METABOLIC CONTROL MECHANISMS OPERATIVE IN NATURAL MICROBIAL POPULATIONS SELECTED BY THEIR ABILITY TO DEGRADE LYSINE

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CHAPTER I

INTRODUCTION

A. Nature and Importance of the Problem

It is well known that the United States of America has become aware of the need for improved water pollution control for it is impossible to watch television, listen to the radio, or read a newspaper or magazine without being exposed to the problem. Unfortunately, as is often the case in a campaign designed to overcome apathy, scare tactics have been used by implying that the United States is running out of water, when in fact it is not. A recent study projected that by the year 2000 the total withdrawal will be four-fifths and the used return about two-thirds of the total U. S. stream flow (1). Thus, the problem is not of quantity so much as quality, and the solution will lie in better treatment allowing water reuse.

Waste water treatment will not be inexpensive. The Federal Water Pollution Control Administration has estimated that $26 to $29 billion will be needed over the next five years in order to clean up the rivers (2). If this cost is to be met, as it must, it will fall upon the shoulders of the people, through taxes and increased consumer costs. In an effort to reduce the economic impact,

Robert L. Coughlin, a senior economist with the FWPCA, has recommended that industrial management consider: 1) applying process control to waste production and waste water discharge, 2) anticipating waste reduction requirements in the design of new facilities, and 3) making use of cooperative waste treatment facilities (2).

While the idea of using cooperative waste treatment facilities is not new, the increasingly stringent enforcement of water pollution control regulations has given additional impetus to it. Within the last decade greater communication has been initiated among government, industry, and designers, through discussions concerning combined treatment of municipal and industrial wastes in which the legal, technical, and economic aspects of such a venture have been analyzed (3) (4). While some cities might have reservations about accepting industrial wastes, others, such as Milwaukee, feel that it is their obligation to provide waste treatment to their industries just as they do for their private citizens, and, therefore, set few restrictions on waste discharge (5). Other cities prefer to design and operate their plants for specific wastes; an example is Kalamazoo, Michigan, which recently opened a new secondary waste treatment facility to treat combined wastes from the city, paper mills, and a pharmaceutical company (6). There are many other examples including plants that accept cannery wastes as well as those that treat packing house, tannery, and laundry wastes (7) (8).

Many advantages are cited for combined waste treatment (9) (10). 1) There is a cost saving to both the industry and the municipality. The industry avoids increasing its tax valuation and funds that would have been required for capital improvements are released for other purposes. (Recent tax laws, such as the one in Oklahoma, however, make it more advantageous for the industry to build its own plant by allowing a complete write-off of construction costs on state income tax.) 2) The costs of waste treatment, paid as sewer charges, are tax deductible. 3) The municipality can borrow construction funds at a lower rate of interest. 4) The municipal waste can often provide nutrients required for treatment of the industrial waste. 5) The people who run the municipal plants are "experts" and this promotes an effluent of superior quality.

Because it is often difficult or inconvenient for the city to serve all of the industries in an area, a new concept for industrial parks has been devised, in which the developer provides the waste treatment in order for the industries to benefit from the economic advantages of cooperative efforts. Bayport, a new heavy industrial district in Harris County, Texas, will encompass many industries, particularly chemical manufacturing and fabrication, and will provide central waste water treatment (11). Activated sludge will be used and all sanitary and amenable industrial wastes will be treated there after

the industries have given pretreatment to their own ultra-high strength or exotic wastes.

The key to all of these combined waste water treatment schemes is compatibility. The wastes that enter a biological treatment process must be compatible, and, thus, there are difficulties involved with the discharge of certain industrial wastes to a combined treatment plant (12). It is obvious that hazardous substances, or substances that can damage the physical plant should not be discharged, nor should substances that can impair the plant processes, such as acids, cyanides, metal ions, oils, and brines. Not so obvious, but just as important, are those wastes which by their strength or character would impose an overload or shock load upon the treatment facility. Shock loads are often given slight consideration when the disadvantages of combined treatment are discussed; however, they can be extremely important to the efficiency and performance of an activated sludge treatment plant.

A shock load is any rapidly occurring or immediate change in the chemical or physical environment of a waste treatment system (13). Such changes, which can seriously affect the established metabolic patterns of the reactor, are generally divided into three major types. A "quantitative shock load", the one ordinarily thought of when the term "shock load" is used, is usually a rapid increase in organic loading due to an inflow of a high concentration of substrate to which the sludge is acclimated or to which

it needs no acclimation, though it could also be a rapid decrease in concentration due to a hydraulic shock. This type of shock load is concerned only with a change in the amount of substrate, not a change in its character. The second type, the "toxic shock load", involves an influx of compounds or elements which inhibit or damage the existing metabolic pathways or disrupt the physiological condition of the microbial population; this type includes pH changes. The final type of shock is the "qualitative" shock, which, as the name implies, involves a change in the chemical structure of the substrate. Since the total organic loading may also increase during the change, this type of shock can involve some of the conditions of the quantitative shock. Qualitative shock loads are particularly important to installations like industrial parks where each contributing industry may be supplying a unique type of compound to the treatment plant, for as process schedules shift and change, so would the character of the loading on the treatment plant.

Since an activated sludge plant is a mixed community of micro-organisms, there are several possible responses when a qualitative shock load is placed upon the system: 1) shift in the predominance of the microbial community to one better suited for degradation of the new compound, 2) the opening of new metabolic pathways for the destruction of the compound by the utilization of enzymes already present or the manufacture of new enzymes, 3) the

prevention of the manufacture of the enzymes necessary for the destruction of some substrate already present in the waste stream, thus allowing its escape in the effluent, or 4) a decrease in the activity of enzymes present within the cells, again allowing the escape of some compound which previously had been metabolized. Thus, it is obvious that qualitative shock loads can have great effects upon the efficiency of the activated sludge process, particularly since industrial wastes may restrict the number of species present. These substrate interactions come about as a result of metabolic control mechanisms, which are operative within microbial cells for the regulation of their energy yielding and requiring processes.

Induction, repression, metabolite repression, and inhibition are important metabolic control mechanisms which are thoroughly documented in the next chapter, but which, by way of introduction, will be briefly mentioned here. Induction is an increase in the differential rate of synthesis of an enzyme when a compound, the inducer, is present. Generally, the inducer is the substrate of the enzyme. Repression is a decrease in the rate of synthesis of an enzyme resulting from the presence in cells of a repressor molecule. If the repression acts on catabolic enzymes and if it is produced by intermediates of catabolism, it is termed metabolite repression. Inhibition is defined as a decrease in the rate of activity of preformed enzymes.

Studies have been in progress for several years in the Bioenvironmental Engineering Laboratories at Oklahoma State University to discover the extent and nature of these mechanisms in natural microbial populations. In 1962 Gaudy (14) found that induction and repression occur in activated sludge systems; later, he and his co-workers showed evidence for an inhibition mechanism operative on degradative enzymes (15) (16). More recently these studies have been extended to systems involving mixtures of carbohydrates and mixtures of carbohydrates and amino acids (17) (18).

Preliminary studies were performed to assess the effect of glucose upon the utilization of amino acids by bacterial populations adapted to the amino acids. The source of the organisms was sewage. Those studies showed that glucose exerted an effect upon the removal of lysine, and this report presents the results of a detailed investigation of that effect as well as the effects of other carbohydrates. This study was performed with cultures of organisms maintained on lysine, because the aim was to understand better the control mechanisms operating on an enzyme system responsible for the utilization of the primary energy source. Since that enzyme system is required for the survival of the population, it could be considered as an example of specialized enzyme systems found in organisms degrading industrial wastes of restrictive nature. In this way, it is hoped that enough basic data can be gathered so that eventually the design engineer will know

what types of interactions to expect in the specialized enzyme systems of the organisms in industrial waste water treatment facilities when those facilities are subjected to shock loads.

B. Purposes of the Study

One purpose of this study was to determine the nature of the control mechanisms operative upon the enzyme systems responsible for the degradation of lysine. This portion of the work was carried out in batch experiments in which cells were grown on lysine mixed with either glucose, fructose or ribose. The experiments were designed to differentiate between repression of enzyme synthesis, inhibition of enzyme activity, and predominance shifts in the population. In the cases where the results indicated the operation of control mechanisms, those mechanisms were characterized further by assessing the effects of preinduction to one substrate and of the removal of ammonia nitrogen from the system upon operation of the mechanisms.

Another purpose was to determine whether the control mechanisms observed under batch growth conditions would also be found under continuous flow conditions. The experiments were performed at two different flow rates using two different growth limiting substances in order to assess the severity of the response as a function of growth conditions. It is hoped that the continuous flow experiments will provide additional evidence as to the importance of control mechanisms in actual waste water treatment systems.

CHAPTER II

THEORETICAL CONSIDERATIONS OF METABOLIC CONTROL MECHANISMS

A. General Comments

Biologists' understanding of metabolic control mechanisms, one aspect of molecular biology, is undergoing rapid and significant changes because of the great research effort being expended on it. No attempt will be made here to review the entire field because that task would be much beyond the scope of this report, but some basic aspects will be presented. For more detailed information on this subject, and as a guide to persons reading in this area for the first time, the author highly recommends the book by J. D. Watson (19) as well as review articles by Maas and McFall (20), Ames and Martin (21), and Vogel and Vogel (22).

Metabolic control mechanisms are the natural result of evolution because they allow more efficient energy utilization within cells. For example, if an energy source were not available to a cell there would be no need for the enzymes required to use that source, and they would not be made since their production would be inefficient. However, as long as the cell possessed the genetic

"blueprint" for the enzymes, they could be made on demand. Enzymes of this type are called "inducible" and the compound that triggers their synthesis is called the "inducer." Likewise, it would be inefficient for a cell to make the enzymes necessary to synthesize a metabolic building block, such as an amino acid, if that building block were supplied exogenously to the cell. Thus, if the amino acid (the end product of a metabolic pathway) were present in the cells it would function as a "corepressor" and prevent (repress) the synthesis of the enzymes necessary to make it. There is another special type of repression, called metabolite repression which is based on the premise that if an easily degradable energy source were present the cell would have no need for the enzymes necessary to degrade a more difficult source, and, thus, their synthesis would be repressed. Mechanisms involved in the manufacture of enzymes are often referred to as "coarse" controls because they affect only the quantities of enzymes but not their activities. Thus, a pathway could continue functioning for some time (until the enzymes were diluted out by growth, or were destroyed) even if the end product were present in excess. Enzyme inhibition, however, is a "fine" control and gives an instant response by altering the rate of activity of enzymes.

All of the information needed to determine the structures of all of the enzymes that can be produced by a cell is perpetuated in the chromosomes, which are made of DNA.

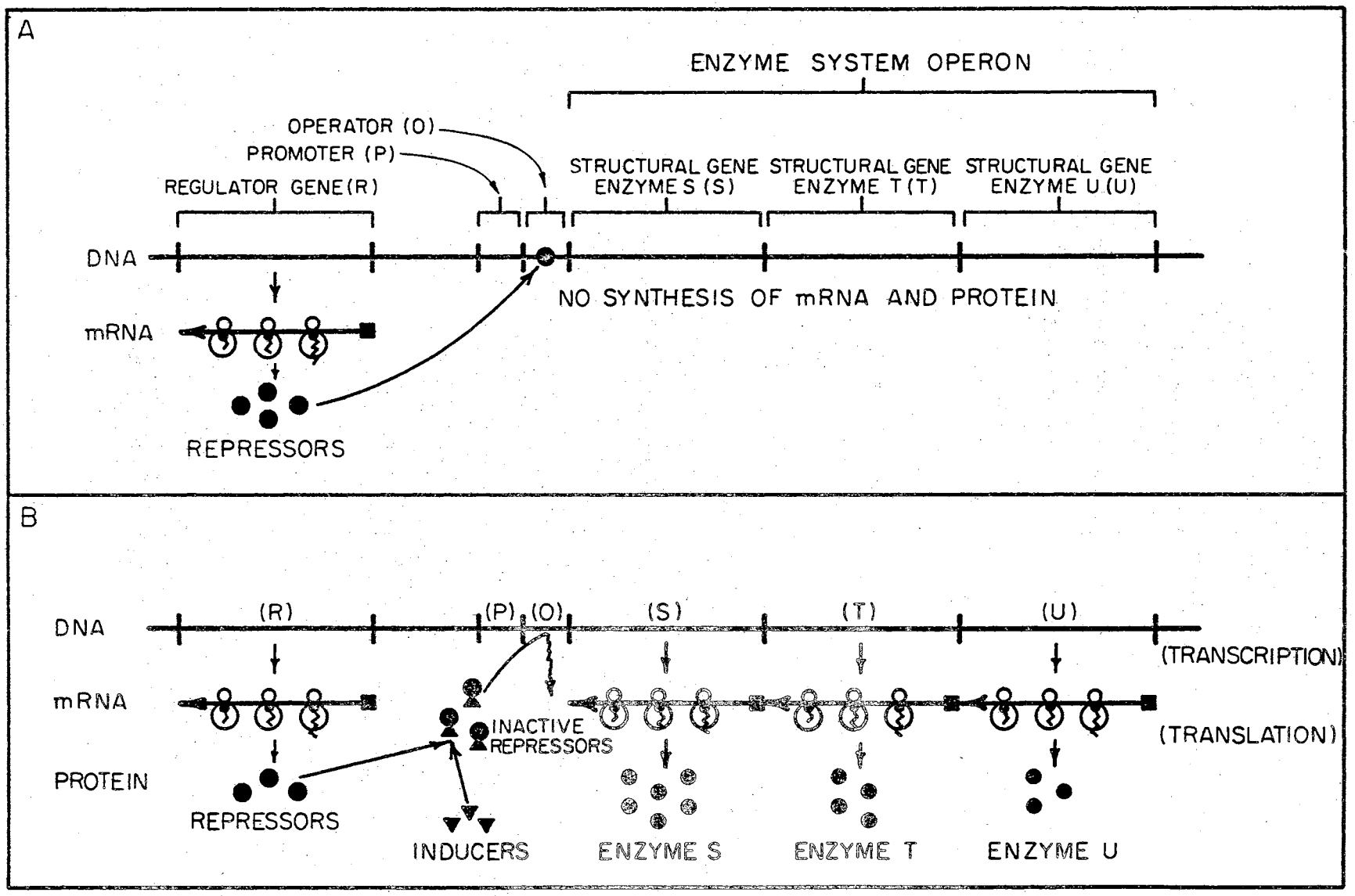
In order to be of use to the cell, it must be possible for that information to be transferred to the enzyme synthesizing devices, called ribosomes. The route of that information transfer has been called the "central dogma" and goes through steps from DNA to messenger RNA to protein (19). In other words, the DNA, the master blueprint, is transcribed onto a working print, the RNA, which is then translated at the ribosomes for production of the final product, the protein (enzyme). In 1961 Jacob and Monod (23) proposed the "operon" model to "summarize and express conveniently the properties of the different factors which play a specific role in the control of protein synthesis." This model was developed from studies on the enzyme system for degradation of lactose, whose genes are collectively called the "lac" operon. Some of the features of the system were based upon speculation and while some have been proven, others remain in the realm of speculation. The basic aspects of the model (amended to bring it up to date) will be presented here merely as a background upon which to build an engineering study of control mechanisms and their engineering application. Many of the speculative and controversial points do not affect the engineering significance and, thus, will not be discussed here. If the reader is interested in those points, he is urged to consult the above reviews.

B. The Operon Model

Figure 1 is a diagrammatic representation of protein synthesis as set forth by the operon model. Ames and Martin (21) have listed certain attributes of the model:

1. The bacterial chromosome (DNA) is organized into clusters of genes, called operons. The enzymes coded for by an operon have related functions, such as individual steps in a pathway, and are regulated together.
2. The information in the operon is transcribed into a molecule of messenger RNA of the same length as the operon.
3. The expression of the genes in the operon is regulated by a small region, called the operator, located at one end of the operon. It is not known whether the operator region is transcribed into RNA.
4. Induction is an increase in the differential rate of synthesis of an enzyme (the rate with respect to synthesis of the total protein in the cell). As shown in Figure 1A, an inducible enzyme is not normally produced (in physiologically significant amounts), because the products of the regulator gene, repressors, interact with the operator and prevent enzyme synthesis. When an inducer enters the cell (Figure 1B)

Figure 1. The Operon Model for Inducible Enzyme Synthesis
A) Under conditions when the inducer is not in the medium the repressors are active and bind with the operator preventing mRNA and protein synthesis.
B) When the inducer is present it combines with the repressors making them inactive and allowing mRNA and protein synthesis. (Modified from Watson) (19).


A
ENZYME SYSTEM OPERON
OPERATOR (O)
PROMOTER (P)
REGULATOR GENE(R)
STRUCTURAL GENE ENZYME S (S)
STRUCTURAL GENE ENZYME T (T)
STRUCTURAL GENE ENZYME U (U)
DNA
NO SYNTHESIS OF mRNA AND PROTEIN
mRNA
REPRESSORS
B
DNA
(R)
(P)
(O)
(S)
(T)
(U)
(TRANSCRIPTION)
mRNA
INACTIVE REPRESSORS
(TRANSLATION)
PROTEIN
REPRESSORS
INDUCERS
ENZYME S
ENZYME T
ENZYME U

it reacts with the repressors, making them inactive allowing protein synthesis. There has been some uncertainty as to whether the repressors act to prevent transcription into RNA or translation into protein, although the former site of action is generally accepted (from an engineering point of view the result is the same).

5. Repression is a decrease in the differential rate of synthesis of enzymes. Under normal conditions the products of the regulator gene for this type of system are inactive and allow protein synthesis, but when the end-product of the biochemical pathway controlled by the operon (corepressor) is present in large amounts it combines with the inactive repressor to form an active repressor and prevent protein synthesis.
6. Coordinate repression: The ratio of the amount of any enzyme to that of any other of the same operon is constant regardless of the degree of repression or induction.

Recently, the repressor molecule for the lac operon was isolated and shown to be a protein (24). Gilbert and Müller-Hill performed the isolation and showed that it was bound to the DNA of the operator except when the inducer was present, when no repressor was found on the DNA (25).

Further experiments showed that no RNA was involved, indicating that the repressor can bind directly with the operator region of the DNA, as proposed by Jacob and Monod in their model.

A region not originally proposed by Jacob and Monod is shown adjacent to the operator in Figure 1. This region was termed the promoter and has been located between the regulator gene and the operator on the lac operon by Ippen, et al. (26). They stated that the site is essential to operon expression, and proposed a scheme whereby it serves as the initiation point for transcription, possibly by acting as a binding site for the enzyme that forms the messenger RNA (RNA polymerase). If this were true, then by binding to the operator, the repressor could directly block the progress of the RNA polymerase into the structural genes of the operon and, thus, the action of repression would be at the level of transcription (26).

C. Allosteric Interactions - Enzyme Inhibition

1. Biosynthetic Inhibition

The control mechanisms mentioned above are all "coarse" controls because they affect only the formation of new enzymes without changing the rates of activity of the enzymes left in the cells. In a biosynthetic pathway, if the end-product of the pathway were added to the medium, new enzyme synthesis would stop (due to repression)

but the enzymes left would continue making the compound unless some other mechanism were available to stop their activity. Such a mechanism has evolved and is termed "feedback" or "end-product" inhibition (27). The end-product of a pathway inhibits the activity of the enzyme at the first committed step (i.e., after the last branch point) in the pathway so that its presence can shut-off the entire pathway after that enzyme. Thus, the control acts in a manner analogous to feedback in an electronic circuit. This mechanism is quite wide spread and its existence in many pathways in micro-organisms has been well documented (28).

This mechanism at first appeared to be paradoxical because the inhibiting compounds bore no structural similarity to the substrates of the enzymes that they inhibited and the inhibition could not be explained by classical enzyme kinetics. Monod and Jacob emphasized the fact that the inhibitor did not need to be a steric analogue of the substrate by referring to the interaction between enzyme and inhibitor as allosteric inhibition (29). The exact nature of this interaction has been the subject of much research and much controversy and is as yet unresolved (29). The model proposed by Monod (30) and his colleagues assumes separate, rather than overlapping, substrate and inhibitor sites with the inhibitor acting to change the conformation of the substrate-binding site.

2. Inhibitions in Energy Metabolism

In addition to biosynthetic pathways allosteric interactions are also involved in energy metabolism. Most examples have been found in non-bacterial systems where ATP, ADP, and AMP are generally the metabolites exerting the control. The enzyme for the phosphorylation of glucose, phosphofructokinase, fructose diphosphate phosphatase, citric acid cycle enzymes, and pyruvate carboxylase are all involved in allosteric interactions but some are activated by the interacting metabolite while others are repressed (29). Some examples in bacterial systems have been found also.

3. Catabolic Inhibition

Gaudy and his co-workers (15) (31), using both heterogeneous populations and a pure culture of *Escherichia coli*, have shown the existence of an inhibition mechanism in catabolic pathways which is possibly analogous to feedback inhibition in biosynthetic pathways. Zwaig and Lin (32) found that fructose-1, 6-diphosphate was a feedback inhibitor of the catabolic enzyme glycerol kinase in *E. coli*. Using both *E. coli* and *Achromobacter* sp., Tsay (33) showed that the introduction of glucose into the growth medium exerted a rapid inhibition of utilization of either sorbitol or glycerol. Thus, it appears that a "fine" control is available in catabolic as well as anabolic pathways, allowing the immediate cessation of the

degradation of one compound when a metabolic product accumulates in the medium or inside the cell, or when a second compound is present whose degradation is easier and leads to the same inhibiting metabolite.

D. Metabolite Repression

There is one final area of control mechanisms which is extremely important to the field of waste water treatment. Metabolite repression causes a decrease in rate of synthesis of the enzymes needed to degrade one compound when another, more easily degraded compound is added to the medium. In 1947 Monod (34) published a paper in which he discussed the historical aspects of the phenomenon and presented evidence for its cause. One of the earliest recorded observations of the phenomenon was made in 1898 when Katz noted that the production of amylase by a Penicillium strain would occur in the absence of any carbohydrate but was slightly decreased in the presence of lactose or maltose and completely inhibited in the presence of sucrose. In 1901, Dienert found that glucose had an inhibitory effect upon the formation of galactozymase by yeast, a fact which was confirmed by Euler and Johannson in 1912 and strongly emphasized by Stephenson and Yudkin in 1936. Finally, in 1941 when Monod (34) discovered "diauxie" the systematic study of these effects was made possible.

