glucose and Other Carbon Sources." Biochem. J., 82, 489-492 (1962).

20. Loomis, W. F. Jr., and Magasanik, B., "Genetic Control of Catabolite Repression of the lac Operon in Escherichia coli." Biochem. Biophys. Res. Commun., 20, 230-234 (1965).

21. Loomis, W. F. Jr., and Magasanik, B., "Glucose-Lactose Diauxie in Escherichia coli." J. Bacteriol., 93, 1397-1401 (1967).

22. Gaudy, A. F., Jr., Komolrit, K., and Bhatla, M. N., "Sequential Substrate Removal in Heterogeneous Populations." J. Water Pollut. Control Fed., 35, 903-922 (1963).

23. Prakasam, T. B. S., and Dondero, N. C., "Observations on the Behavior of a Microbial Population Adapted to a Synthetic Waste." Proc. 19th Ind. Waste Conf., Purdue Univ., Ext. Ser. 117, 835-845 (1964).

24. Gaudy, A. F., Komolrit, K., and Gaudy, E. T., "Sequential Substrate Removal in Response to Qualitative Shock Loading of Activated Sludge Systems." Applied Microbiol. 12, 280-286 (1964).

25. Experimental Biochemistry, Ed. by John M. Clark, Jr., W. H. Freeman and Company, San Francisco (1964).

26. Krishnan, P., and Gaudy, A. F. Jr., "Studies on the Response of Activated Sludge to Shock Loadings." Biotech. and Bioeng. 7, 455-470 (1965).

27. Gaudy, A. F. Jr., Komolrit, K., Gaudy, E. T., and Bhatla, M. N., "Multicomponent Substrate Removal by Activated Sludge and by Pure Culture Systems." Presented at the 63rd Annual Meeting of the American Society for Microbiology, Cleveland, Ohio, (1963).

28. Bhatla, M. N., and Gaudy, A. F. Jr., "Sequential Substrate Removal in a Dilute System by Heterogeneous Microbial Populations." Applied Microbiol. 13, 345-347 (1965).

29. Komolrit, K., and Gaudy, A. F. Jr., "Substrate Interaction During Shock Loadings to Biological Treatment Processes." J. Water Pollut. Control Fed., 38, 1259-1272 (1966).

30. Grady, C. P. L. Jr., and Gaudy, A. F. Jr., "Substrate Interactions in Natural Populations." Proc. Okla. Acad. Sci., XLVII (1966 - in press).

31. Yu, T., "Studies on the Effect of Bacterial Growth Rate on Substrate Removal in Multicomponent Synthetic Wastes." Doctoral Thesis, Oklahoma State University, Stillwater, Okla. (1969).

32. Yu, T., Personal Communication (1968).

33. Su, J. J., "Utilization of Mixtures of Carbohydrates by Heterogeneous Populations." Masters Thesis, Oklahoma State University, Stillwater, Oklahoma. (1968).

34. Chian, S. K., and Mateles, R. I., "Growth of Mixed Cultures on Mixed Substrates, I. Continuous Culture." Applied Microbiol., 16, 1337-1342 (1968).

35. Gaudy, A. F. Jr., Gaudy, E. T., and Komolrit, K., "Multicomponent Substrate Utilization by Natural Populations and a Pure Culture of Escherichia coli." Applied Microbiol., 11, 157-162 (1963).

36. Tsay, S. S., "Feedback Inhibition of Catabolic Pathways." Masters Thesis, Oklahoma State University, Stillwater, Okla. (1968).

37. Tsay, S. S., Personal Communication (1969).

38. Luria, S. E., in The Bacteria, I, Gunsalus, I. C., and Stanier, R. Y., editors, page 13, Academic Press Inc., New York (1960).

39. Bustamante, R. B., "Studies on Bacterial Predominance Patterns in Mixed Cultures." Doctoral Thesis, Oklahoma State University, Stillwater, Okla. (1968).

40. Standard Methods for the Examination of Water and Wastewater. 12th ed. American Public Health Association, New York (1965).

41. "Glucostat for the Enzymatic Determination of Glucose." Worthington Biochemical Corporation, Freehold, New Jersey (1963).

42. "Galactostat - A Coupled Enzyme System for the Determination of Galactose." Worthington Biochemical Corporation, Freehold, New Jersey.

43. Komolrit, K., "Biochemical Response of Activated Sludge Processes to Organic Shock Loads." Doctoral Thesis, Oklahoma State University, Stillwater, Okla. (1965).