1. Diauxie

During an investigation of the influence of carbohydrates on bacterial growth rates, Monod (34) observed that with some mixtures of compounds the growth curves exhibited two successive complete growth cycles, separated by a lag. He labeled this phenomenon "diauxic growth". A similar effect by carbohydrates on breakdown of proteins was noted and summarized by Gale in 1943 and termed the "glucose effect" by Epps and Gale. In further studies of diauxie Monod found that the carbohydrates could be divided into two groups, "A" and "B", but that the composition of the groups varied somewhat depending upon the bacterial species being used. "A" compounds resulted in diauxic growth when placed in a medium with a "B" compound, with the "A" compound being used first. A few special compounds, such as fructose and mannose, when used for growth of E. coli strain H, did not give diauxie with glucose, and also did not exert it upon any of the "B" compounds. Monod (34) drew several conclusions from his work: 1) Classification in the "A" or "B" series did not appear to be associated with the configuration of the compound. For example, in E. coli H, glucose, fructose, mannose, and mannitol were all A compounds while galactose, arabinose, xylose, rhamnose, maltose, lactose, sorbitol, and dulcitol were all B compounds. 2) The A compounds were attacked by constitutive enzymes while the B compounds were attacked by inducible ones. 3) Each cycle in

diauxic growth corresponded to exclusive utilization of one of the two compounds. 4) A compounds were attacked during the first cycle and B during the second. 5) Adaptation to B did not suppress diauxic growth but it sometimes shortened the lag. 6) The effects could be understood only as a result of a repression by A compounds of the synthesis of the inducible enzymes required for the utilization of the B compounds.

2. Catabolite Repression

During the next fourteen years much work was done in the field of metabolic control mechanisms so that in 1961 the annual symposium at Cold Spring Harbor was on that subject. At that meeting Magasanik (35) presented a paper in which he summarized the work that had been done on the glucose effect. He also presented a theory for the mechanism responsible for the observed reduction in the rate of formation of enzymes sensitive to the effect when glucose was placed in the medium. Since the effect was not specific for glucose but could be caused by any energy source that could serve efficiently as a source of intermediate metabolites, he suggested the name catabolite repression.

a. Previous Hypotheses. Before presenting his own theory Magasanik (35) discussed the various hypotheses that had been proposed for catabolite repression. In 1953, Cohn and Monod ascribed the inhibitory effect of glucose on the formation of β-galactosidase to interference with

the transport mechanism for the inducer. However, Magasanik pointed out that the mechanism could not explain the effect on other enzymes, such as histidase, which was repressed even when histidine was inside the cell. In 1955, Spiegelman and his coworkers attempted to explain the effect by suggesting that during growth on glucose the formation of glucose-degrading enzymes preempts the cell's internal supply of amino acids and nucleotides. However, in 1956, Neidhardt and Magasanik showed that supplementation of these constituents caused an acceleration of protein synthesis but did not relieve the glucose effect. Another hypothesis, proposed by Englesberg in 1959, attempted to explain the effect by assuming that metabolism of glucose lowers the level of inorganic phosphate in the cell and, thus, prevents the synthesis of inducible enzymes. This theory assumed that the synthesis of RNA for inducible enzymes was more sensitive to a lack of inorganic phosphate than that for constitutive enzymes, but Magasanik pointed out that there was no evidence for that. Besides, not all inducible enzymes are glucose-sensitive (repressed by glucose) nor are all glucose-sensitive enzymes inducible.

b. Magasanik's Theory. The first fact cited in support of Magasanik's theory of catabolite repression was that all glucose-sensitive enzymes are capable of converting their substrates to metabolites which the cell can also obtain independently and more readily by metabolism

of glucose (35). The second assertion was that those metabolites are normally formed from glucose at a rate more than sufficient to saturate the capacity of the cell to convert them to synthetic products, so that a cell growing on a mixture of glucose and some more slowly degradable compound would not profit from the manufacture of the enzymes needed for the breakdown of the latter. A cell capable of dispensing with the manufacture of extra enzymes would have an economic advantage, particularly if the conserved compound were something like an amino acid which could be used directly in synthesis. Magasanik and his coworkers noted the similarity between this catabolic control mechanism and repression exerted by the ultimate product of a biosynthetic pathway on the enzymes which catalyze the individual reactions of the pathway. In 1957, they formulated the concept that catabolites which are formed rapidly from glucose accumulate in the cell and repress the formation of enzymes whose activities would only augment the already large pool of those compounds. This interpretation led to the name "catabolite repression."

c. Supporting Concepts. If the theory of catabolite repression were valid, then any condition leading to a decrease in the rate of biosynthesis of protein or nucleic acid without a comparable reduction in the rate of catabolism should lead to an intracellular accumulation of metabolites and an increased repression of catabolic

enzymes. That compounds normally degraded too slowly to exert catabolite repression can repress under those conditions was demonstrated by Magasanik and his coworkers (35) using guanine-requiring mutants of Aerobacter aerogenes and E. coli. The validity of this point was confirmed by Mandelstam (in 35) with nitrogen-starved cells (whose continued slow synthesis of protein was due to turnover) that could produce β-galactosidase only in the absence of an energy source and with cells grown under nitrogen-limitation in continuous culture (36).

One fundamental question concerning the theory was whether different metabolites were responsible for the repression of different enzymes. When cells were placed in ammonia-free medium with glucose and histidine, the repression of histidine degrading enzymes was lifted although the synthesis of inositol dehydrogenase was still repressed, indicating different corepressors (35). On this same point, McFall and Mandelstam (37) demonstrated that different metabolites served as corepressors for three different enzymes and that glucose repressed only when it gave rise to sufficient amounts of the specific corepressor. They advocated expanding the concept somewhat by changing the name to "metabolite repression."

Since Magasanik's paper (35) was presented at the same conference in which the operon model was discussed, there was speculation concerning the involvement of the regulator gene in metabolite repression. During the

discussion, Brown (in 35) presented data demonstrating that cells with mutations in the regulator gene for β-galactosidase formed the enzyme at a maximal rate in the absence of the inducer but were still sensitive to the repression exerted by glucose in the absence or presence of inducer. Although this indicated that the product of the regulator gene was not involved in metabolite repression, it did not negate the possibility that the mechanism works by acting eventually on the operator gene. McFall and Mandelstam (36) (37) also presented evidence that the repression is exerted independently of the regulator gene.

3. Basic Mechanism

Nakada and Magasanik have studied the basic mechanisms of metabolite repression. By using 5-fluorouracil, which is incorporated into m-RNA, causing the subsequent synthesis of inactive protein, and by separating the induction phase from the phase of enzyme synthesis, they obtained evidence that induction stimulated the formation of m-RNA specific for β-galactosidase while metabolite repression slowed the formation of the m-RNA (38). Once induction had occurred, the subsequent synthesis of β-galactosidase in inducer-free medium was not affected by the presence or absence of repressing metabolites (39). This, therefore, excluded the possibility that repression by metabolites was due to the inhibition of the release of enzyme from ribosomes as postulated by Hauge, McQuillan,

Cline, and Halvorson (in 39). If induction took place in the presence of glucose but before the metabolic products of glucose had begun to accumulate, the cells acquired their full capacity for β-galactosidase synthesis, demonstrating directly that the corepressor was not glucose itself but a metabolic product (39). McFall and Mandelstam (40) had suggested that glucose exerted its effect on the lac system by virtue of its rapid conversion to galactose, but by using the same type of experiment that they had used with glucose, Nakada and Magasanik (39) showed that it was also necessary for galactose to be metabolized to exert its effect.

Nakada and Magasanik (39) summarized the nature of metabolite repression as observed in the lac system. The metabolites from glucose inhibit, but do not completely block, the synthesis of β-galactosidase m-RNA, and the level of those metabolites determines the rate at which the RNA is synthesized. The level of m-RNA, in turn, depends only upon the rate of synthesis because it decays exponentially at the same rate irrespective of the presence or absence of inducer or metabolite corepressor and irrespective of the rate of protein synthesis or rate of energy metabolism. Since the differential rate of β-galactosidase synthesis is determined by the proportion of m-RNA specific for it in the total cellular m-RNA, the level of metabolite corepressors will determine the rate of enzyme synthesis.

4. Relationship to the Operon Model

One area of continued interest is the relationship between metabolite repression and the operon model. Evidence had been presented that the repression was exerted independently of the regulator gene product postulated to be involved in induction. In 1964, Loomis and Magasanik (41) used mating experiments with *E. coli* to confirm that fact. The experiments were designed to rule out the possibility of any allosteric character of the regulator gene product and showed that the repression appeared to depend upon the level of the metabolite (corepressor) but not upon the level of the regulator gene product.

In 1965 a mutant was isolated with insensitivity to metabolite repression (42). The character of the mutant suggested the presence of another regulator gene which determined the metabolite-sensitive repression of the lac operon. This gene (labeled the CR gene) controlled a cytoplasmic factor which was specific for the lac operon and controlled the rate of synthesis of both β-galactosidase and β-galactoside permease (involved with transport into the cell); thus, it resembled the regulator gene postulated in the operon model (43). Carrying the analogy further, the operon model would predict a genetic region contiguous to the lac operon serving as an operator for the CR gene product. Since an O^c mutant strain (one which has a deletion mutation in the operator region

making it insensitive to normal repression) was still sensitive to metabolite repression, the product of the CR gene probably does not interact with the classic operator (41). Studies are currently underway in Magasanik's laboratory to determine the nature and location of the operator region for metabolite repression.

5. Involvement of the Reduction of Internal Inducer Concentration in Diauxie

Additional studies with the lac system revealed another method by which glucose could affect the rate of expression of the operon when it was discovered that glucose could reduce the internal concentration of inducers for the operon (44). Pre-induction or a high concentration of lactose (8×10^{-2} M) could overcome diauxie caused by glucose. Both results are consistent with an increased level of lactose in the cells, in the first case from increased permease activity, and in the second from the higher external concentration of lactose. Loomis and Magasanik (44) pointed out that other groups had shown that pre-induction of permease would help to overcome the severe repression exerted by glucose. They suggested that diauxie was actually caused by two phenomena acting together. Metabolite repression reduced the basal level of permease so that insufficient activity was present to overcome the ability of glucose to reduce the internal concentration of inducer.

Adhya and Echols (45) presented evidence that inhibition at the level of inducer transport is a possible primary mechanism of diauxie in the case of the galactose degrading enzymes. They stated that glucose did not inhibit induction of the galactose enzymes if glucose and galactose were produced intracellurlarly by the hydrolysis of lactose. However, the data presented in their Table 4 for a galactoside permease constitutive mutant does not verify this because it shows that the level of transferase in cells grown on lactose plus glucose was 40% of the level in cells grown on galactose and that the level in cells grown on galactose plus glucose was 50% of the unrepressed level (45). Though it appears obvious that a repression had occurred in both cases, it is possible that their statement was based on the failure to obtain a complete repression of synthesis. The growth curve on galactose plus glucose for the mutant was not diauxic as it was in the wild type. Probably, therefore, the effect that they observed was similar to that reported by Loomis and Magasanik (44), wherein the severe repression characterized by diauxic growth on glucose plus lactose was caused by a combination of the effect on transport and metabolite repression. Thus, six years after Magasanik (35) had dismissed the proposal of Cohen and Monod that interference with the transport mechanism of the inducer was involved in diauxie, new evidence has been presented showing that it is partially, but not entirely, the cause in some cases.

6. Attempts to Identify the Metabolite Corepressor

Some attempts have been made to identify the metabolite that acts as the corepressor to trigger metabolite repression. In the β-galactosidase system of E. coli, glucose, galactose, gluconate, or their direct derivatives were not required for repression because when anabolism was reduced in an isomerase-negative strain repression was caused by fructose, lactate, xylose, and succinate (the former group of compounds could not be formed from the latter in that mutant) (46). Also, compounds of the Krebs cycle did not appear to be required since glucose gave rise to repression in a mutant under conditions where the formation of Krebs cycle compounds was severely restricted. Loomis and Magasanik (46) suggested that if metabolite repression were caused by a single compound that it would probably be related to the pentoses and trioses of intermediate metabolism.

Dobrogosz (47) (48) and Okinaka and Dobrogosz (49) showed an association between metabolite repression of β-galactosidase and the oxidative decarboxylation of pyruvate. When cells were placed under anaerobic conditions, metabolite repression was shut off for one to one and one-half generations, then switched back on, but if nitrate or pyruvate were provided when the anaerobic conditions were imposed, then the repression continued. When oxygen, nitrate, or hydrogen ions were available as electron acceptors, pyruvate decarboxylation proceeded at a

rapid differential rate and repression occurred, but in the absence of the acceptors, the decarboxylation proceeded slowly and there was no repression. Their interpretation of those results was that repression increased in proportion to the efficient dissimilation of pyruvate to acetate and CO_2 with the accompanying production of ATP and other "high-energy" compounds. Based on several considerations they postulated that one or more of the energy rich compounds might function directly or indirectly as the primary signal or corepressor for initiating metabolite repression. This is an interesting proposal, especially in light of the fact that ATP is thought to be involved with allosteric interactions of some enzymes, as discussed previously. If ATP is involved in such interactions, then a similar allosteric activation of an inactive CR gene product would provide a satisfying unity to control mechanisms. If this were true, however, then it would appear that many cases would have been reported in which a single compound serves as an effective repressor for several enzymes. Instead, as was pointed out earlier, both Magasanik (35) and McFall and Mandelstam (37) have shown that different metabolites were required for different enzymes. This question will probably not be resolved for some time because the level of metabolites and level of ATP in the cell are related in such a way that when the level of metabolites is high, the level of ATP is apt to be high also.

Mandelstam and Jacoby (50) presented evidence for the involvement of specific compounds in the repression of the first three enzymes of the mandelate degrading pathway. The first three enzymes in the pathway were determined by an operon and were inducible by mandelate, but they were repressible by benzoate (the end-product to which they give rise), catechol (the end-product of the second group in the pathway), and by succinate (the final product of the pathway).

7. Transient Repression

There is one other area that should be briefly mentioned in the theoretical considerations of metabolite repression, and that is "transient repression." When cells from certain strains of *E. coli* were transferred from a medium containing glycerol to one containing glucose and an inducer of β-galactosidase, there was no delay in the resumption of growth, but the cells did not make detectable amounts of β-galactosidase for a period of approximately one generation, after which they recovered the ability to form enzyme (51) (52). This effect was also observed for galactokinase and tryptophanase. The extent of recovery was to about 15% of the differential rate of synthesis established when glucose was not in the medium (53). The cause of the severe transient repression was thought to be the high internal level of metabolites that was produced when glucose was added to the system

(53). Following the addition of glucose, there was a rapid increase in the concentrations of glucose-6-phosphate, 6-phosphogluconate, fructose-1, 6-diphosphate, and NADPH, but as these pool sizes decreased the severe repression was released and the less severe repression took over.

Genetic studies showed that transient repression was distinct from metabolite repression because it required a functional operator, and could be genetically abolished in strains that still showed metabolite repression (54). Tyler, Loomis, and Magasanik (55) found transient repression to be independent of a mutation in the CR gene which made the mutant insensitive to metabolite repression. Palmer and Moses (56) used two strains with mutations in the regulator gene to demonstrate that when the cell was made constitutive transient repression was abolished, although metabolite repression had been shown to be active under those conditions. They postulated that perhaps transient repression was caused by interaction of the metabolite corepressor and the regulator gene product so that the affinity of the repressor for the operator was increased.

Other differences between metabolite and transient repression are that the phosphorylated compounds implicated in transient repression presumably do not enter the common metabolic pool involved in metabolite repression, and that transient repression appears to be effective as soon as the new compound enters the cell while at 37° about

eight minutes of protein synthesis elapse before metabolite repression begins (55). Therefore, Tyler et al. (55) concluded that transient repression is a general phenomenon which effects the differential rate of β-galactosidase synthesis immediately upon the addition of many compounds to cells growing in another carbon source; does not result from a reduction in the internal level of inducer; and is apparently elicited by a mechanism distinct from that which mediates metabolite repression.

8. Summary

In general, it appears that metabolite repression works in a manner analogous to the control mechanisms of induction and repression postulated in the operon model. The evidence indicates that inducible catabolic enzymes are controlled by two regulator genes, one of which (CR gene-type) is sensitive to the level of a corepressor whose structure is unknown, so that when the level of the corepressor (probably an intermediary metabolite) is high the CR gene product is activated causing a decrease in the rate of inducible enzyme synthesis. It is currently not known how the reduction is caused. Regardless of the particulars of the mechanism, the effect is to reduce the rate of synthesis of the enzymes needed to degrade one compound when another more easily degraded compound is placed into the medium.

CHAPTER III

LITERATURE REVIEW

All of the concepts presented in the previous chapter were developed using pure cultures of microorganisms; however, bioenvironmental engineers must often work with natural or heterogeneous microbial populations, particularly in waste water treatment. As Gaudy (31) pointed out in the introductory remarks of a paper presented before the American Society for Microbiology, working with such populations had perhaps limited basic research into microbial mechanisms and fostered a dependence on certain "platitudes" by engineers. One such over-simplification that had been prevalent for some time was that because waste waters have a variety of carbon sources and organisms, each species will pick out the substrate it can metabolize best, and removal of all exogenous carbon sources will proceed concurrently. However, exposure to Monod's concepts of diauxie led Gaudy to question that "platitude" and initiate experiments which have shown that the control mechanisms that apply to pure cultures have broad applicability and are exerted in natural populations as well. This literature review is concerned chiefly with the expanding area of research into control mechanisms as found in natural

populations plus additional information on pure cultures that is applicable to continuous flow operation.

A. Batch Systems

The preferential utilization of glucose by a sorbitol-acclimated culture was the first published example of metabolic control mechanisms in heterogeneous populations (14). Glucose was totally removed from the system before the metabolism of sorbitol began, but the lag between the utilization phases was not severe enough to cause diauxic growth or diphasic oxygen uptake.

The effect of cell age (defined by operational parameters) on the response of the system was studied next (16). Old cells (those that had been grown in a batch unit with wastage of one-third of the mixed liquor every day for at least 21 days) failed to show the sequential substrate removal exhibited by young cells (2 ml of seed used to reseed 60 ml of fresh medium every day for three days) while intermediate-age cells (a small portion of old cells were washed and resuspended in fresh medium, then fed like the old cells for three days) showed an intermediate substrate removal response. In the last system sorbitol removal continued in the presence of glucose but at a decreasing rate until all glucose was gone, then proceeded at an increasing rate. Old cells that had been seeded into flasks and grown under "new cell" conditions gave the new cell substrate removal response, indicating that the

change in response of the system with age was not due to a shift in the population away from cells possessing the responsible control mechanism. The cause of the cell age phenomenon is still under investigation.

In the experiments in which the cell age phenomenon was discovered, the initial cell concentration was high, yet sorbitol metabolism was stopped in spite of the prior acclimation to the substrate (16). In order for that to occur, the activity of the enzymes already present must have been altered. This implied that an inhibition of enzyme activity was occurring as well as a repression of synthesis. The results of two other types of experiments led to the postulation of the catabolic inhibition mechanism discussed in the previous section. First, in both heterogeneous and pure cultures, sequential substrate removal occurred in a large inoculum under non-proliferating conditions. Since little or no enzyme synthesis could occur under those conditions, the only possible relevant mechanism was inhibition (15). The second type of experiment involved a "severe" shock load in which the shock compound was injected into a culture actively metabolizing another substrate (57) (58). Using young cells it was shown that the metabolism of sorbitol, mannitol, or dulcitol was blocked immediately by the addition of glucose. When a unit degrading sorbitol was shocked with galactose, there was an initial blockage of sorbitol utilization, followed by concurrent removal of the two

substrates with sorbitol removal occurring at a diminished rate. A lactose shock had no effect upon the removal of sorbitol, and lactose removal did not start until all sorbitol was gone. When a ribose degrading unit was shocked with glucose, there was no immediate effect upon ribose metabolism, but as the rate of glucose metabolism increased, the rate of ribose metabolism decreased. The results of these experiments were additional evidence for the existence of the catabolic inhibition mechanism and emphasized the broad character of control mechanisms in general.

Su (17) recently completed a study on the utilization of mixtures of carbohydrates by heterogeneous populations in which he investigated 18 different combinations of substrates. Experiments were run with cells acclimated to each substrate in each pair. He observed four different patterns of substrate removal: 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 could characterize the growth patterns, 20 were of type 1, 11 of type 2, one of type 3, and one of type 4. Thus, it is clear that substrate interactions caused by metabolic control mechanisms is a widespread phenomenon in natural populations.

In a study preliminary to this report, it was shown that glucose slightly decreased the rates of removal of glutamic acid and aspartic acid (18). In both cases the substrate removal was concurrent, although it was obvious that an interaction had occurred. Because amino acids generally were removed concurrently with glucose, it was necessary to develop a more sensitive method for detecting a repression or inhibition effect in mixtures of carbohydrates and amino acids. The method developed for the present studies will be discussed in the next chapter.

Prakasam and Dondero (59) (60) acclimated sewage seed to sorbitol in a manner identical to that used by Gaudy (14) in order to check his report of sequential substrate removal. Using the young cell technique, they confirmed independently the effect of glucose on sorbitol removal. Since the results depended on an indirect determination of sorbitol by calculation, they also performed experiments using radioactively labeled sorbitol which substantiated their previous data. They also performed labeled sorbitol utilization experiments with activated sludge directly from a sewage treatment plant (60). Removal of sorbitol occurred more quickly in the mixed unit than it did in the control, but since no data were given for glucose utilization in the mixture or for sludge growth it is difficult to evaluate their results. However, in light of Gaudy's (16) findings of the sludge age effect, it is not surprising that concurrent removal appears to have occurred,

since cells in activated sludge are often quite old. In fact, the oxygen uptake data for the activated sludge indicates very low activity, characteristic of an old sludge. Although they were aware of the cell age phenomenon, they made no mention of it in connection with this finding.

Prakasam and Dondero evaluated the diversity of the microbial communities in the sewage with which they started and in the adapted cultures with which they ran the sorbitol removal experiments. As would be anticipated, they found a divergence between the types of organisms in the two systems. They then said (60):

> The simplification of the population during adaptation provided an explanation for the repression of sorbitol metabolism by glucose. ... In a truly mixed population, whatever enzyme repression takes place appears to affect only a part of the population, leaving the remainder free to attack the available substrates immediately.

The author must disagree with the implications of these statements. Actually, a population selected for growth on one compound offers the most severe test of the importance of the repression or inhibition phenomena. In such a selected population, the only organisms present are those that can grow on the original substrate or its excreted metabolic products. Those that have been "discarded" are those which cannot use the substrate. Since only extraneous organisms are missing, their absence should have no effect upon the operation of the control mechanisms involved with a substrate that they cannot use. In a "truly mixed population" the only way that the

"remainder" can be free to attack the substrate is if that remainder has the genetic capability to use that substrate. If it has that ability, it will be present in the selected population, and, thus, its effect will still be measured. It is much more likely that the concurrent removal observed in the activated sludge was due to the sludge age phenomenon instead of the divergence in population that existed between it and the adapted culture.

Stumm-Zollinger (61) (62) has also studied metabolic control mechanisms in heterogeneous populations. In her first paper (61) she considered the kinetics of substrate utilization as related to growth, but unfortunately her final equation was incorrect. She integrated $-dS/dt = (\mu/y)B_o e^{\mu t}$ and obtained $-\Delta S = (B_o/y)(1 + e^{\mu t})$; however, the correct equation is $-\Delta S = (B_o/y)(1 - e^{\mu t})$. Although this could have been a typographical error, her use of the equation is open to question. She said that a plot of $\log(-\Delta S)$ versus time gives a linear relationship for $t >> 0$; however, in order for $(1 - e^{\mu t})$ to be approximately (less than 5% error) equal to $(-e^{\mu t})$ (the condition needed to make a simple log plot) t must be greater than 6 hours, a condition rarely met in her experiments. Even more critical is her statement that "the rate of bacterial growth, as an approximate measure of the rate of enzyme formation, is determined over a period of several generation times" (61). That growth rate could represent the rate of enzyme formation would be a valid assumption for

enzymes in a single substrate system or for the total enzyme complement in a multi-component system, but it is not valid for the specialized enzymes needed to degrade one compound in a multi-component system. It cannot be assumed that the rate of growth will represent the rate of synthesis of a particular enzyme when the object of the study is to determine the existence of a control mechanism (repression) which exerts its effect by altering the rate of synthesis with respect to the rate of growth.

Because of the above statement, it would appear necessary for readers to evaluate independently the data from Stumm-Zollinger's growing systems, but unfortunately not enough data were presented to allow this. The data showed that glucose exerted no inhibitory effects upon the activity of benzoate degrading enzymes, but, in the growth experiment, no substrate removal data were presented for the control reactors so that it is impossible to determine whether any repression occurred in the mixed reactor. Glucose exerted an inhibitory effect upon the removal of galactose in cells acclimated to galactose, but the experiment performed with glucose-acclimated cells was inconclusive. The same time was required for the initiation of galactose removal in the galactose control as in the mixed unit, so it was concluded that glucose had no effect upon the formation of galactose degrading enzymes. Unfortunately, since all glucose was removed from the mixed reactor before the initiation of galactose removal in the

control, it is impossible to determine whether glucose exerted any effect. In such a mixture, where an inhibition of activity had been shown to occur, an assay for galactose degrading ability in the absence of glucose would be necessary in order to distinguish between inhibition and repression. This same argument applies for the growth experiment which showed diauxic growth. Such an assay will be discussed in the next chapter. Glucose had no effect upon the activity of pre-formed phenylalanine degrading enzymes but did repress the synthesis of those enzymes in a growing system. Although it was claimed that benzoate exerted the same effect upon phenylalanine degrading enzymes, only a diphasic growth curve was presented and this could be interpreted as showing growth on phenylalanine first. In an interesting experiment, it was shown that phenylalanine served as the nitrogen source for growth on glucose in an ammonia-free medium, and that a lag period was required before phenylalanine degradation commenced after the utilization of glucose.

Although Stumm-Zollinger's experiments could have made a contribution to the bioengineering knowledge of control mechanisms, they were ruined by her failure to realize the importance of comparing the change in removal rate with respect to a unit change in cell mass in the mixture to the corresponding change in the control. It also appears that she did not realize that metabolite repression can result merely in a decrease in the rate of synthesis of an

enzyme, and need not necessarily cause a complete cessation. When only a decrease occurs, concurrent removal will still be found, although repression is still operative. Methods of evaluating results to take this into account will be discussed in the next chapter.

The same general comments apply to Stumm-Zollinger's second paper (62). She said that in a growing culture repression is indicated if the rate of substrate utilization stays constant despite an increase in solids, again not recognizing that repression does not have to be a complete cessation of synthesis. Her results suffer from her failure to consider the fact that her controls and mixtures had different concentrations of organisms, particularly in the tryptophan experiment. All of the experiments presented on galactose used substrate and cell concentrations such that little growth could occur, so that they were measurements of inhibition of activity, and as such agree with her previous paper. The reconstituted bacterial river water community exhibited a delay in the utilization of galactose in the presence of glucose, which perhaps was caused by a repression mechanism, but no explanation was offered. Stumm-Zollinger's conclusions agree with Prakasam and Dondero (60) that acclimation leads to enrichment of a few species; however, as pointed out in the discussion of their results, this is not necessarily a disadvantage.

Because Toerien and Kotze (63) thought that metabolite

repression might play an important role in anaerobic digestion of sewage and industrial wastes, they isolated a strain of Bacillus cereus from a laboratory anaerobic digester and studied the effects of glucose, fructose, galactose, and soluble starch on the activities of protease and various intermediary metabolic enzymes. The experiments were performed in shaker flasks, presumably under aerobic conditions, so that, in light of the work of Dobrogosz (47) (48), the application of the results to anaerobic conditions is questionable, although they might offer some information concerning metabolite repression in general. Protease activity was measured at the end of 24, 48, 72, and 96 hours, while the other enzymes were determined after 72 hours. The growth rate constants were the same in all units, but the duration of exponential growth was not noted and, therefore, it is impossible to assess at what points in the growth cycle the enzyme assays were performed. Since Bacillus cereus grows quite rapidly, it is probable that some of the analyses were performed after all of the substrates were exhausted, in which case they would not particularly be meaningful in relation to the repression mechanism. Protease units were plotted versus time in all of the reactors. At 24 hours only the glucose reactor differed from the control (which contained only bacto-peptone) and its enzyme level was lower, indicating repression. At 48, 72, and 96 hours, there was no difference between the galactose reactor and

the control, but the protease levels in the fructose and starch reactors were higher than the controls. Growth curves were not presented and the activities were expressed as units per ml of test solution so it is not possible to determine the specific activities for direct comparisons among the units. The data were discussed in terms of possible mechanisms for the repression based on the analyses of the activities of EMP, pentose shunt, and TCA enzymes, but since no data were presented indicating the nature of the culture when the analyses were performed the significance of the discussion is limited. About the only conclusion that could be confirmed from the data was that glucose repressed protease synthesis, but fructose, galactose and starch did not.

The last three sets of papers discussed have emphasized three shortcomings of bioenvironmental engineers working in the area of metabolic control mechanisms using batch cultures: 1) The failure to recognize that metabolite repression can result in only a decrease in the rate of enzyme synthesis and does not always cause a complete cessation; 2) the failure to provide adequate controls in the experiments; and 3) the failure to evaluate data in growth experiments on the basis of the amount of change in substrate removal rate that occurs with a unit change in solids. The experiments presented in this report were designed to take these considerations into account and the methods employed will be discussed in the next chapter.

B. Continuous Flow Systems

1. Heterogeneous Populations

The most important type of shock load (from the standpoint of control mechanisms) to a continuous flow reactor is the qualitative one because it can cause substrate interactions within the cells. The only published research report of this type of shock is the paper by Komolrit and Gaudy (64) in which they studied the responses of completely mixed, continuous flow reactors growing on sorbitol or glycerol at four hour detention times to glucose shock loads. When the shock was strictly qualitative (i.e., no change in the total organic loading), the system responded quite well with very little washout of substrate into the effluent. When the load was applied by adding the second compound in addition to the first, there was a disruption of treatment in proportion to the magnitude of the shock. For example, when the influent was changed from 1000 mg/l sorbitol to 1000 mg/l sorbitol plus 1000 mg/l glucose, there was no washout of either sorbitol or glucose although the total COD in the effluent did reach almost 300 mg/l due to excreted metabolic products. When the influent was changed from 1500 mg/l sorbitol to 1500 mg/l sorbitol plus 1500 mg/l glucose, the sorbitol concentration in the effluent rose to 200 mg/l and the total COD to 600 mg/l, indicating a disruption of sorbitol utilization and a large production of metabolic intermediates. After a

transient period the compounds were again removed in the reactor. Similar results were obtained for the glycerol reactor when glucose was added to the feed. When 1500 mg/l of glucose was added to the feed of a reactor growing on 500 mg/l glycerol, there was a build-up of glucose for two hours before the culture was able to metabolize it, but once utilization started it caused a build-up of glycerol in the reactor.

Komolrit and Gaudy also shocked the reactors under conditions of nitrogen deficiency at which time the cells could not respond by greatly increasing the biological solids concentration as in the previous experiments. Under the most severe nitrogen limitation, the cells used the added glucose in preference to the sorbitol or glycerol, causing the latter two to be washed out in the effluent with little or no removal. In the less severe experiments of this type, there was an increase in solids, probably due partly to stored carbohydrate material, allowing less wash-out in the effluent. No explanation was offered for the drastic alteration in the pattern of response under the more severe nitrogen limitation, but they did speculate that possibly the synthesis of enzymes required for rapid formation of polysaccharides could occur under the less severe nitrogen limitation but not under the greater. Part of the difference between the systems may lie in the fact that before the shock the less severely limited systems had a BOD:N ratio of 10:1, while

the one subjected to the severe limitation had BOD:N of 20:1. As the shock loads on the first system (10:1) show, there was probably excess nitrogen in the system prior to the shock so that when the influent substrate concentration was changed to give a ratio of 20:1 the system was brought into balance with all nitrogen being used. The basis for this conjecture is that if more nitrogen had been available, the solids would not have stabilized while the effluent still contained 300 mg/l of COD. Prior to the severe nitrogen-limited shock the BOD:N ratio was 20:1, and the control curves given show that the effluent COD was 200 mg/l, indicating that the system was under nitrogen-limited conditions, not carbon-limited. Thus, when the shock was added (giving BOD:N of 40:1) there was no excess nitrogen to allow more solids production, and the response was that of a system that is not carbon-limited. It would be interesting to see the response of a system taken from BOD:N of 10:1 to the level of 40:1 by the addition of substrate. The final steady state values would probably be similar to those reported but the transient conditions would be different, with an increase in solids until nitrogen became limiting.

2. Pure Cultures

Because their interests are different from those of the waste water treatment engineer, the basic scientists have not approached the problem of metabolite repression

from the aspect of "shock loads". Instead, when they have used continuous flow conditions, repression has been studied by adding the inducer and following the rate of increase of enzyme when some other carbon source was in the medium. Boddy et al., (65) followed the level of amidase specific activity when acetamide was added to the feed of continuous flow reactors growing on succinate and found that both the rate of increase and the steady state levels were functions of flow rate. At the low flow rate ($D = 0.22\ hr^{-1}$), the amidase level reached 100, but at $D = 0.76$, it reached a level only slightly higher than the basal level found on succinate alone. When the levels of amidase in cultures growing on acetamide or acetamide plus succinate were compared, it was found that the steady state activity in the mixed substrate system decreased more rapidly than that in the acetamide culture as the growth rate was increased (66). It was postulated that the production of metabolic intermediates caused the more rapid decrease in amidase synthesis with increasing flow rate in the mixed system (65). At the low growth rate, the metabolites were used and the internal carbon pool depleted, so that when acetamide was added to the feed the conditions were favorable for induction and the rate of synthesis was regulated by the supply of inducer. At the higher growth rates, however, the pools were probably less depleted, causing a shift in the balance between inducer and catabolite repressor in favor of repression

until at the highest growth rate the amidase synthesis was almost completely stopped.

Baidya, Webb, and Lilly (67) studied the response of the two-stage chemostat when the feed was shifted from glucose to glucose plus lactose. The volume of the second stage was 1.7 times the volume of the first. At the lowest flow rate studied (D = 0.148 in first stage), 45 hours were required for the initiation of lactose utilization, with degradation starting in the second reactor, and following shortly in the first reactor so that when the new steady state was reached both substrates were removed in the first reactor. At higher flow rates, the time between introduction of lactose and commencement of utilization increased, and steady states were reached, which lasted for long periods, in which only glucose was removed in the first reactor and lactose in the second. Under the conditions when both substrates were utilized in the first reactor, if the lactose was removed from the feed, kept out long enough for the glucose steady state to be re-established, and then returned to the medium, lactose utilization in the first reactor was immediate. Because of this finding and because the delay in utilization when lactose was first introduced was much longer than the diauxic lag observed in batch, they postulated the involvement of the permease effect known to occur in lactose utilization (i.e., the protection offered by preinduction against repression in the lac operon). Since the cells

were preinduced and the permease is fairly stable, when the lactose was added again it could be taken inside the cell to cause further induction and allow utilization immediately. When the cells were not preinduced, the level of intermediates from glucose utilization was evidently high enough to prevent entrance of lactose and synthesis of the β-galactosidase system.

Harte and Webb (68) studied the response of a two-stage reactor to the introduction of maltose. Maltose and glucose gave diauxic growth in batch, but when maltose was added to the feed of a continuous flow reactor at low flow rate (D up to 0.38 in first reactor) the response was the same as an increase in the glucose concentration: there was no lag before utilization began. At medium flow rates (D = 0.46 to 1.03), there was a distinct lag before maltose utilization began which increased in duration as the flow rate increased. Growth due to maltose occurred initially in the second reactor, followed after a long delay by the removal of all maltose in the first reactor. At fast flow rates (D = 1.055 to 1.135), maltose removal was accomplished only in the second reactor. These results indicate that repression of synthesis of maltose degrading enzymes in the first reactor became more severe at the higher flow rates.

The literature on both heterogeneous and pure cultures in continuous flow has shown that metabolic control mechanisms influence the response of organisms to a change in

environment. In the heterogeneous culture experiments a glucose shock load disrupted sorbitol metabolism. The pure culture experiments showed that repressing-type compounds influenced the induction of enzymes for degradation of new compounds in the medium and that the response was a function of the flow rate. The experiments that will be presented in this report were designed to measure the influence of several carbohydrates on lysine removal in continuous flow, to determine whether that influence was due to metabolite repression, and to ascertain the effect of flow rate upon the response.

CHAPTER IV

MATERIALS AND METHODS

A. General Conditions

1. Batch Experiments

a. Organisms. The organisms used in all experiments were obtained from a laboratory batch reactor initially seeded with primary clarifier effluent from the municipal sewage treatment plant at Stillwater, Oklahoma.

b. Daily Growth Conditions - Stock Reactor. The culture was maintained in a 100 ml aeration vessel (stock reactor) by transferring five per cent of the growing culture into fresh medium every twelve hours. The substrate was L-lysine at a concentration of 5000 mg/l COD. Buffer and inorganic salts were provided in the following concentrations: 1.0 M potassium phosphate buffer, pH 7.0, 50 ml/l; $(NH_4)_2SO_4$, 2500 mg/l; $MgSO_4 \cdot 7H_2O$, 500 mg/l; $FeCl_3 \cdot 6H_2O$, 2.5 mg/l; $MnSO_4 \cdot H_2O$, 50 mg/l; $CaCl_2$, 30 mg/l; tap water, 200 ml/l. The remainder of the volume was distilled water.

c. Experimental Growth Conditions. Experiments were performed at room temperature ($22^\circ \pm 1.5^\circ$) in batch

reactors of 1500 ml volume, aerated at a rate of 3000 ml/min. Seed was taken from the stock reactor in volume sufficient to provide an initial biological solids concentration of approximately 30 mg/l. Buffer and salt concentrations were the same in all experiments: 1.0 M potassium phosphate buffer, pH 7.0, 33 ml/l; $(NH_4)_2 SO_4$, 1666 mg/l; $MgSO_4 \cdot 7 H_2O$, 333 mg/l; $FeCl_3 \cdot 6 H_2O$, 167 mg/l; $MnSO_4 \cdot H_2O$, 33.3 mg/l; $CaCl_2$, 20 mg/l; and tap water, 200 ml/l. The remainder of the volume was distilled water. The substrates and their concentrations were changed for each experiment, and will be appropriately noted in the results section.

2. Continuous Flow Experiments

a. Organisms. Primary clarifier effluent from the municipal sewage treatment plant at Stillwater, Oklahoma was used to seed a batch reactor in which 5000 mg/l lysine (COD) was the sole carbon source. The population was allowed to grow overnight and then a 250 ml aliquot was transferred to each continuous flow reactor with 2000 mg/l lysine COD. After two hours growth, the feed medium pumps were started.

b. Media. Reactor A was operated under carbon-limited conditions, with 1000 mg/l lysine, as COD, serving as the sole carbon source. Reactor B was operated under magnesium-limited conditions with 2000 mg/l lysine, as COD, serving as the sole carbon source. The lysine

concentration in reactor B was higher than that in A to insure that carbon was not limiting. The growth media were made of the constituents in Table I. The concentrations of the components of these media are similar to those normally used in the Bioenvironmental Engineering Laboratories for 2000 mg/l COD of carbon source, except for the $MgSO_4 \cdot 7H_2O$ in reactor B (64). Tap water was not added because it contains magnesium in variable quantities, and the $Na_2MoO_4 \cdot 2H_2O$ was used to provide molybdenum. When the shock loads were applied to the systems, 1000 mg/l of COD due to a carbohydrate was added to the feed media.

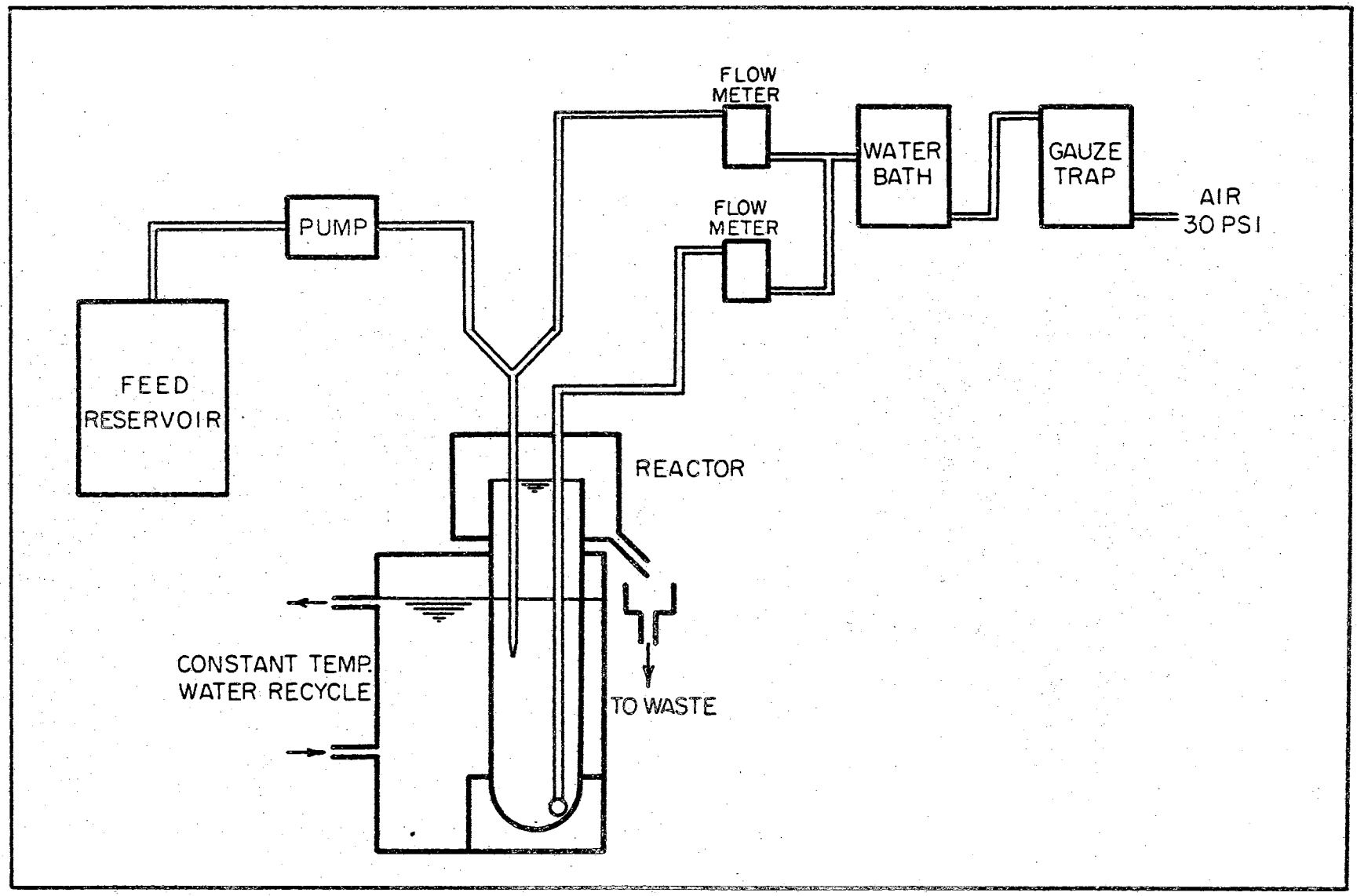
c. Apparatus. A diagram of the continuous flow reactor used in this study is shown in Figure 2. Since the objective was to study the metabolic control mechanisms operative when a carbohydrate was added to the feed, a single stage, completely mixed, homogeneous, open-type reactor was employed (69). The reactor was cylindrical, with a nominal volume of 1 liter, had effluent discharge around the entire upper edge, and was submerged in a constant temperature bath maintained at 25° C ± 0.5°. Aeration and mixing were provided by compressed air (30 psi) which was passed through a gauze trap and then through a water bath before entering the reactor at a rate of 3500 ml/min through a stone diffuser. Feed was delivered to the reactors by a peristaltic pump (Sigmamotor Model AL-2E) and was injected below the liquid surface to

TABLE I

CONTINUOUS FLOW GROWTH MEDIA

Component	Reactor A	Reactor B
1.0 M potassium phosphate buffer, pH 7.0	20 ml/l	20 ml/l
$(NH_4)_2SO_4$	1000 mg/l	1000 mg/l
$Na_2MoO_4 \cdot 2H_2O$	2.0 mg/l	2.0 mg/l
$FeCl_3 \cdot 6H_2O$	1.0 mg/l	1.0 mg/l
$CaCl_2$	15 mg/l	15 mg/l
$MnSO_4 H_2O$	20 mg/l	20 mg/l
$MgSO_4 \cdot 7H_2O$	200 mg/l	5.0 mg/l(D = 0.167) 1.5 mg/l(D = 0.083)
L-lysine-HCl (measured as COD)	1000 mg/l	2000 mg/l
Distilled water	to volume	to volume

Figure 2. Schematic Representation of a Continuous Flow Reactor


FLOW
METER
WATER
BATH
GAUZE
TRAP
AIR
30 PSI
PUMP
FLOW
METER
FEED
RESERVOIR
REACTOR
CONSTANT TEMP.
WATER RECYCLE
TO WASTE

insure against short circuiting. The injection tube was kept clear of reactor liquid by passing a stream of air (250 ml/min) through it, and was cleaned daily with a chromic acid cleaning solution as was the feed line downstream from the pump. The feed jug and line upstream from the pump were cleaned every other day and no difficulty was encountered with contamination of either. Dilute-in curves confirmed that the reactor was completely mixed. Two such units were run concurrently.