44. Steel, R. G. D., and Torrie, J. H., Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., New York (1960).

45. McGrew, S. B., and Mallette, M. F., "Energy of Maintenance in Escherichia coli." J. Bacteriol. 83, 844-850 (1962).

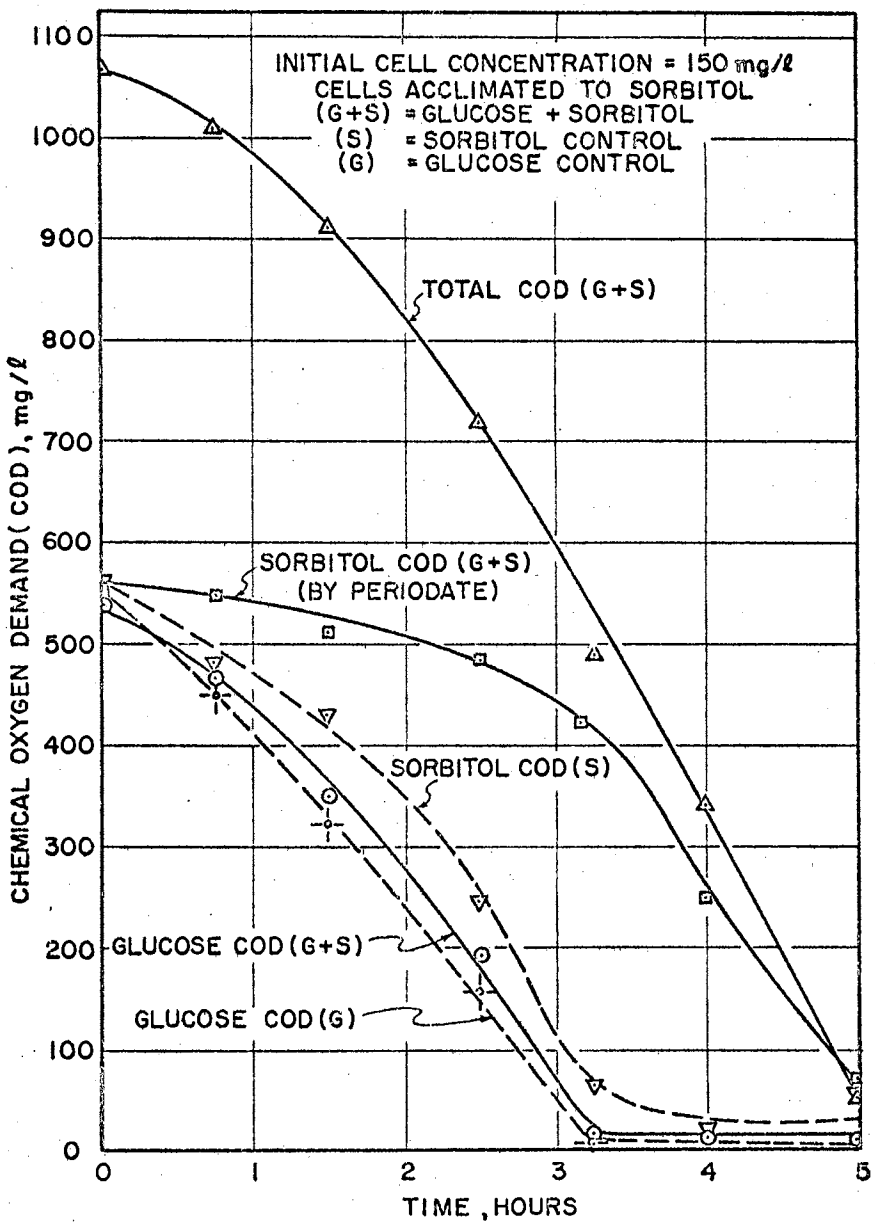
46. Clifton, C. E., "Aging of Escherichia coli." J. Bacteriol. 92, 905-912 (1966).

47. Komolrit, K., and Gaudy, A. F. Jr., "Biochemical Response of Continuous Flow Activated Sludge Processes to Qualitative Shock Loadings." J. Water Pollut. Control Fed., 38, 85-101 (1966).

48. Harte, M. J., and Webb, F. C., "Utilization of Mixed Sugars in Continuous Fermentation - 2." Biotechnol. Bioeng., 9, 205-221 (1967).

49. Neidhardt, F. C., "Mutant of Aerobacter aerogenes Lacking Glucose Repression." J. Bacteriol. 80, 536-543 (1960).

APPENDIX



INHIBITION OF SORBITOL METABOLISM BY GLUCOSE IN A GLUCOSE-SORBITOL MEDIUM WITH A HETEROGENEOUS POPULATION. (Reproduction of a selected figure previously presented by Gaudy et al. (27).

VITA

3

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