B. Experimental Protocol

1. Batch Experiments

Immediately prior to running an experiment, seed was removed from the stock reactor and centrifuged for fifteen minutes at 25,400 RCF in a Sorvall RC2-B refrigerated centrifuge. The supernatant was discarded, and the cells were resuspended in potassium phosphate buffer by blending for ten seconds at low speed in a Waring blender. The suspension was used to seed a lysine control reactor, carbohydrate control reactor, one or more reactors containing mixtures of lysine and carbohydrate, and a series of shaker flasks for the determination of maximum growth rate, μ_m. At zero hour, the substrates were added to the reactors and 40 ml samples were removed and centrifuged for twenty minutes at 18,400 RCF in a Sorvall SS-1 centrifuge. At the same time samples were removed for optical density determination at 540 nm using 19 mm tubes in a

Bausch and Lomb Spectronic 20. After centrifugation, the supernatant was carefully removed; 20 ml were used for determination of COD and 10 ml were frozen for later analysis for lysine and carbohydrate. Two samples were also used for measurement of biological solids concentration. This procedure was repeated at each sample point. In some experiments, aliquots were also removed for replica plating with the initial and final samples. As will be noted in the results section, a specific enzymatic capability determination was performed in some of the experiments. In those cases an extra sample was removed from each reactor, and all samples were centrifuged ten minutes at 37,000 RCF in the RC2-B refrigerated centrifuge.

2. Continuous Flow Experiments

The first set of experiments was performed at a detention time of six hours ($D = 0.167\ hr^{-1}$). A detention time of four hours had been tried, but the population was not stable at that flow rate. After all units had been shocked at $D = 0.167$, the flow rates were changed and the second set was run at $D = 0.083$, or a detention time of twelve hours. The reactors were allowed to stabilize and run at "steady state" for several detention times and then the feeds were changed by adding 1000 mg/l COD due to a carbohydrate (either glucose, fructose, or ribose). One 25 ml and two 10 ml samples were removed periodically, directly from the reactor, and centrifuged at 37,000 RCF

for 10 minutes in a Sorvall RC2-B refrigerated centrifuge. After centrifugation, the supernatant was carefully removed from the 25 ml sample; 10 ml were used for the determination of COD and 10 ml were frozen for later analyses of lysine and carbohydrate. The remainder was filtered in order to determine the biological solids concentration. The supernatants were discarded from the two 10 ml samples and the cells used for the determination of the specific enzymatic capability and the protein concentration. This procedure was followed until the new "steady state" was reached. The carbohydrate was then removed from the feed and the sampling procedure was followed until the system had again achieved "steady state".

C. Methods of Analysis

Except where noted, the procedures used in the batch and continuous flow experiments were the same.

1. Biological Solids

Gravimetric determination of biological solids was performed by filtration through membrane filters (0.45 μ pore size, Millipore Filter Corp., Bedford, Mass.) as described in Standard Methods (70). By plotting biological solids versus the corresponding optical density, a standard curve was prepared for each batch experiment. This curve was then used to convert the optical density readings of other samples to biological solids

concentration so that all results are reported as biological solids concentration. The gravimetric procedure was used on all samples in the continuous flow experiments. Biological solids concentration, mg/l, is represented by X in the equations and figures.

2. Cell Protein

The protein content of the cells was determined only in the continuous flow experiments. After the supernatant was discarded, the cells from one 10 ml sample were resuspended in 10 ml of distilled water by repeated rapid aspiration with a pipette and stored at -15°C for later analysis. Protein was determined on the suspension by the Folin-Ciocalteau method (71).

3. Chemical Oxygen Demand

The COD procedure employed was the alternate method given in paragraph 4.5, Section IV, of Standard Methods (70) for 40 ml sample size (batch experiments) or 20 ml sample size (continuous flow experiments). Mercuric sulfate and silver sulfate were used at all times. A check of the procedure using lysine, glucose, fructose, and ribose yielded COD values that were greater than 95% of the theoretical values, thus validating the applicability of the COD test.

4. Lysine

The concentration of lysine in the samples was determined by use of an acidic ninhydrin test as proposed by Chinard (72). Ammonia nitrogen does not interfere with the test, and proline, ornithine, and hydroxylysine are the only other amino acids which react significantly. All reaction volumes were doubled, and the absorbance was read at 450 nm. The linear correlation coefficient between lysine concentration and optical density was 0.999. For ease of comparison, results were expressed as equivalent COD values (1 mg/l lysine = 1.53 mg/l COD).

5. Glucose

The concentration of glucose in the samples was determined by the enzymatic Glucostat test (Worthington Biochemical Corp., Freehold, N. J.). In the continuous flow experiments, for concentrations below 60 mg/l, the dilute sample procedure was employed wherein the reagent was made to 50 ml instead of 90, and equal portions of reagent and sample were used. Results are expressed as equivalent COD (1.00 mg/l glucose = 1.06 mg/l COD).

6. Fructose

The resorcinol test was used for the determination of the fructose concentration in the samples and the results are expressed as equivalent COD (1.00 mg/l fructose = 1.06 mg/l COD) (73).

7. Ribose

The ribose concentration in the samples was determined by the orcinol test (71). (1.00 mg/l ribose = 1.07 mg/l COD).

8. Replica Plating

A replica plating technique (74) was used to ascertain whether there had been a shift in the population during a batch experiment. At the initial and final samples, aliquots were removed from each reactor, diluted, and plated on minimal medium containing all of the salts of the experimental medium. Because the substrate concentration was 1000 mg/l COD (and in order to prevent precipitation) the salts were at one-third the concentration of the liquid medium (which had substrate concentrations up to 3200 mg/l). A spread plate technique was used. The initial plating was done on agar containing both lysine and a carbohydrate and the plates were incubated at 25°C. After about thirty-six hours, the plates were replicated, using sterile pads, onto lysine agar, carbohydrate agar, and the mixed substrate agar. The colonies on the single substrate plates were compared to each other and to the mixed substrate replica to determine whether all colonies could use both substrates. This technique was used only as a rough check, and statistical analysis was not attempted.

9. Specific Enzymatic Capability

a. Batch Experiments. At various sample points, aliquots were centrifuged, the supernatant discarded, and the cells resuspended in a solution containing lysine, 500 mg/l (as COD); 1.0 M potassium phosphate buffer, pH 7.0, 33 ml/l; chloramphenicol, 100 mg/l; and actidione, 100 mg/l. The sample size and the amount of test solution used to resuspend the cells were adjusted so that the cell concentration for the test was between 200 and 300 mg/l dry weight. After aeration for one hour, the samples were centrifuged again and the supernatant analyzed for lysine. The final lysine concentration was subtracted from the initial value and the results were expressed as mg/l lysine COD removed per hour per mg/l cell mass. Since the chloramphenicol and actidione prevented further protein synthesis, and since no glucose was present to exert an inhibitory effect, the test was a measure of the enzymatic capability of the cells at the time that they were removed from the experimental reactor (75). A series of tests was run at various cell concentrations to determine whether lysine removal was linear during the one hour incubation time. The results showed that there was an immediate uptake (within the first minute) followed by linear substrate removal. Both the immediate uptake and the linear removal rate were proportional to the cell concentration. Because the significant consideration is the total amount of lysine removed, it was decided to include

the initial uptake in the enzymatic capability value, thus giving a relative test, not an absolute one. The initial lysine concentration was determined from analysis of the test solution.

b. Continuous Flow Experiments. Ten ml aliquots were centrifuged, the supernatant discarded, and the cells resuspended in a solution like that used in the batch experiments. After aeration for one hour the suspensions were filtered directly through membrane filters (0.45 μ pore size) and the supernatant analyzed for lysine. Usually the liquid passed through in one minute, but the maximum filtration time allowed was five minutes. (Since the majority of the liquid passed in the first minute, allowing five minutes for filtration contributes much less than the apparent eight per cent error.) The amount of test solution used to resuspend the cells was varied so that there was always at least 100 mg/l lysine COD left in the solution at the end of the one hour period; this tended to insure that lysine would not become rate limiting. A test was run to check for lysine adsorption on membrane filters and it was found that the lysine concentration in the filtrate was 87.2% of the unfiltered value. This value is based on a total of 56 samples, 28 filtered and 28 unfiltered. After correction for lysine adsorption, the final lysine concentration was subtracted from the initial value and the results were expressed as mg lysine COD removed/hr/mg cell protein (EC/P).

10. Maximum Specific Growth Rate

In order to determine any variation in the kinetic traits of the seed culture, the maximum specific growth rate (μ_m) on lysine was determined for every batch experiment. Four flasks containing 100, 200, 400, and 800 mg/l lysine COD, respectively, were seeded with the same culture as the experimental reactors. Growth was followed by measuring optical density of 540 nm as described previously. The growth rate, μ, at each substrate concentration, L, was obtained by measuring the slope of the straight line portion of a semi-log plot. The maximum specific growth rate (Monod kinetics) (76) was obtained by the use of an Eadie plot (77) of L/μ versus L. The same procedure was used to determine μ_m on glucose, fructose, and ribose.

D. Methods of Calculating and Plotting

1. Batch Experiments

Biochemists and microbiologists determined some time ago that the study of the synthesis of a cellular constituent as a function of time is difficult to interpret because the rate of synthesis depends upon both specific and nonspecific metabolic factors. Therefore, instead of considering the time rate of enzyme (E) synthesis, dE/dt, they consider the rate relative to the total rate of protein (P) synthesis, $dE/dt \,/\, dP/dt = dE/dP$. The term "physiological time" (dP) is substituted for "absolute

time" (dt), and the relationship dE/dP is called the differential rate of synthesis, S (78). Usually, since the protein content of a culture is a constant percentage during the exponential growth phase, the bacterial mass, X, is measured instead of P, and the expression dE/dX is used. When a growing culture is being studied, samples are removed periodically for the determination of cell concentration and enzyme content. The method of determining the enzyme content, E, may involve whole cells, purified enzyme, or whatever means is suitable. E is then plotted versus the bacterial concentration, X, at the time the sample was taken, and the slope, dE/dX, or the differential rate of synthesis, S, measured.

Metabolite repression has been defined as a decrease in the rate of synthesis of a particular enzyme in relation to the rate of formation of other proteins (35). If metabolite repression is being investigated, two reactors are used, one with the inducer only (the control) and one with the inducer plus a second compound whose effect is being studied (the mixture). A plot of enzyme content versus solids is made for each reactor and the differential rate of synthesis, S, is calculated for each system. If $S_{control}$ is greater than $S_{mixture}$, then metabolite repression has occurred.

In biological process engineering, a similar approach must be taken in order to determine whether one substrate in a reactor has interfered with the removal of another;

i.e., changes in a culture in relation to physiological time must be considered instead of absolute time. The following text should serve to amplify the approach taken in the present study. First, consider an absolute time plot of substrate concentration in a reactor. The point slopes of the curve, R, are the rates at which the particular substrate is being removed by the culture and are a measure of the total over-all enzyme activities of the culture at the times of measurement. The important difference between this total over-all activity and an enzyme assay is that an assay is a measurement of enzyme content, unaffected by any compounds in the growth medium which may inhibit their activity. The substrate removal curve, however, measures the activities of the enzymes in relation to all other substances in the cell and in the medium. If the substrate removal rates, R, are plotted versus the biological solids concentrations in the medium at the corresponding times, a plot similar to that used by biochemists is obtained, and the slope, dR/dX, could be called the <u>differential rate of activity</u>, A. If $A_{mixture}$ is less than $A_{control}$, then some metabolic control mechanism has been operating because each unit solids change in the mixed reactor has not resulted in the same change in total enzyme activity as a corresponding unit solids change in the control reactor. Since the substrate removal rate, R, measures the total over-all activity and not actual quantity of enzyme, a decrease in the

differential rate of activity, A, could be the result of either an inhibition of pre-formed enzyme activity or a repression of new enzyme synthesis.

The "specific enzymatic capability" of the cells is measured with cells that have been removed from the reactor and washed, so that they are no longer influenced by any inhibitors in the medium. Since this test is similar to a whole cell assay (79), the enzymatic capability may be thought of as being roughly equivalent to the enzyme content of the cells. If the specific enzymatic capability (mgCOD/hr/mg cells) were multiplied by the concentration of solids in the reactor at the time the sample was taken, the result would be the "total enzymatic capability" of the culture, EC(mgCOD/hr/l). If the value of EC were plotted versus the biological solids, the slope of the resulting curve, dEC/dX, would be analogous to the differential rate of synthesis, S. As with an enzyme assay, if $S_{mixture}$ is less than $S_{control}$, then repression of synthesis has occurred.

Using the reasoning above, the curves shown in the results section were obtained in the following way. The substrate curves were plotted arithmetically versus time and then graphically differentiated to obtain the slope, R. R was then plotted versus the biological solids, X, at the corresponding time in order to determine dR/dX, the differential rate of activity, A. Similarly, the total enzymatic capability of the culture, EC, was plotted

versus the solids, X, in order to obtain the differential rate of synthesis, S. Thus, it was possible to distinguish between substrate interactions involving repression of enzyme synthesis and inhibition of enzyme action.

The COD due to excreted metabolic intermediates was calculated by subtracting the sum of the lysine and carbohydrate COD values from the total COD.

2. Continuous Flow Experiments

In order to show the response of the reactors to the shock load, time plots of the various parameters were used. The actual lysine removal rates in the reactors (specific substrate removal rate) were calculated and plotted for comparison with the specific enzymatic capability of the cells. The balance equation for substrate in a completely mixed, continuous flow reactor is:

$$\text{Change} = \text{Input} - \text{Output} - \text{Consumption}$$

or:

$$dL/dt = (f/V)L_1 - (f/V)L - \text{Consumption}$$

$$\text{Consumption} = (f/V)(L_1 - L) - dL/dt$$

(69). Since consumption is the total substrate used per unit time, the specific substrate removal rate with respect to cell protein is the consumption divided by the protein concentration, P. Thus, the specific substrate removal rate is $(RR/P) = \frac{(f/V)(L_1 - L) - dL/dt}{P}$, where f is the flow rate, V is the volume, L_1 is the influent lysine

concentration, L is the lysine concentration in the reactor (and consequently in the effluent), and dL/dt is the rate of change of lysine concentration in the reactor, measured by graphically differentiating the plot of lysine versus time. During steady state operation, dL/dt is zero.

E. Chemicals

L-Lysine was obtained as L-lysine-HCl from Nutritional Biochemicals Corp., Cleveland, Ohio. Chloramphenicol and actidione were obtained from Calbiochem, Los Angeles, California. Glucostat is produced by Worthington Biochemical Corp., Freehold, N. J. Fructose and ribose were from Eastman Organic Chemicals, Rochester, N. Y. All other chemicals were reagent grade.

CHAPTER V

RESULTS

A. Preliminary Experiments

Twenty-six preliminary experiments were run to screen for an effect of glucose upon the rates of removal of amino acids. Fourteen amino acids and one amide were studied. The experimental methods used in those experiments differed somewhat from the methods outlined in Chapter IV and have been presented elsewhere (18). The seed for each experiment was started from sewage and grown in shaker flasks through three transfers on the particular amino acid under study. The experimental protocol itself was essentially the same as that in Chapter IV, except that growth was followed by optical density only. In the first sixteen experiments, the amino acid concentration was obtained by subtracting the glucose COD from the total COD, and was verified roughly by paper chromatography. In the last ten experiments, the amino acid concentrations were determined quantitatively using a ninhydrin test on samples pretreated to remove ammonia nitrogen.

1. Experiments in Which the Amino Acid Concentrations Were Determined by Calculation

The amino acids screened in this preliminary series were glycine, alanine, valine, serine, leucine, isoleucine, aspartic acid, glutamic acid, threonine, lysine, histidine, arginine, cysteine, and phenylalanine. The amide asparagine was also studied. Glucose had no effect upon the removal of valine, serine, isoleucine, arginine, or histidine so no additional experiments were performed with these compounds. Some degree of interaction was found for all of the others, and the experiments were repeated using the ninhydrin test so that more definitive results could be obtained.

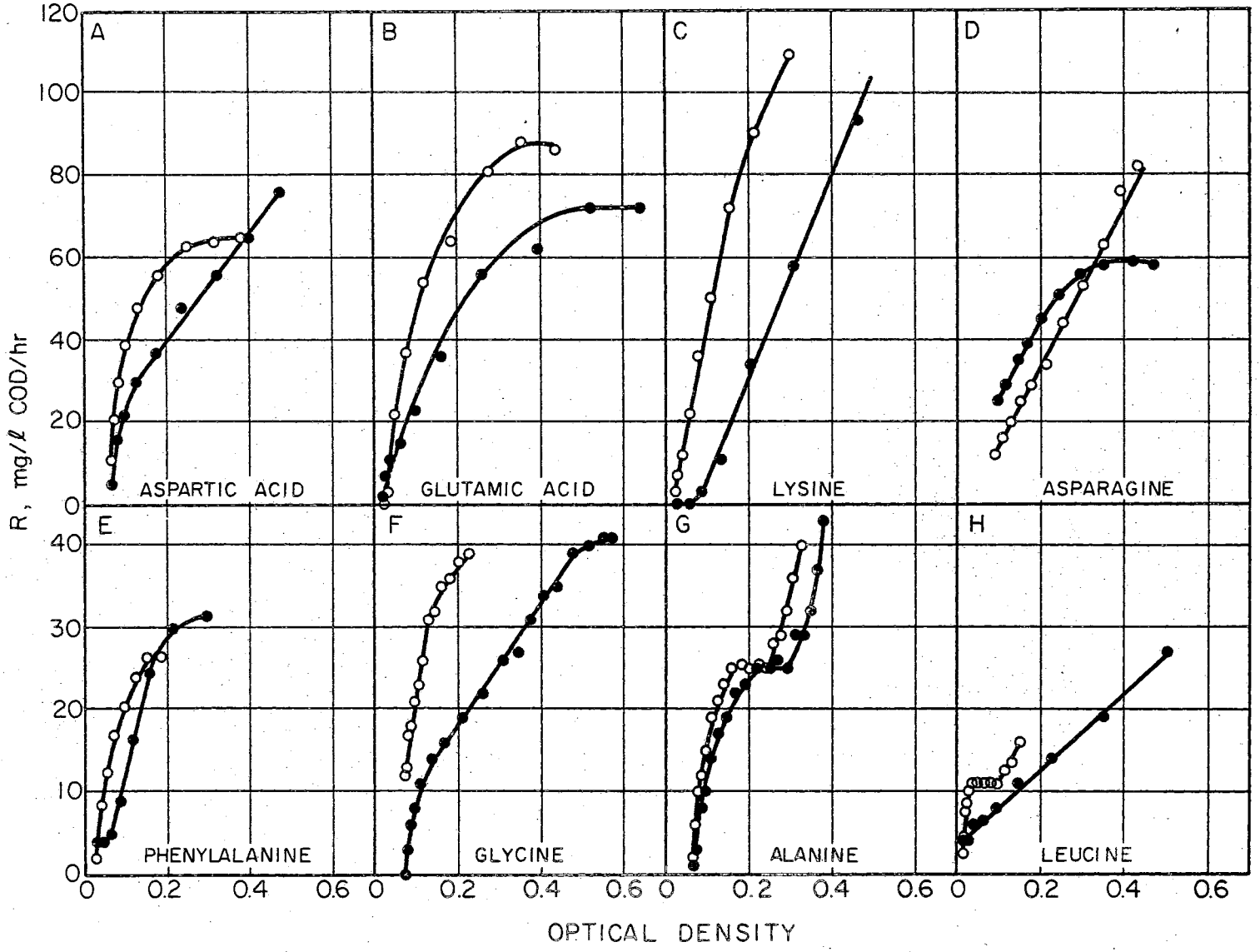
2. Experiments in Which the Amino Acid Concentrations Were Determined by Ninhydrin

Figure 3, plots of amino acid removal rate versus growth (i.e., biological solids as optical density) for eight amino acids, shows that glucose exerted the strongest effect upon the differential rates of activity (A) for lysine, glycine, and leucine. Curves are not shown for two substrates, cysteine, and threonine. Glucose and cysteine had a mutually antagonistic effect upon each other, but the ninhydrin results were not satisfactory and, thus, no curve is presented. Growth on threonine was extremely slow with only 200 mg/l being removed in 48 hours. Lysine was chosen for the more detailed

Figure 3. Effects of Glucose on the Rates of Removal (R) of Amino Acids by Cells Growing on Both the Amino Acid and Glucose
(The seed was started from sewage and grown through three transfers on the amino acid. A) Aspartic acid, B) Glutamic acid, C) Lysine, D) Asparagine, E) Phenylalanine, F) Glycine, G) Alanine, H) Leucine.

o = Removal rate of amino acid, R, in the control reactor.

● = Removal rate of amino acid, R, in the mixed reactor.)


A
B
C
D
E
F
G
H
ASPARTIC ACID
GLUTAMIC ACID
LYSINE
ASPARAGINE
PHENYLALANINE
GLYCINE
ALANINE
LEUCINE
R, mg/ℓ COD/hr
OPTICAL DENSITY
0
10
20
30
40
60
80
100
120
0.2
0.4
0.6

investigation because it showed a significant effect with glucose (Figure 3c) and grew at a rate fast enough to allow experiments of reasonable duration. Growth on glycine was very slow and growth on leucine could have been complicated by the relationships between isoleucine, valine, and leucine synthesis.

B. Batch Experiments

1. Inducibility of Lysine Degrading Enzyme System

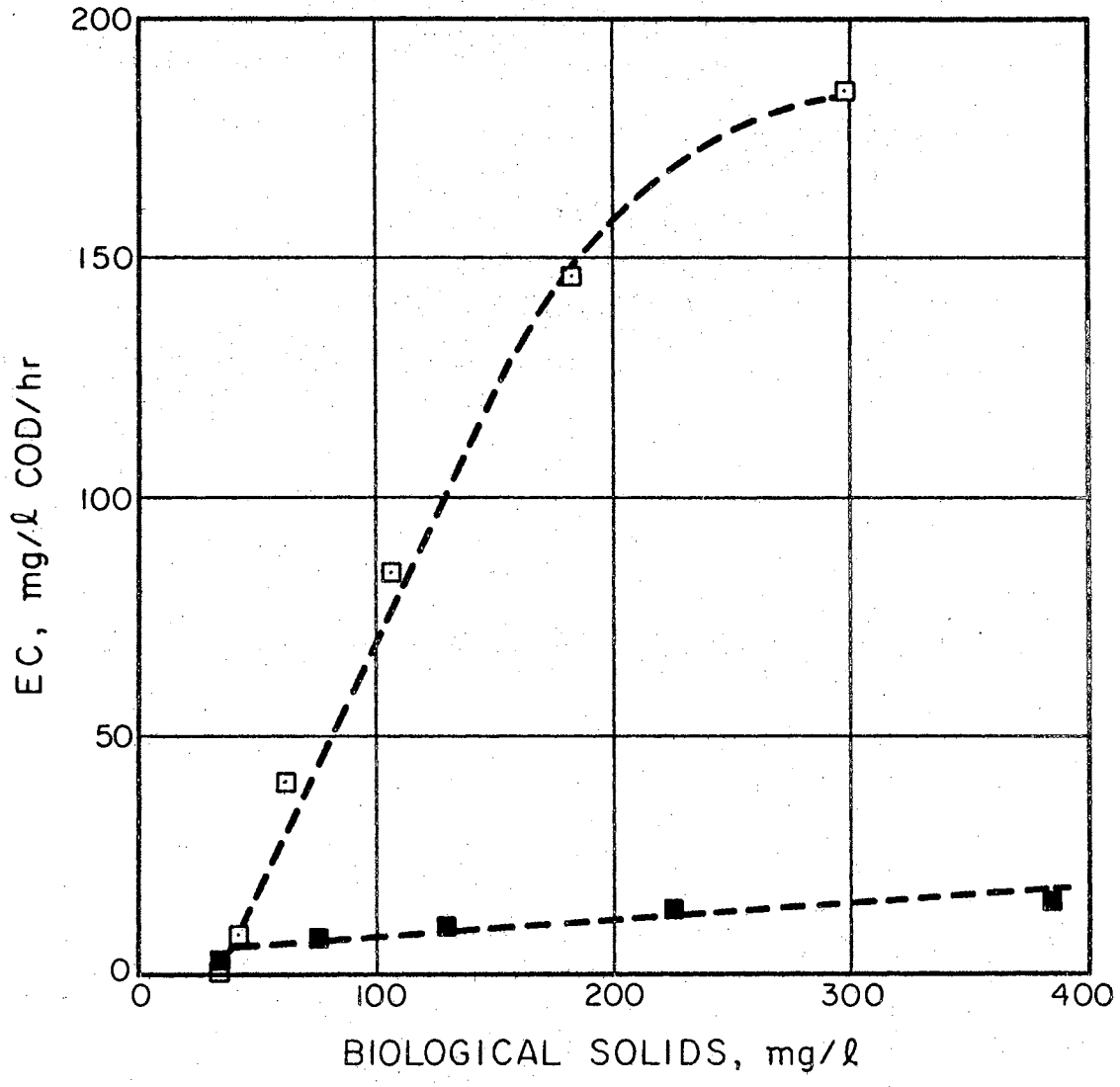
Because metabolite repression is involved in the control of synthesis of inducible catabolic enzymes, it was necessary to show that the lysine degrading enzymes are inducible (37). So that the lysine degrading enzymes already present could be "diluted out", cells were removed from the stock reactor and transferred to a reactor containing only glucose as a carbon source. After twelve hours, these cells were used to seed two experimental reactors, one containing lysine and the other glucose. Samples were removed at various times as described in Materials and Methods and the cells were analyzed for their enzymatic capability to remove lysine. The results, shown in Figure 4, demonstrate that the cells placed in the presence of lysine formed the enzymes for lysine degradation much more rapidly than those not in the presence of lysine. Thus, the lysine degrading enzyme system fits the definition of an "adaptive" or "inducible" enzyme system (34).

Figure 4. Demonstration of the Inducibility of the Enzymes Degrading Lysine
(The seed was grown through one transfer on glucose.

μ_m on lysine = 0.38.

□ = Enzymatic capability of the culture growing on lysine, S = 1.05.

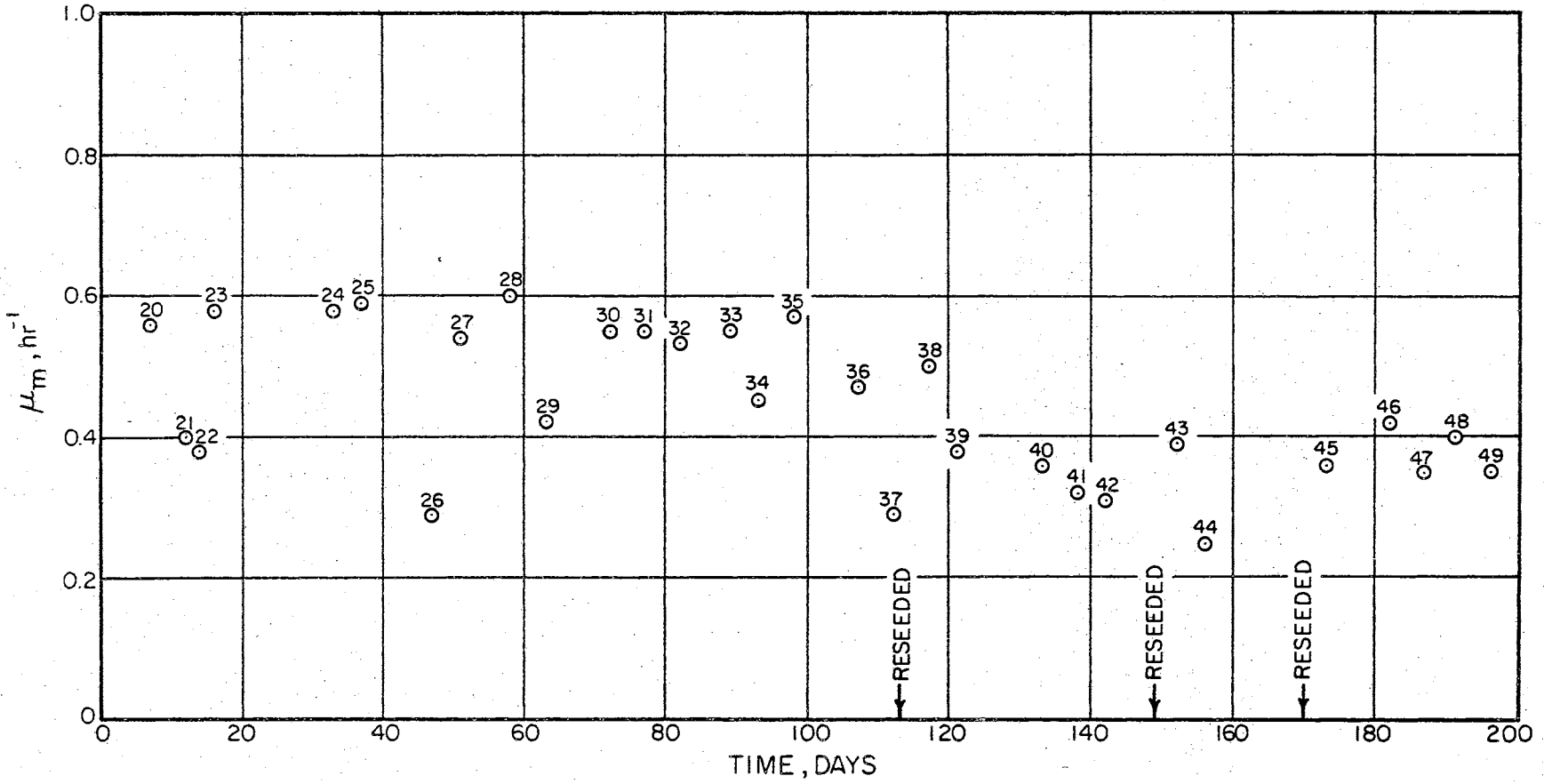
■ = Enzymatic capability of the culture growing on glucose, S = 0.036.)


200
150
100
50
0
EC, mg/ℓ COD/hr
0
100
200
300
400
BIOLOGICAL SOLIDS, mg/ℓ

2. Changes in the Kinetic Characteristics of the Stock Reactor

Figure 5 is a plot of the maximum specific growth rate, μ_m, on lysine of the experimental cultures, as determined in shaker flasks, during the course of this study. The numbers above the points refer to experiment numbers and are provided as an aid in the interpretation of the results. The values for μ_m began to decrease on the 100th day and after the 109th day the culture no longer turned green, a color that had been characteristic of the mixed liquor since initiation of the culture. Plates made on the 112th day indicated a predominance of fungal colonies, so the culture was discarded and the reactor reseeded with slants made on the 93rd day. The growth rate was higher after the reseeding, but started decreasing again, and from the 140th until the 149th day only light green pigment was produced. There was no evidence that fungi were present in the culture at this time, because on the 138th day there were several types of bacterial colonies present on the plates. Growth curves measured on the 148th day had a very short exponential growth phase followed by a very long declining phase so the reactor was reseeded again the next day using a "day-93" slant. The culture was discarded on the 158th day, but when more experiments were necessary it was restarted from a "day-93" slant and used for three more experiments. The last two experiments were performed on

Figure 5. Variation in the Maximum Specific Growth Rate, μ_m, on Lysine in Shaker Flasks During the Course of the Experiments


1.0
0.8
0.6
0.4
0.2
0
μ_m, hr^{-1}
0
20
40
60
80
100
120
140
160
180
200
TIME, DAYS
RESEEDED
RESEEDED
RESEEDED
20
23
24
25
28
27
30
31
32
33
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29
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22
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36
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45
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47
48
49

cells taken from slants and grown in batch through one transfer on lysine and three on the carbohydrates.

It appears, then, that the culture was subjected to a "fungal take-over" on day 112, and that after reseeding a predominance shift or mutant take-over occurred. As will be pointed out later, the substrate removal patterns were altered after μ_m decreased in the culture. However, since experiment 48 was done with glucose and gave the same metabolic response as the previous glucose experiments, the control mechanisms operative appear to be the same.

3. Experiments With Glucose

a. Effect of Glucose Upon the Lysine Removal Rate.

The object of this set of experiments was twofold: to ascertain whether glucose had an effect upon the differential rate of activity, A, of the experimental culture and to determine whether that effect was dependent upon the concentrations of glucose and lysine in the reactors. Four experiments were run in this set, each with a different concentration of lysine, and within each experiment, four glucose concentrations were employed. The nominal concentrations of glucose and lysine were 400, 800, 1200, and 1600 mg/l, as COD. Plots of lysine removal rates, R, versus solids concentration, X, are presented in Figure 6 and demonstrate clearly that the presence of glucose in the medium decreased the differential rate of activity, A, of the cultures. Arrows are used to denote the points at

Figure 6. Effect of glucose on the Lysine Removal Rate (R) for Cells Growing in the Presence of Both Lysine and Glucose
(The seed was grown on lysine. The slopes of these curves are the differential rates of activity, A.

○ = lysine only; △= lysine + 400 mg/l glucose COD;

□ = lysine + 800 mg/l glucose COD;

▽ = lysine + 1200 mg/l glucose COD;

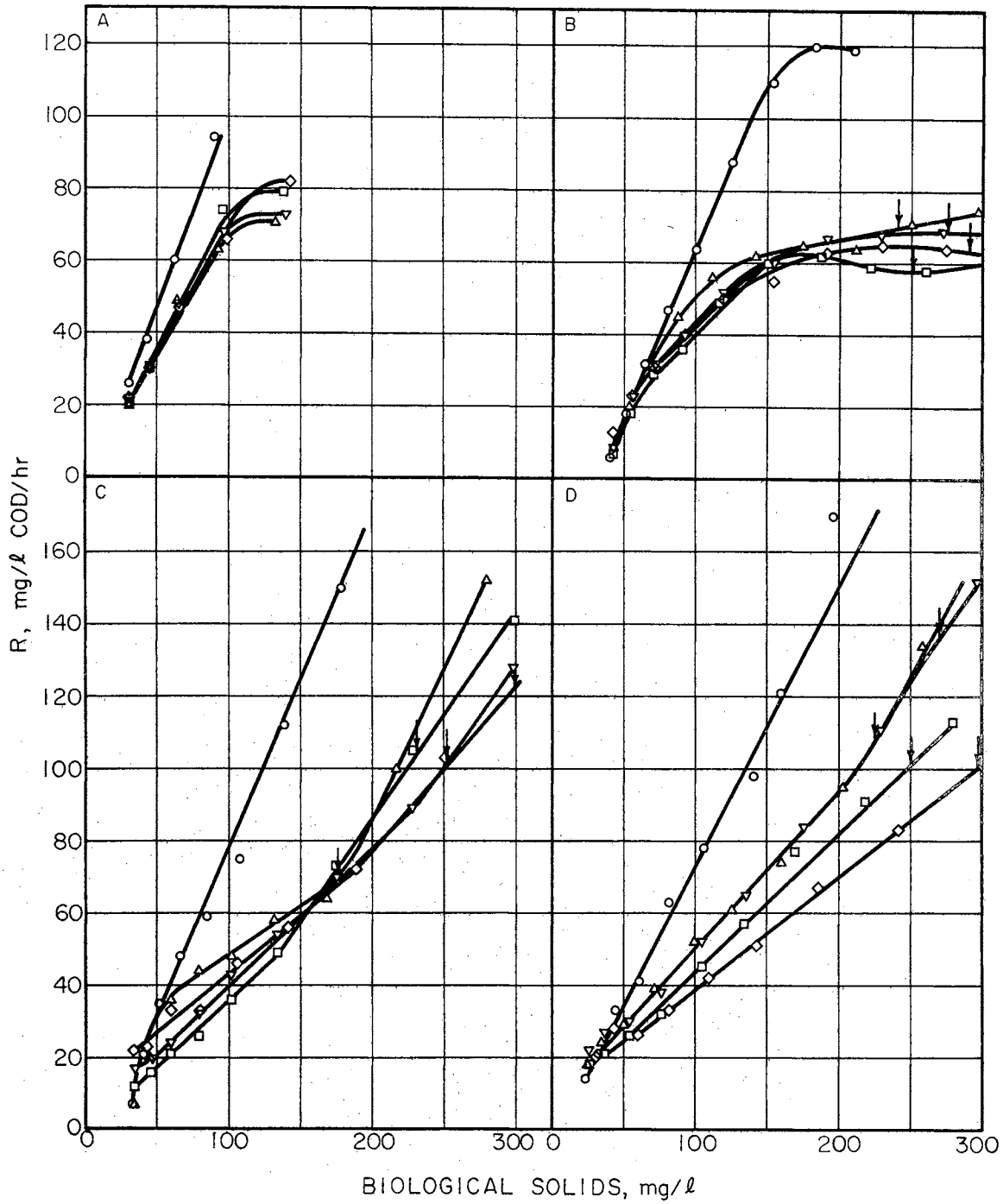
◇ = lysine + 1600 mg/l glucose COD.

a. Expt. No. 20
Lysine concentration = 400 mg/l COD.
μ_m on lysine = 0.56.

b. Expt. No. 21
Lysine concentration = 800 mg/l COD.
μ_m on lysine = 0.40.

c. Expt. No. 23
Lysine conc ntration = 1200 mg/l COD.
μ_m on lysine = 0.58.

d. Expt. No. 22
Lysine concentration = 1600 mg/l COD.
μ_m on lysine = 0.38.)


A
B
C
D
R, mg/ℓ COD/hr
BIOLOGICAL SOLIDS, mg/ℓ
0
20
40
60
80
100
120
140
160
0
100
200
300

which all glucose had been removed from the medium.

In order to evaluate the effect of substrate concentration on the differential rate of activity of lysine degrading enzymes, the slopes (A) of the straight line portions prior to the removal of glucose were calculated. These values are presented in Table II, as are the substrate concentrations in the reactors. There was no straight line portion for the reactor containing 800 mg/l lysine COD plus 400 mg/l glucose; therefore, the value of A could not be calculated for the system. An examination of Table II and of Figure 6 shows that there was variation in the A values for the four control reactors. This variation was probably caused by population changes in the stock reactor between experiments; it is noted that the μ_m values varied also. In order to compare experiments, the A value for each mixed reactor was divided by the corresponding value for its control. The ratios are presented in the last column of Table II. A linear correlation coefficient was calculated for $A_{mixture}/A_{control}$ versus lysine COD/glucose COD, and as expected it was quite low, 0.255. Examination of the data for each experiment and for the entire set shows that glucose did have an effect upon the differential rate of activity, A, in the mixtures, but that there is little or no correlation between the ratio of lysine COD/glucose COD and the extent of the effect.

Replica plating was performed in this set of

TABLE II

EFFECT OF THE CONCENTRATIONS OF LYSINE AND GLUCOSE ON THE DIFFERENTIAL RATE OF ACTIVITY, A, OF LYSINE DEGRADING ENZYMES IN CULTURES GROWING ON BOTH COMPOUNDS

Figure No	Lysine mg/l COD	Glucose mg/l COD	$\frac{\text{Lysine COD}}{\text{Glucose COD}}$	A	$\frac{A_{mixture}}{A_{control}}$
6a	333	0	Control	1.10	----
6a	336	390	0.86	0.69	0.63
6a	342	758	0.45	0.77	0.70
6a	342	1182	0.29	0.70	0.64
6a	350	1542	0.23	0.69	0.63
6b	715	0	Control	0.95	----
6b	727	416	1.75	n.d.	n.d.
6b	720	784	0.92	0.40	0.42
6b	737	1213	0.61	0.42	0.44
6b	734	1572	0.47	0.40	0.42
6c	1120	0	Control	0.92	----
6c	1147	407	2.80	0.27	0.29
6c	1120	784	1.43	0.37	0.40
6c	1158	1165	0.99	0.38	0.41
6c	1183	1562	0.76	0.32	0.35
6d	1428	0	Control	0.77	----
6d	1450	384	3.78	0.43	0.56
6d	1459	788	1.85	0.39	0.51
6d	1459	1172	1.24	0.44	0.57
6d	1470	1483	0.98	0.32	0.42

experiments, and the results showed that there were no drastic shifts in predominance in the reactors. In general, all colonies that could grow on lysine could grow on glucose, and vice-versa; therefore, the response of the population was an "en masse" one.

b. Inhibition Effects of Glucose Upon Lysine Removal. After it had been established that glucose had an effect upon the ability of the cells to remove lysine, experiments were designed to gain an insight into the nature of that effect. Six reactors were prepared, three without glucose and three with 1600 mg/l glucose COD; one reactor of each type contained 400 mg/l lysine COD, one 800, and one 1200. The normal medium was used with the addition of 100 mg/l of chloramphenicol and 100 mg/l of actidione to prevent further protein synthesis (75). Each reactor was seeded with a microbial population grown on lysine; the initial biological solids concentration in each reactor was 180 mg/l. Since no new enzymes could be formed, all substrate removal would be due to enzymes already present in the cultures and any retardation of lysine removal would be due solely to inhibtion of activity of those enzymes. Samples were removed and analyzed in the standard manner and it was found that glucose had no inhibitory effect upon the activity of pre-formed enzymes.

In two common organisms, Aerobacter aerogenes and Pseudomonas aeruginosa, it has been shown that at least one pathway of glucose catabolism is inducible (35) (80).

If this were the case in the culture under study (which had predominantly gram-negative organisms), the effect of the inducible glucose pathway could not have been measured because chloramphenicol would have prevented the synthesis of the pathway. Thus, a second experiment was performed in which the lysine-acclimated cells were allowed to grow through one transfer in a mixture of glucose and lysine prior to use. The results are shown in Figure 7, and demonstrate that glucose had a very minor inhibitory effect upon the activity of pre-formed lysine degradative enzymes.

c. Effect of Glucose Upon the Differential Rate of Syntheis of Lysine Degrading Enzymes. Since only a minor portion of the glucose effect was due to an inhibition of the activity of pre-formed enzymes, the effect observed was in all probability due to metabolite repression which reduced the rate of synthesis of the lysine degrading enzymes. It is very difficult to prepare a representative enzyme extract from a non-pure culture, due to differences in efficiency of rupturing different species, so a whole cell assay, the enzymatic capability test, was used. In this test no inhibitory effects should be measured since during the test procedure the cells are no longer in contact with glucose or its extracellular metabolic products. Therefore, a change in the enzymatic capability should be proportional to a change in the level of lysine degrading enzymes within the cells. There are limitations involved in the use of whole cell assays, but as long as these are

Figure 7. Effect of Glucose on Lysine Removal Under Non-Proliferating Conditions (Expt. No. 26) (The seed was grown through one transfer on lysine plus glucose. Initial biological solids in all reactors was 325 mg/l.

○ = 400 mg/l lysine COD;

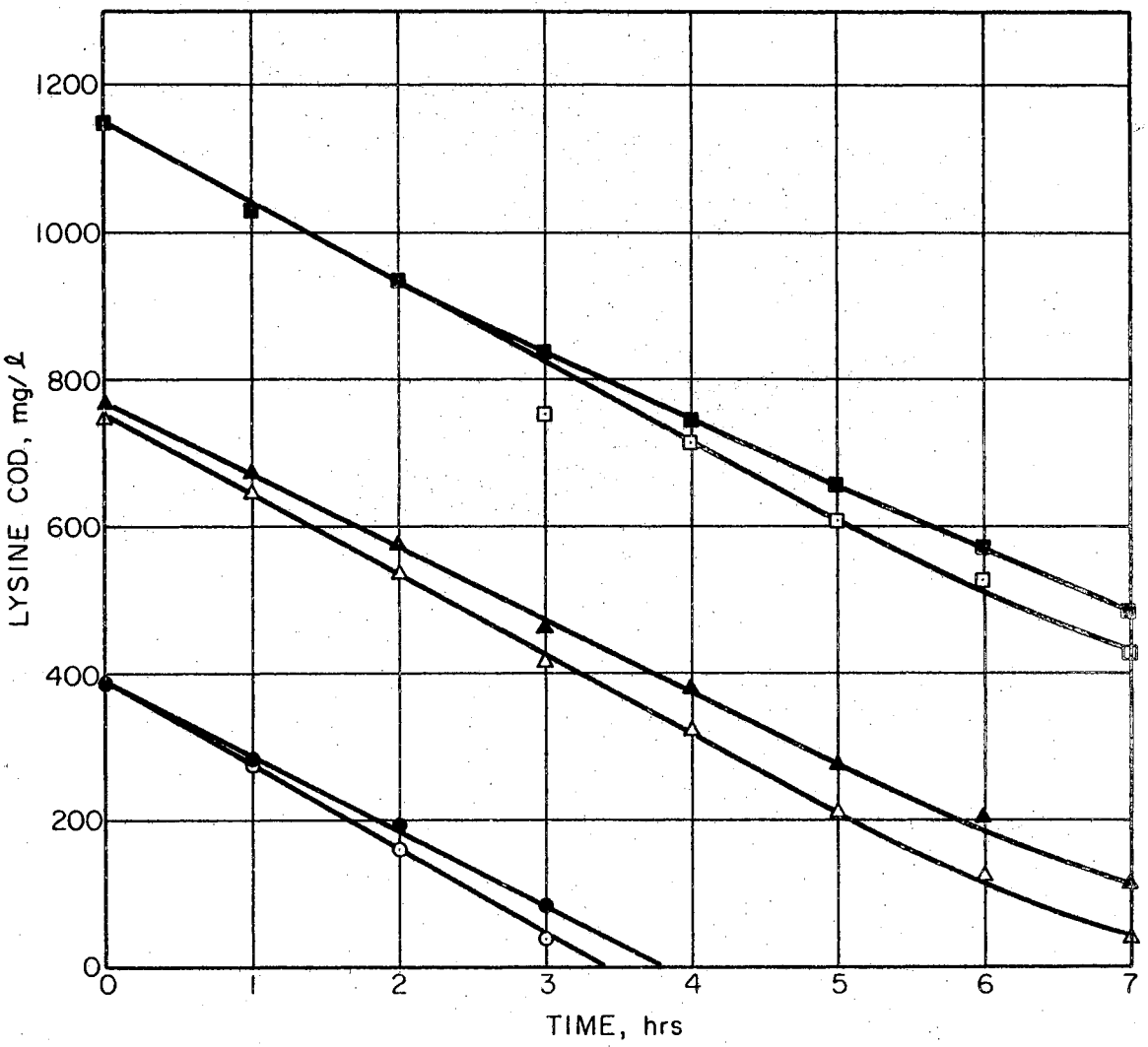
△ = 800 mg/l lysine COD;

□ = 1200 mg/l lysine COD;

● = 400 mg/l lysine COD + 1600 mg/l glucose COD;

▲ = 800 mg/l lysine COD + 1600 mg/l glucose COD;

■ = 1200 mg/l lysine COD + 1600 mg/l glucose COD.)


LYSINE COD, mg/ℓ
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
TIME, hrs

recognized the test should allow a comparison of the enzymatic capability at one time to that at another in the same reactor (79).

Three reactors were used in the first experiment: one with 1200 mg/l lysine COD, one with 400 mg/l glucose COD, and one with 1200 mg/l lysine COD plus 400 mg/l glucose COD. The lysine removal rates, R, were calculated from the lysine removal curves and plotted versus the biological solids in the reactors. The enzymatic capabilities of the cells, EC, were also determined in accordance with procedures previously outlined and plotted as dashed lines in Figure 8. Although the enzymatic capability was also determined in the glucose control, the values are not shown on the graphs for the sake of clarity. These curves show clearly that the effect of glucose is due to a decrease in the differential rate of synthesis, S, of lysine degrading enzymatic capability. The arrow shows the point at which all of the glucose had been removed from the mixed reactor, and after that point the differential rate of synthesis, S, (i.e., slope of E curve) became almost equal to that in the control reactor. This occurred even though the actual removal rate, R, or removal ability of the culture decreased (the decrease was due to the fact that the lysine concentration in the reactor was becoming small). The decrease in enzymatic capabilities in the two cultures occurred after the lysine was eliminated and indicates that a degradation of lysine catabolic enzymes

Figure 8. Effect of Glucose on the Removal Rate (R) and Enzymatic Capability (EC) for Lysine in a Culture Grown on Lysine (Expt. No. 28) (Lysine COD = 1200 mg/l; Glucose COD = 400 mg/l. A is the slope of the R versus solids plot; S is the slope of the EC versus solids plot.

○ = Lysine removal rate, R, in the control reactor.

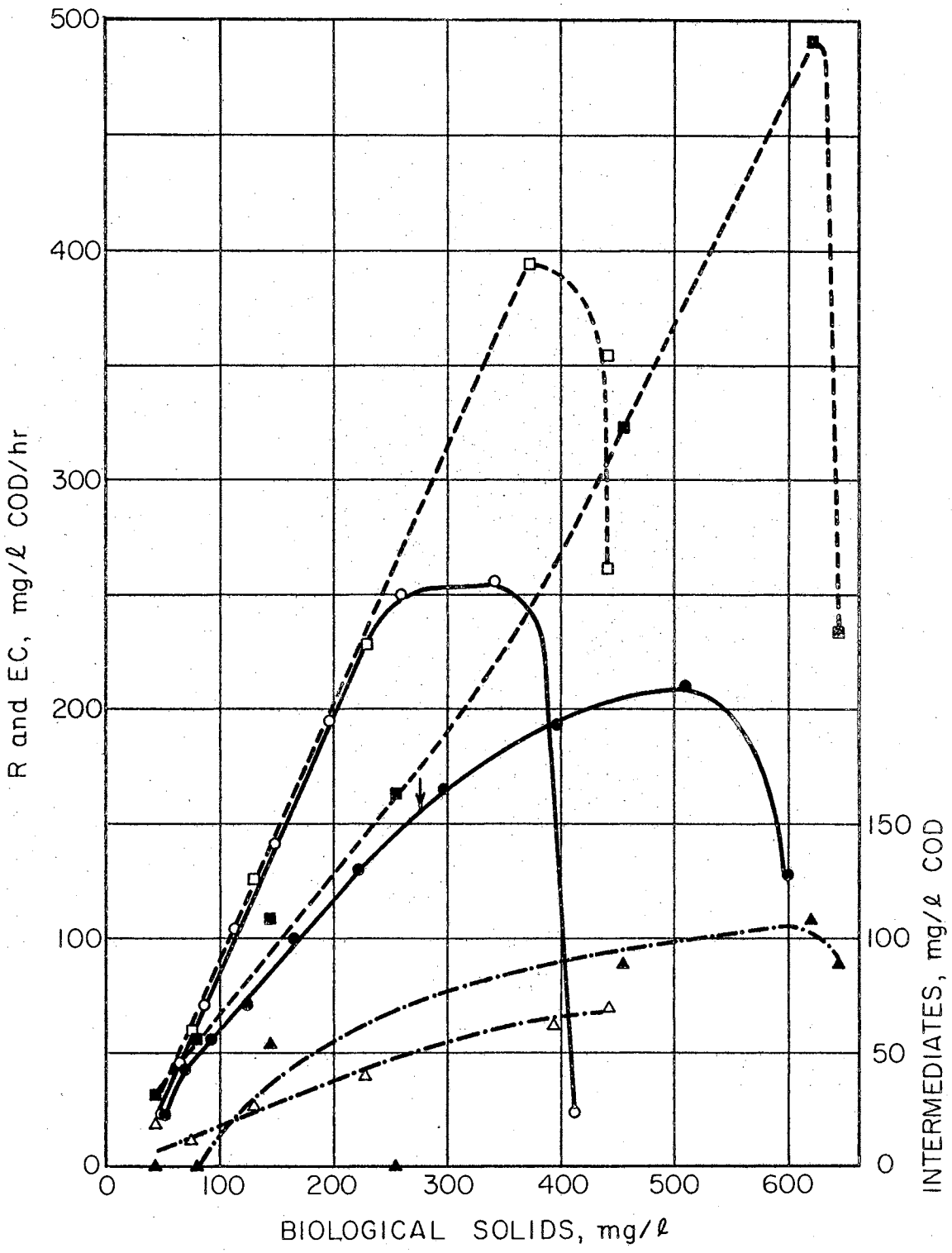
□ = Lysine enzymatic capability, EC, in the control reactor.

△ = Exogenous intermediates in the control reactor.

● = Lysine removal rate, R, in the mixed reactor.

■ = Lysine enzymatic capability, EC, in the mixed reactor.

▲ = Exogenous intermediates in the mixed reactor.)


500
400
300
200
100
0
R and EC, mg/ℓ COD/hr
0
100
200
300
400
500
600
BIOLOGICAL SOLIDS, mg/ℓ
150
100
50
0
INTERMEDIATES, mg/ℓ COD

occurred when there was no longer exogenous substrate in the medium. A comparison of the total COD and lysine-plus-glucose COD values revealed the accumulation of only minor amounts of exogenous metabolic intermediates.

In the previous experiment, there was an eight hour lag in the glucose control reactor before growth occurred, even though glucose utilization started immediately in the mixed reactor. To avoid this lag and to determine whether prior acclimation to glucose would influence the results, the seed for the next experiment was grown through 12 hours on a mixture of glucose and lysine. The experiment was then performed in the same way as the previous one except that the glucose concentration was increased to 800 mg/l. The results are shown in Figure 9. Again, glucose caused a decrease in the differential rate of synthesis, S, and in the differential rate of activity, A, of the lysine degrading enzymes. In this case, almost 500 mg/l COD due to intermediates of glucose metabolism, had been excreted into the medium of the mixed reactor by the time of glucose removal and these products continued to exert the same effect upon the rate of formation of enzymatic capability as had the glucose. After the majority of the intermediates had been removed, the differential rates of activity, A, and synthesis, S, increased. There was no lag in the glucose control, indicating that the cells contained glucose degrading enzymes prior to the experiment.

Figure 9. Effect of Glucose on the Removal Rate (R) and Enzymatic Capability (EC) for Lysine in a Culture Grown Through One Transfer on Lysine Plus Glucose (Expt. No. 29)
(Lysine COD = 1200 mg/l, Glucose COD = 800 mg/l.

○ = Lysine removal rate, R, in the control reactor.

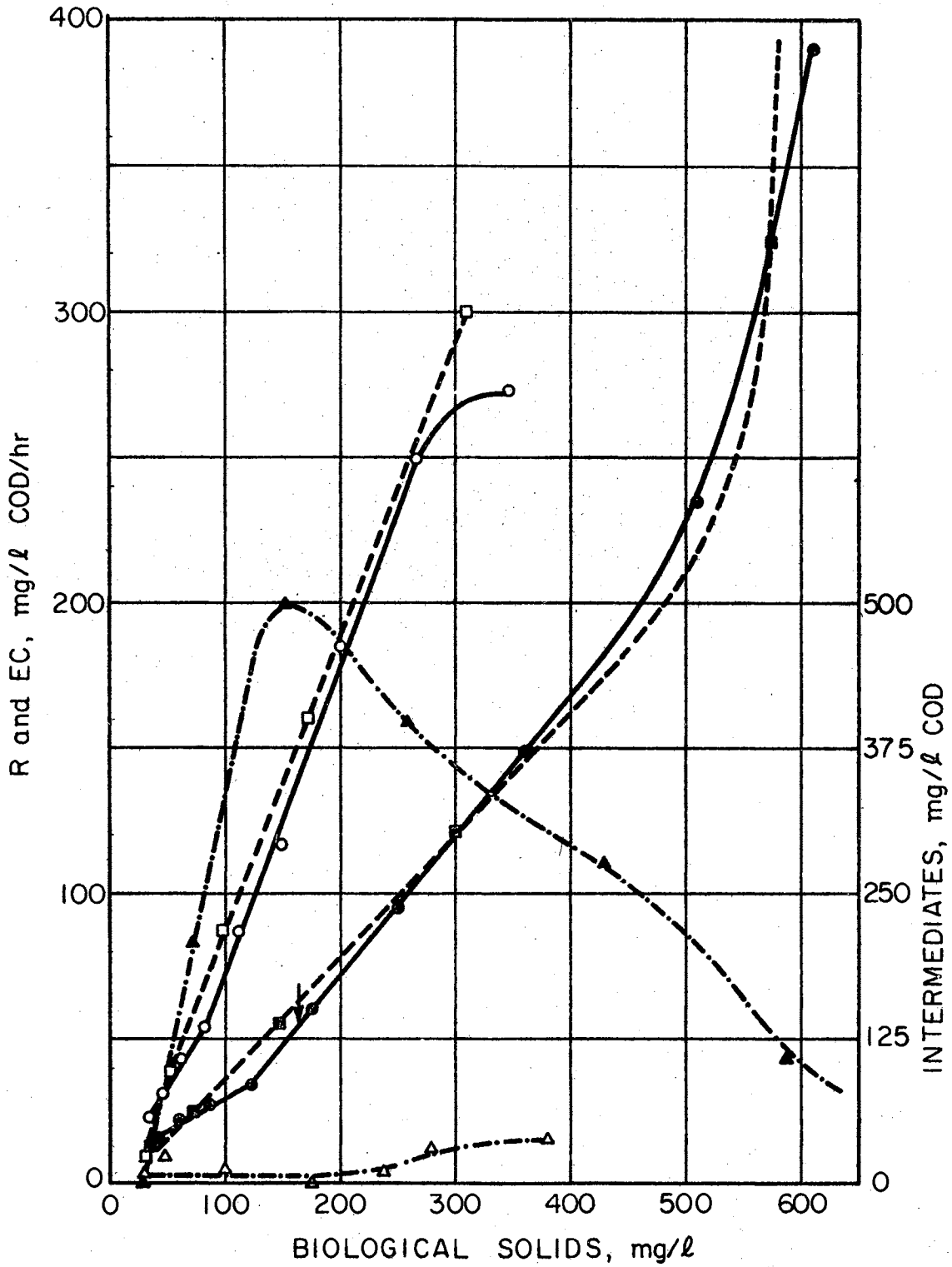
□ = Lysine enzymatic capability, EC, in the control reactor.

△ = Exogenous intermediates in the control reactor.

● = Lysine removal rate, R, in the mixed reactor.

■ = Lysine enzymatic capability, EC, in the mixed reactor.

▲ = Exogenous intermediates in the mixed reactor.)


400
300
200
100
0
R and EC, mg/ℓ COD/hr
500
375
250
125
0
INTERMEDIATES, mg/ℓ COD
0
100
200
300
400
500
600
BIOLOGICAL SOLIDS, mg/ℓ

It has been shown that pre-induction offers a degree of protection against metabolite repression in the lac operon (44). In order to investigate the effect of pre-induction for the lysine system, cells were removed from the stock reactor and grown on glucose before use so that they would not be pre-induced for lysine. The results shown in Figure 10 were obtained using cells that had been grown through one transfer on glucose. In the experimental reactors, the lysine concentration was 1000 mg/l COD and the glucose was 1500 mg/l. Again, a high concentration of metabolic intermediates was excreted into the medium from glucose utilization, and the rate of formation of enzymatic capability (S), did not increase appreciably until the concentration decreased. For the experimental results shown in Figure 11, the cells were grown through three transfers on glucose before use. The lysine concentration in the experiment was 1000 mg/l COD, as was the glucose. Again, it was necessary that a decrease in the concentration of intermediates occur before the differential rate of synthesis, S, of enzymatic capability for lysine started to increase.

The results obtained from the last three experiments are summarized in Table III. In order to compare results, it was necessary to calculate the ratio $S_{mixture}/S_{control}$ because the values for the three controls were different, as were the μ_m values. Comparing these ratios for the three experiments, it can be seen that pre-induction with

Figure 10. Effect of Glucose on the Removal Rate (R) and Enzymatic Capability (EC) for Lysine in a Culture Grown Through One Transfer on Glucose (Expt. No. 39)
(Lysine COD = 1000 mg/l. Glucose COD = 1500 mg/l.

○ = Lysine removal rate, R, in the control reactor.

□ = Lysine enzymatic capability, EC, in the control reactor.

△ = Exogenous intermediates in the control reactor.

● = Lysine removal rate, R, in the mixed reactor.

■ = Lysine enzymatic capability, EC, in the mixed reactor.

▲ = Exogenous intermediates in the mixed reactor.)

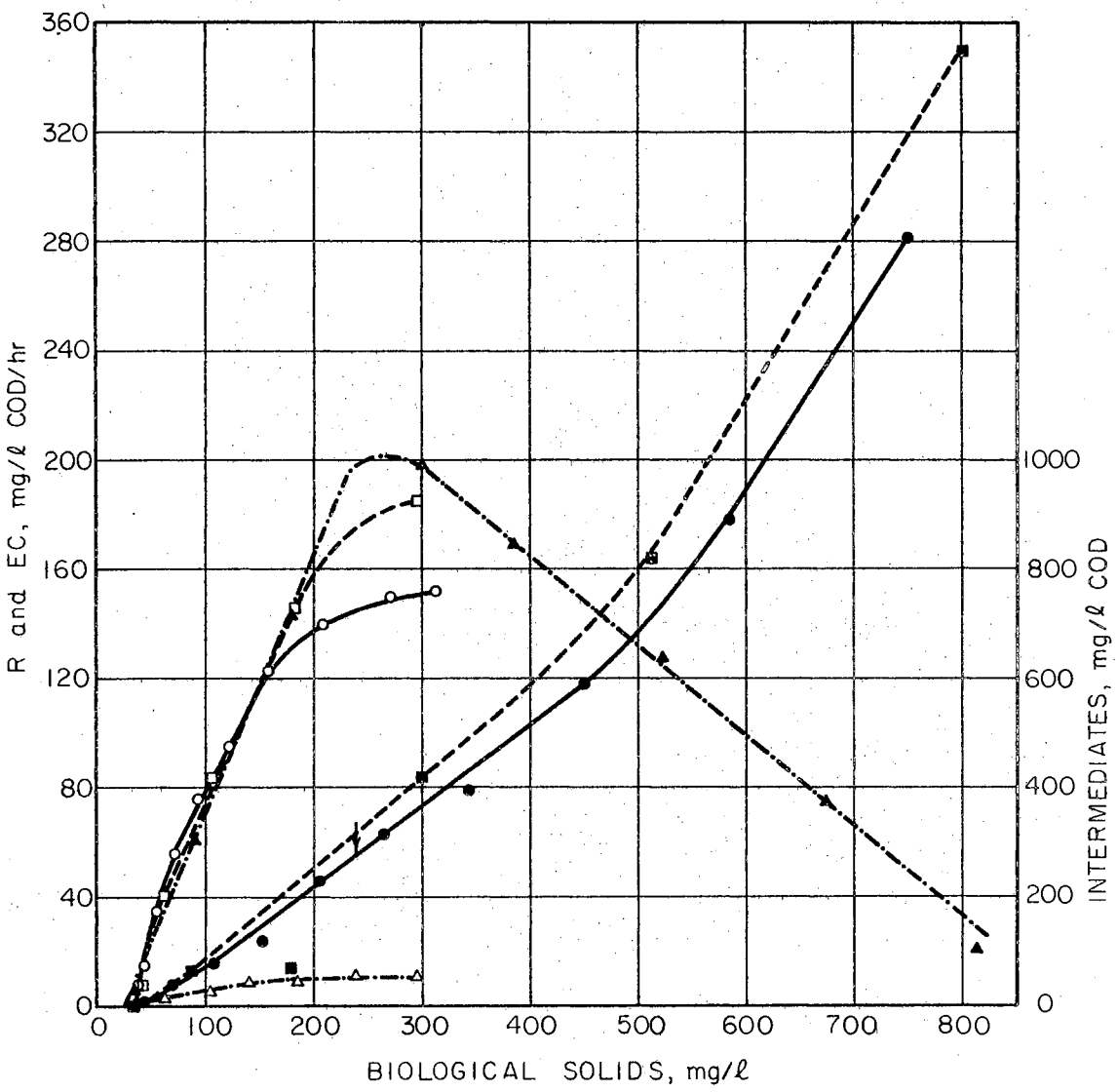

360
320
280
240
200
160
120
80
40
0
R and EC, mg/ℓ COD/hr
0
100
200
300
400
500
600
700
800
BIOLOGICAL SOLIDS, mg/ℓ
1000
800
600
400
200
0
INTERMEDIATES, mg/ℓ COD

Figure 11. Effect of Glucose on the Removal Rate (R) and Enzymatic Capability (EC) for Lysine in a Culture Grown Through Three Transfers on Glucose (Expt. No. 48)
(Lysine COD = 1000 mg/l. Glucose COD = 1000 mg/l.

○ = Lysine removal rate, R, in the control reactor.

□ = Lysine enzymatic capability, EC, in the control reactor.

△ = Exogenous intermediates in the control reactor.

● = Lysine removal rate, R, in the mixed reactor.

■ = Lysine enzymatic capability, EC, in the mixed reactor.

▲ = Exogenous intermediates in the mixed reactor.)

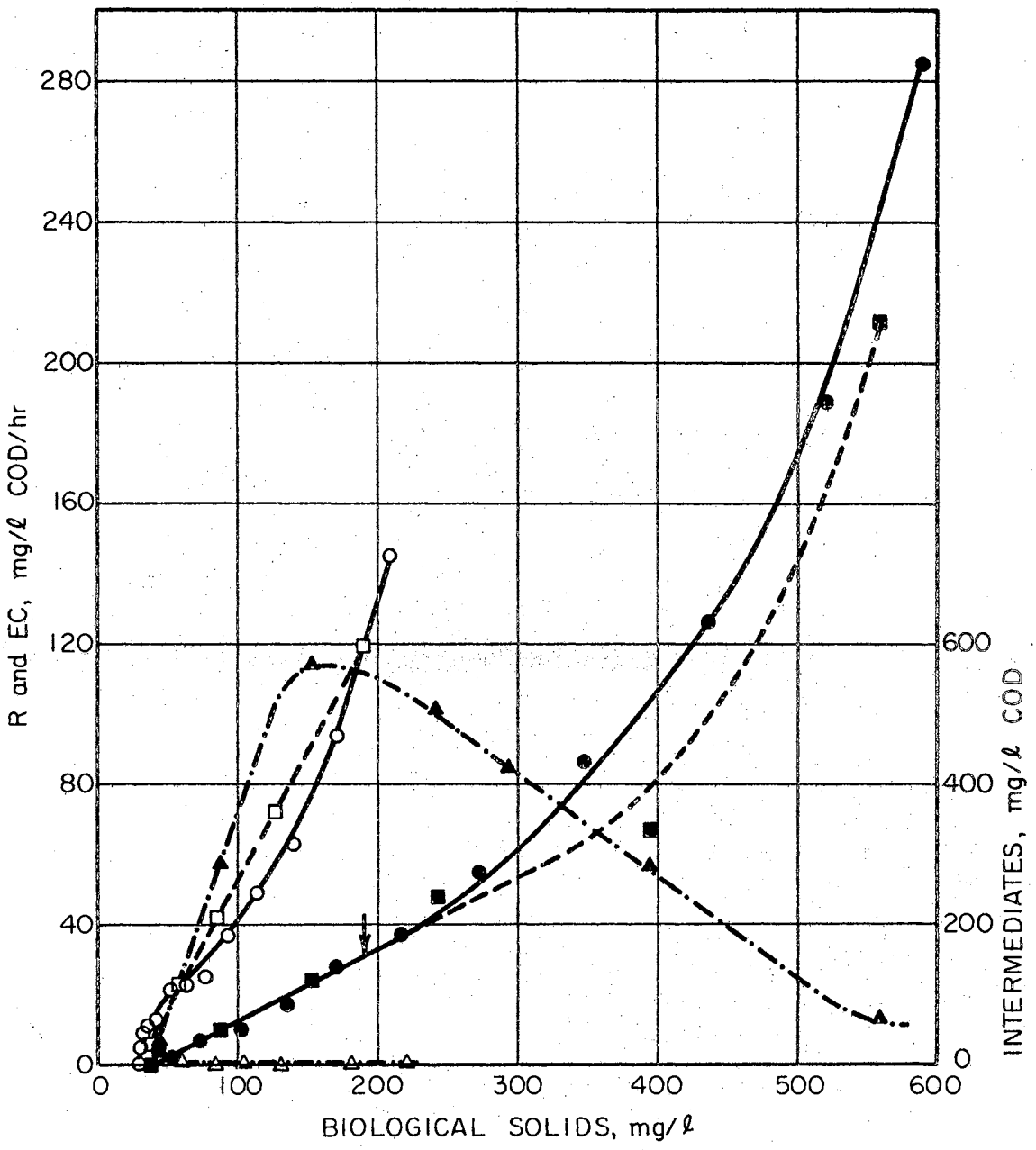

R and EC, mg/ℓ COD/hr
INTERMEDIATES, mg/ℓ COD
BIOLOGICAL SOLIDS, mg/ℓ
0
40
80
120
160
200
240
280
0
200
400
600
0
100
200
300
400
500
600

TABLE III

EFFECT OF PRE-INDUCTION ON
METABOLITE REPRESSION

Figure	Seed Growth Medium	μ_m (Lysine)	$S_{control}$	$S_{mixture}$	$\frac{S_{mixture}}{S_{control}}$
9	Lysine + Glucose (1)*	0.42	1.00	0.43	0.43
10	Glucose (1)	0.38	0.88	0.33	0.37
11	Glucose (3)	0.36	0.74	0.19	0.26

* = number of transfers

lysine did offer a small degree of protection against metabolite repression. Although there was a change in the maximum specific growth rate, μ_m (lysine) for the three experiments, the change in the ratio $S_{mixture}/S_{control}$ was greater. Replica plating was not performed on any of these systems, but there were no gross (morphological) indications of changes in predominance during the individual experiments.

In general, the four enzymatic capability experiments showed that the effect exerted by glucose was due to metabolite repression of synthesis of the lysine degrading enzyme system. The results also indicate that glucose itself was not causing the effect because the repression was maintained as long as a high level of intermediates (due to glucose metabolism) was present in the reactor.

d. Effect of the Removal of NH_4 Nitrogen Upon Metabolite Repression. Neidhardt and Magasanik (81) showed that if ammonia nitrogen were removed from a culture of Aerobacter aerogenes growing in a mixture of histidine and glucose, the cells regained a partial ability to make histidase because histidine was the only source of nitrogen. To determine if similar conditions would overcome the metabolite repression exerted by glucose on the synthesis of lysine degrading enzymes in mixed microbial populations, four reactors were set up, two with ammonia nitrogen and two without. All reactors contained lysine, and one of each type contained glucose.

As can be seen in Figure 12, the removal of ammonia from these systems did not aid in overcoming the glucose effect. Moreover, after the glucose had been removed from the system, there was no increase in the lysine removal rate of the culture without ammonia in spite of a high level of lysine still in the system. In the lysine reactors, the removal of ammonia nitrogen had little effect upon the lysine removal rate until late in the experiment when a decrease in the differential rate of activity, A, occurred even though there was still lysine in the medium.

A second experiment was performed in a similar manner, except that the cells were grown through three transfers on glucose prior to their use; in addition, the enzymatic capability was measured. Figure 13a is a plot of the removal rate, R, versus biological solids, and Figure 13b is a plot of the enzymatic capability, EC, in the reactors. The differential rate of activity, A, and the differential rate of synthesis, S, were initially greater in the mixed reactor without ammonia than in the one with ammonia, indicating that the cells were dependent upon lysine for nitrogen. However, once a minimal level of lysine degrading ability was present, the rates of formation of enzymatic capability in the reactors without ammonia were the same as those in the reactors with ammonia. It appears that although the cells without ammonia had to use lysine as a nitrogen source, the normal rate of formation of enzymatic capability in the glucose-lysine reactor was

Figure 12. Effect of the Removal of Ammonia Nitrogen on the Repression Exerted by Glucose Upon the Removal Rate (R) of Lysine in a Culture Grown on Lysine (Expt. No. 27)

○ = 1200 mg/l lysine COD + NH_4^+;

● = 1200 mg/l lysine COD + 400 mg/l glucose COD + NH_4^+;

□ = 1200 mg/l lysine COD;

■ = 1200 mg/l lysine COD + 400 mg/l glucose COD.

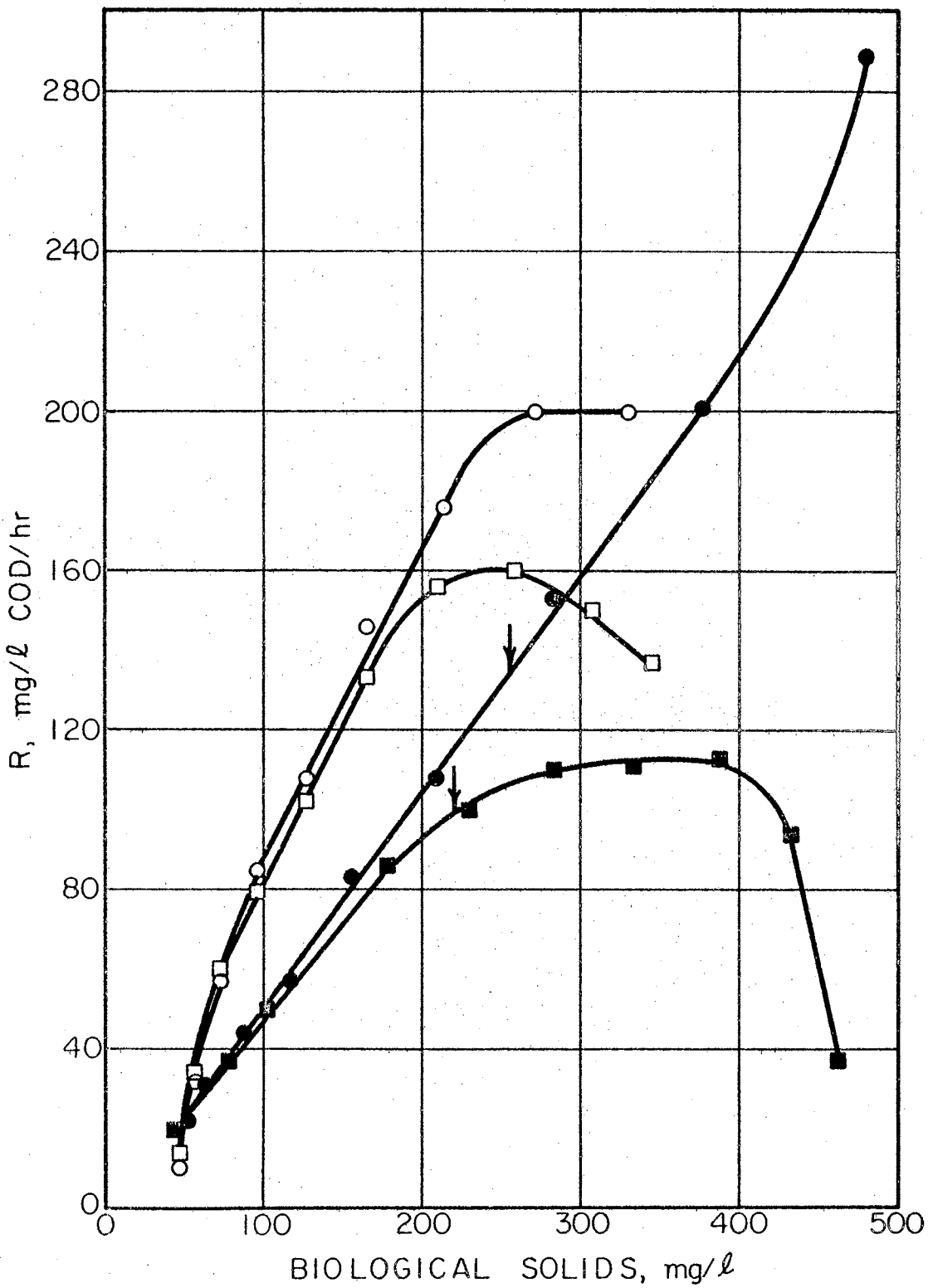

280
240
200
160
120
80
40
0
R, mg/ℓ COD/hr
0
100
200
300
400
500
BIOLOGICAL SOLIDS, mg/ℓ

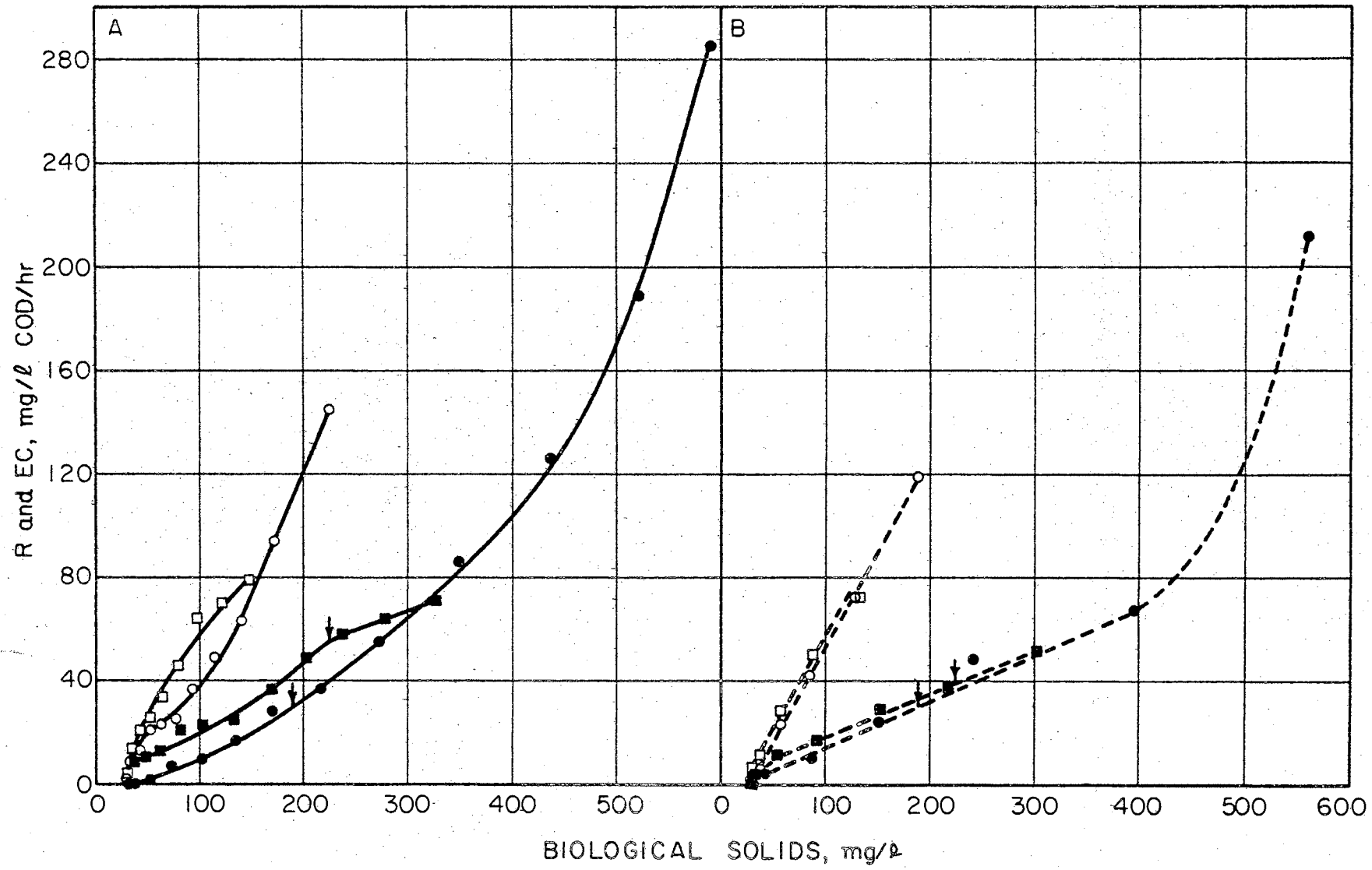
Figure 13. Effect of the Removal of Ammonia Nitrogen on the Repression Exerted by Glucose Upon (a) the Removal Rate (R) and (b) Enzymatic Capability (EC) for Lysine in a Culture Grown Through Three Transfers on Glucose (Expt. No. 48)

○ = 1000 mg/l lysine COD + NH_4^+;

● = 1000 mg/l lysine COD + 1000 mg/l glucose COD + NH_4^+;

□ = 1000 mg/l lysine COD;

■ = 1000 mg/l lysine COD + 1000 mg/l glucose COD.


A
B
280
240
200
160
120
80
40
0
R and EC, mg/ℓ COD/hr
0
100
200
300
400
500
0
100
200
300
400
500
600
BIOLOGICAL SOLIDS, mg/ℓ

sufficient to supply the culture with nitrogen, and, thus, there was no further relief from metabolite repression. In the histidine degrading system studied by Neidhardt and Magasanik, there was a complete repression of histidase in the presence of glucose and ammonia nitrogen (81). Since there was a 40% derepression in the absence of ammonia nitrogen but in the presence of glucose, they concluded that the repressor was a nitrogenous compound and that the partial derepression was necessary for the production of the repressor. In the lysine degrading system, it has been shown that complete repression did not occur. Since nitrogen could be provided to the cells from this "normal partial derepression", no more derepression was necessary when ammonia nitrogen was removed from the medium.

4. Experiments With Fructose

a. Effect of Fructose Upon the Lysine Removal Rate. As in the previous experiments on glucose, the first task was to ascertain whether fructose had an effect upon the differential rate of activity (A) of the experimental culture and to determine whether that effect was dependent upon the concentrations of fructose and lysine in the reactors. Plots of removal rate, R, versus biological solids gave straight lines, and the values for A are given in Table IV with the substrate concentrations. These values demonstrate that fructose had a minor effect upon the differential rate of activity and that the effect was

TABLE IV

EFFECT OF THE CONCENTRATIONS OF LYSINE AND FRUCTOSE ON THE DIFFERENTIAL RATE OF ACTIVITY, A, OF LYSINE DEGRADING ENZYMES IN CULTURES GROWING ON BOTH COMPOUNDS

(The seed was grown on lysine alone. Average $A_m/A_c = 0.87$)

Expt. No.	Lysine conc. mg/l COD	Fructose conc. mg/l COD	$\frac{\text{Lysine COD}}{\text{Fructose COD}}$	A	$\frac{A_{mixture}}{A_{control}}$
30	498	0	Control	1.04	----
30	490	482	1.01	0.90	0.86
30	498	939	0.53	0.96	0.92
30	502	1402	0.36	0.90	0.86
31	985	0	Control	0.84	----
31	1000	482	2.08	0.65	0.77
31	985	965	1.02	0.77	0.92
31	985	1375	0.72	0.77	0.92
32	1462	0	Control	0.85	----
32	1517	477	3.18	0.72	0.85
32	1540	1010	1.52	0.72	0.85
32	1462	1510	0.97	0.72	0.85

not dependent upon the ratio of lysine COD/fructose COD. Replica plating showed no shift in predominance during the experiments.

In the previous set of experiments, there was a long lag in the fructose control reactors before growth began, indicating the fructose was degraded by an inducible enzyme system. Therefore, a similar set of experiments was performed with cells grown through one transfer on both substrates. The results of these experiments are given in Figure 14 and Table V. The effect of fructose was a little more severe under these conditions because the average value for $A_{mixture}/A_{control}$ was 0.82 versus 0.87 for the first set. Again, the ratio of concentrations had no effect. During these experiments, much of the fructose was still in the medium at the time of lysine removal and less than 125 mg/l COD due to intermediates had been excreted.

b. Inhibition Effects of Fructose Upon Lysine Removal. Experiment 36 was performed with cells grown through one transfer on fructose and lysine. The cells were harvested and 225 mg/l were placed in the normal growth medium with the addition of chloramphenicol and actidione to prevent further protein synthesis. Figure 15 shows that fructose had little, if any, inhibitory effect upon the utilization of lysine by pre-formed enzymes, implying that the reduction in the differential

Figure 14. Effect of Fructose on the Lysine Removal Rate (R) for Cells Growing in the Presence of Both Lysine and Fructose
(The seed was grown through one transfer on lysine + fructose.

○ = lysine only;

△ = lysine + 500 mg/l fructose COD;

□ = lysine + 1000 mg/l fructose COD;

▽ = lysine + 1500 mg/l fructose COD.

a. Expt. 33, lysine conc. = 500 mg/l COD

b. Expt. 34, lysine conc. = 1000 mg/l COD

c. Expt. 35, lysine conc. = 1500 mg/l COD.)

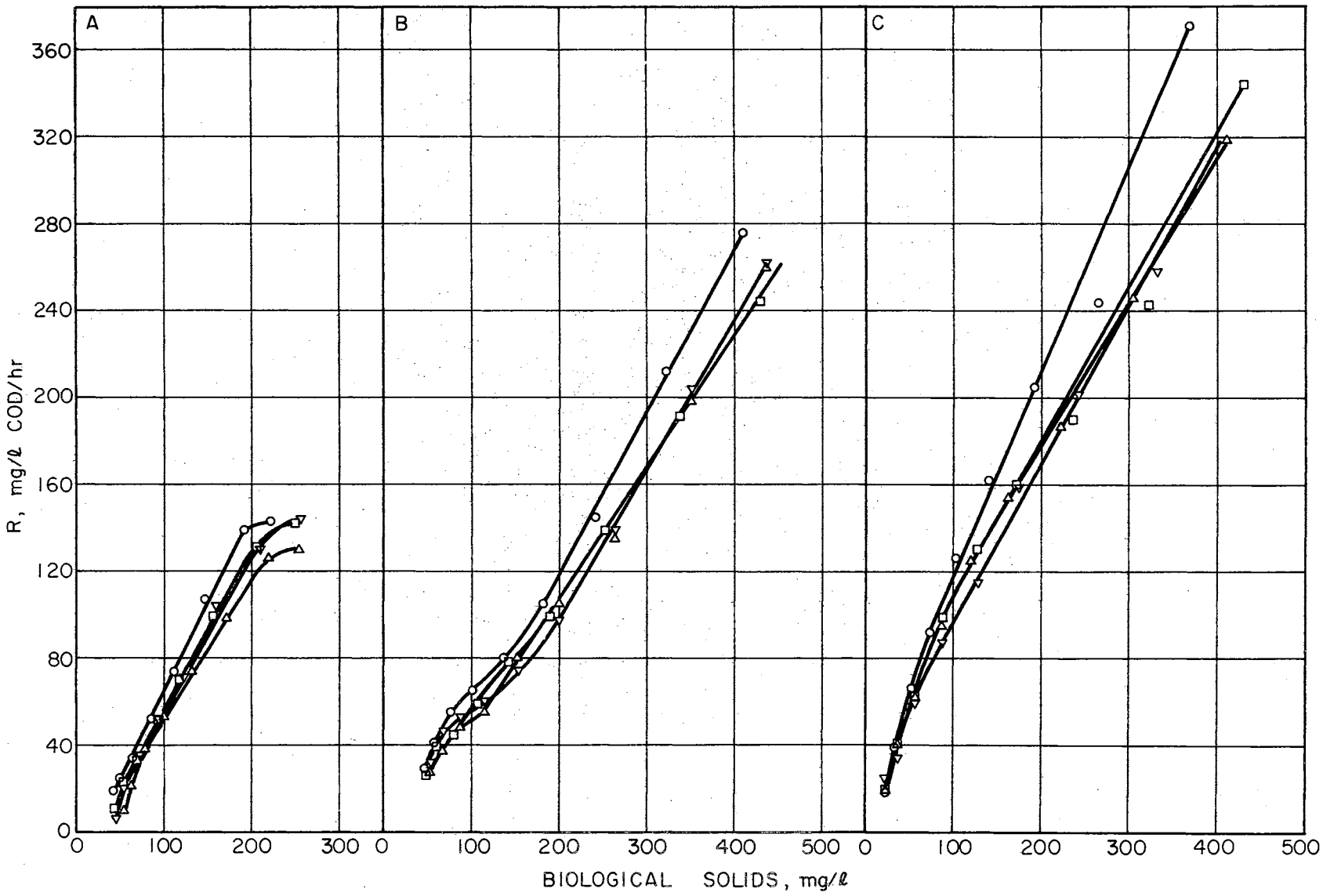

A
B
C
R, mg/ℓ COD/hr
0
40
80
120
160
200
240
280
320
360
0
100
200
300
400
500
BIOLOGICAL SOLIDS, mg/ℓ

TABLE V

EFFECT OF THE CONCENTRATIONS OF LYSINE AND FRUCTOSE ON THE DIFFERENTIAL RATE OF ACTIVITY, A, OF LYSINE DEGRADING ENZYMES IN CULTURES GROWING ON BOTH COMPOUNDS

(The seed was grown through one transfer on lysine + fructose)

Expt. No.	Lysine conc. mg/l COD	Fructose conc. mg/l COD	Lysine COD / Fructose COD	A	$A_{mixture}/A_{control}$
33	494	0	Control	0.81	----
33	484	471	1.03	0.65	0.80
33	490	914	0.54	0.72	0.89
33	490	1424	0.34	0.72	0.89
34	980	0	Control	0.75	----
34	967	460	2.10	0.61	0.81
34	967	944	1.03	0.61	0.81
34	980	1392	0.70	0.69	0.92
35	1525	0	Control	0.95	----
35	1570	490	3.20	0.67	0.71
35	1570	948	1.66	0.74	0.78
35	1587	1453	1.08	0.74	0.78

Average A_m/A_c = 0.82

Figure 15. Effect of Fructose on Lysine Removal Under Non-Proliferating Conditions (Expt. 36) (The seed was grown through one transfer on lysine + fructose. Initial biological solids in the reactors was 225 mg/l.

○ = 500 mg/l lysine COD;

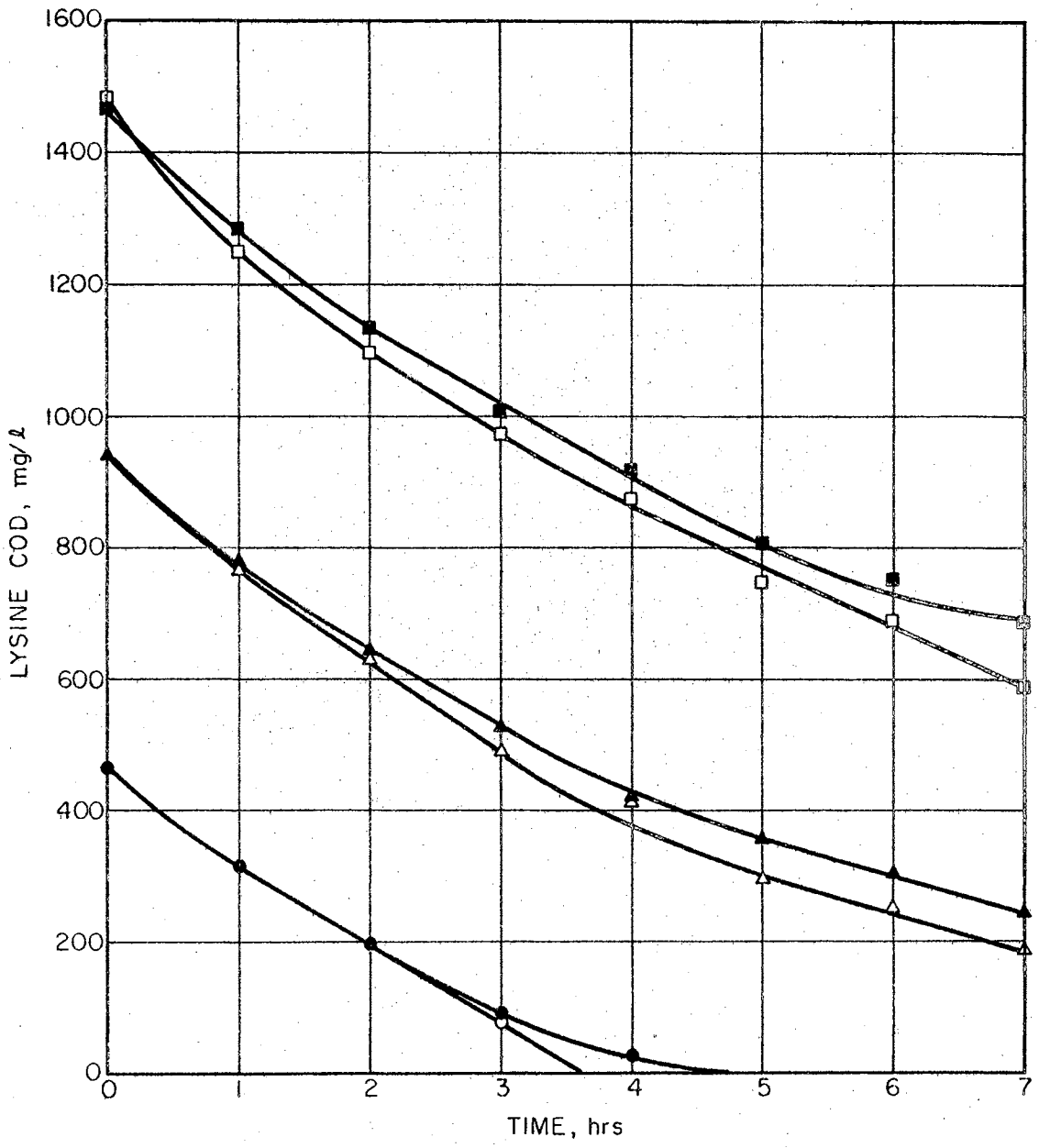
△ = 1000 mg/l lysine COD;

□ = 1500 mg/l lysine COD;

● = 500 mg/l lysine COD + 1000 mg/l fructose COD;

▲ = 1000 mg/l lysine COD + 1000 mg/l fructose COD;

■ = 1500 mg/l lysine COD + 1000 mg/l fructose COD.)


1600
1400
1200
1000
800
600
400
200
0
LYSINE COD, mg/ℓ
0
1
2
3
4
5
6
7
TIME, hrs

rate of activity is probably due to a repression of synthesis.

c. Effect of Fructose Upon the Differential Rate of Synthesis of Lysine Degrading Enzymes. The changes that took place in the stock culture (shift to fungal predominance) occurred before any experiments were run on the enzymatic capability of the cells. The results of one such experiment (Experiment 38), run after the change occurred and reseeding of the system was accomplished, are shown in Figure 16. The seed had been grown through one transfer on lysine plus fructose. The most obvious difference between this experiment and the previous ones was the failure of the R versus solids plots to show linearity, so that A is not constant. Also, it will be noted that there was no difference in $A_{control}$ and $A_{mixture}$ below 125 mg/l solids. There was, however, a difference in the differential rate of synthesis, S, in the two reactors, which indicates that fructose exerted the small effect seen previously by slightly decreasing the rate of formation of lysine degrading enzymes. It is possible that the divergence between the R and EC plots in the individual reactors was caused by an inhibition of activity within the cells by some metabolite of lysine, because the effect was present in the control reactor and was relieved when the cells were removed from the medium. The concentration of extracellular intermediates and end products in the control was less than 50 mg/l when the effect was exerted,

Figure 16. Effect of Fructose on the Removal Rate (R) and Enzymatic Capability (EC) for Lysine in a Culture Grown Through One Transfer on Lysine Plus Fructose (Expt. 38)
(Lysine COD = 1500 mg/l. Fructose COD = 1000 mg/l. A is the slope of the R versus solids plot; S is the slope of the EC versus solids plot.

○ = Lysine removal rate, R, in the control reactor.

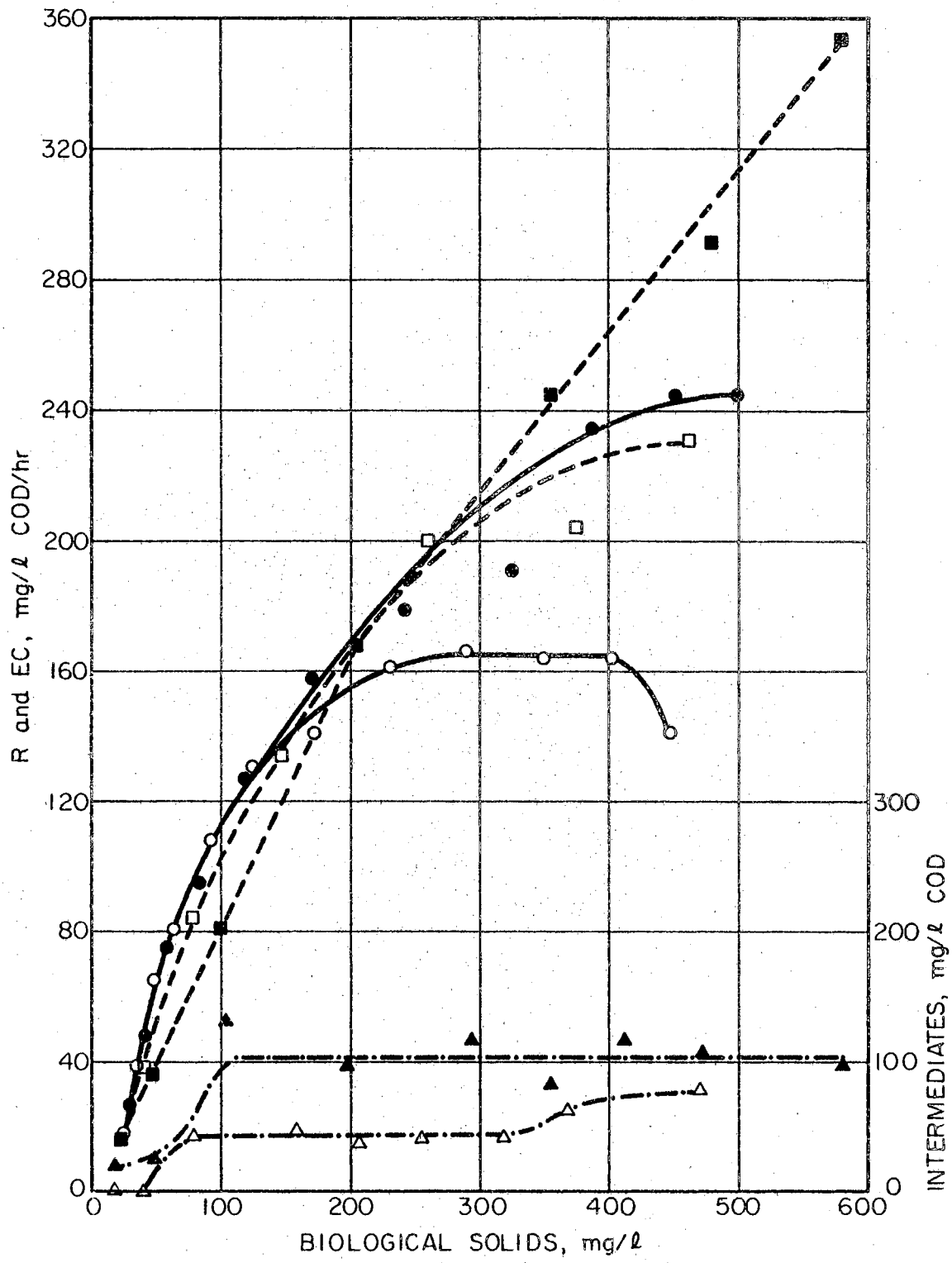
□ = Lysine enzymatic capability, EC, in the control reactor.

△ = Exogenous intermediates in control reactor.

● = Lysine removal rate, R, in the mixed reactor.

■ = Lysine enzymatic capability, EC, in the mixed reactor.

▲ = Exogenous intermediates in mixed reactor.)


360
320
280
240
200
160
120
80
40
0
R and EC, mg/ℓ COD/hr
0
100
200
300
400
500
600
BIOLOGICAL SOLIDS, mg/ℓ
300
200
100
0
INTERMEDIATES, mg/ℓ COD

however, no more than had been found in previous experiments. Since the enzymatic capability test is a whole cell test, caution must be used in assessing the significance of small deviations between plots of R and EC. However, in this case, the magnitude of the divergence is quite large so the difference probably is significant.

Another experiment (No. 46) was run later in a similar manner and it indicated a very slight decrease in the differential rate of synthesis. $A_{mixture}$ was also slightly smaller than $A_{control}$, and the divergence between R and EC was much smaller than in the other experiment. There were fewer intermediates present than in the last experiment.

Experiment 49 was performed with seed that had been taken from a "day 93" slant, grown through one transfer on lysine, then through three on fructose. This experiment shows essentially the same thing as the others; i.e., fructose exerted its slight effect through a decrease in the differential rate of synthesis of lysine degrading enzymes. There was again a large divergence between the R versus X and EC versus X plots, although less than 20 mg/l of intermediates were detected. Since the intermediate COD calculation is based on addition of two experimental values and then subtraction from a third it is possible that more intermediates were present, but were not detected due to experimental error.

The value of μ_m in shaker flasks on fructose was approximately 0.17 during these experiments.

5. Experiments With Ribose

a. Effect of Ribose Upon the Lysine Removal Rate.

All of the ribose experiments were performed after the change in the seed culture, and as the results in Figure 17 show, the plots of R versus X in the controls were curvilinear, with smaller slopes than before the culture changed. Unlike glucose or fructose, however, the effect of ribose was to increase the lysine removal rates, R, in the cultures. No numerical values for the differential rates of activity, A, were calculated due to the difficulty of obtaining meaningful slopes from the controls. It does appear, however, that the higher the ribose concentration, the greater the effect upon A. Little ribose was used during the period of lysine removal, generally less than 100 mg/l COD, but the series was not repeated with seed grown on both compounds because replica plating indicated the likelihood of a predominance shift in the culture if this were done. There were several types of colonies on the lysine plates, but only two types made colonies of normal size on the ribose plates in the same time period, and, thus, it was feared that there would be a predominance of those organisms in the culture if the seed were grown on lysine and ribose. The existence of several types of colonies on the lysine plates is taken as evidence that the culture was not a single species, although it was indeed restricted.

Figure 17. Effect of Ribose on the Lysine Removal Rate (R), for Cells Growing in the Presence of Both Lysine and Ribose
(The seed was grown on lysine.

○ = lysine only;

△ = lysine + 500 mg/l ribose COD;

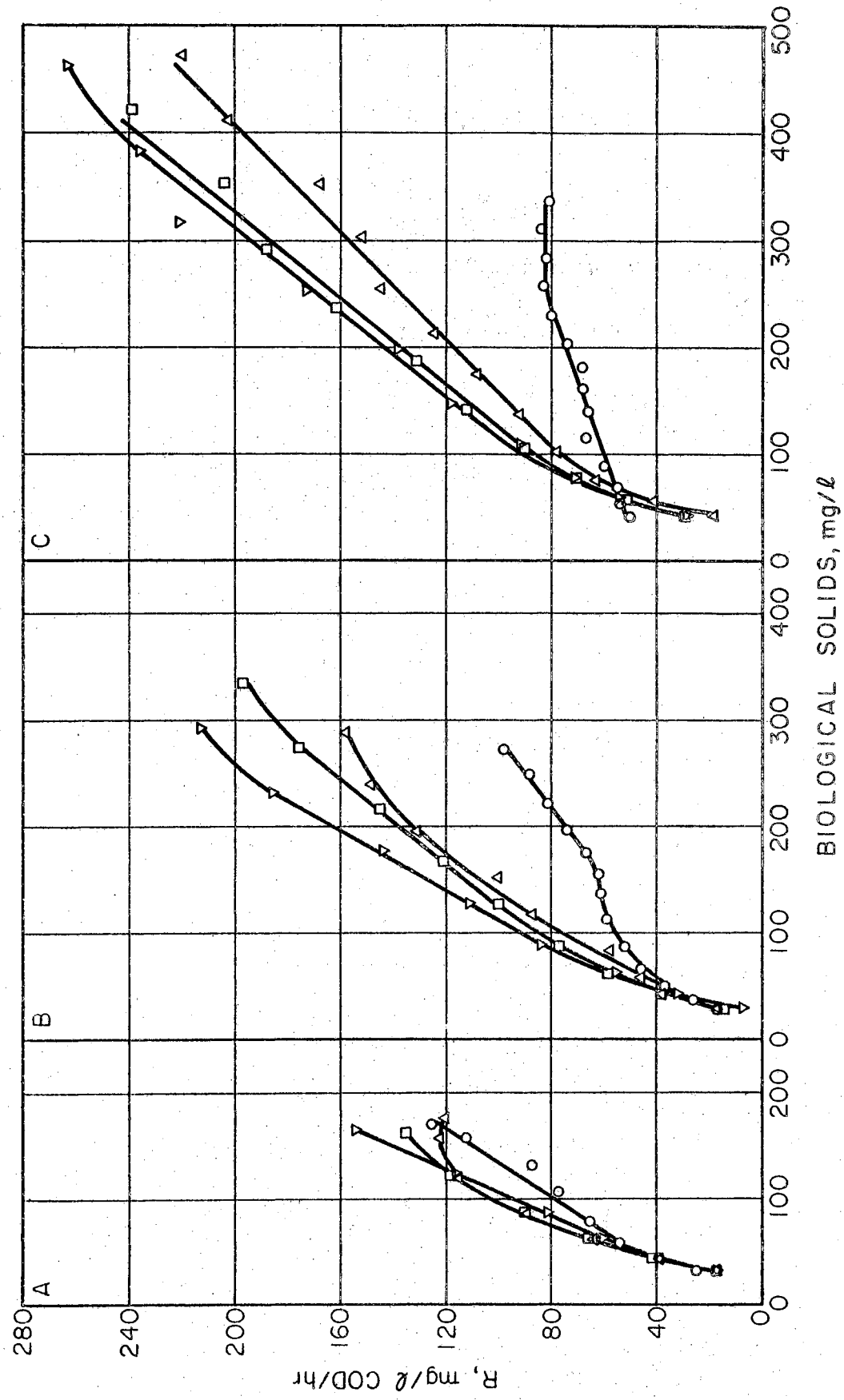
□ = lysine + 1000 mg/l ribose COD;

▽ = lysine + 1500 mg/l ribose COD.

a. Expt. 40, lysine conc. = 500 mg/l COD

b. Expt. 41, lysine conc. = 1000 mg/l COD

c. Expt. 42, lysine conc. = 1500 mg/l COD.)


R, mg/ℓ COD/hr
280
240
200
160
120
80
40
0
A
B
C
0
100
200
300
400
0
100
200
300
400
500
BIOLOGICAL SOLIDS, mg/ℓ

The maximum specific growth rate, μ_m, on ribose in shaker flasks was 0.04.

b. Effect of Ribose on Lysine Removal by Cells Under Non-Proliferating Conditions. Figure 18 (Expt. 44) shows that, when growth was prevented by chloramphenicol and actidione, ribose had no effect upon the rate of lysine removal; therefore, ribose had no influence upon the rate of activity of pre-formed enzymes.

c. Effect of Ribose Upon the Differential Rate of Synthesis of Lysine Degrading Enzymes. Three experiments (Nos. 43, 45, 47) were run to determine the effects of ribose on the rate of formation of enzymatic capability by cells grown on lysine. The concentrations of ribose in these experiments were 500, 50, and 1000 mg/l COD, respectively. In all of the experiments, ribose increased the differential rate of synthesis, S, with the highest concentration of ribose causing the greatest effect. In Expt. 45, Figure 19, the concentration of ribose was 50 mg/l so that an accurate measurement of the amount removed could be made. Only 15 mg/l was removed during the experiment indicating that the ribose was probably being removed for synthesis of macromolecules rather than for use as an energy source. There was no significant difference in the amounts of intermediates accumulated in the two reactors.

Thus, ribose appears to increase the rate of synthesis

Figure 18. Effect of Ribose on Lysine Removal Under Non-Proliferating Conditions (Expt. 44) (The seed was grown on lysine. Initial biological solids in the reactors was 212 mg/l.

○ = 500 mg/l lysine COD;

△ = 1000 mg/l lysine COD;

□ = 1500 mg/l lysine COD;

● = 500 mg/l lysine COD + 500 mg/l ribose COD;

▲ = 1000 mg/l lysine COD + 500 mg/l ribose COD;

■ = 1500 mg/l lysine COD + 500 mg/l ribose COD.)

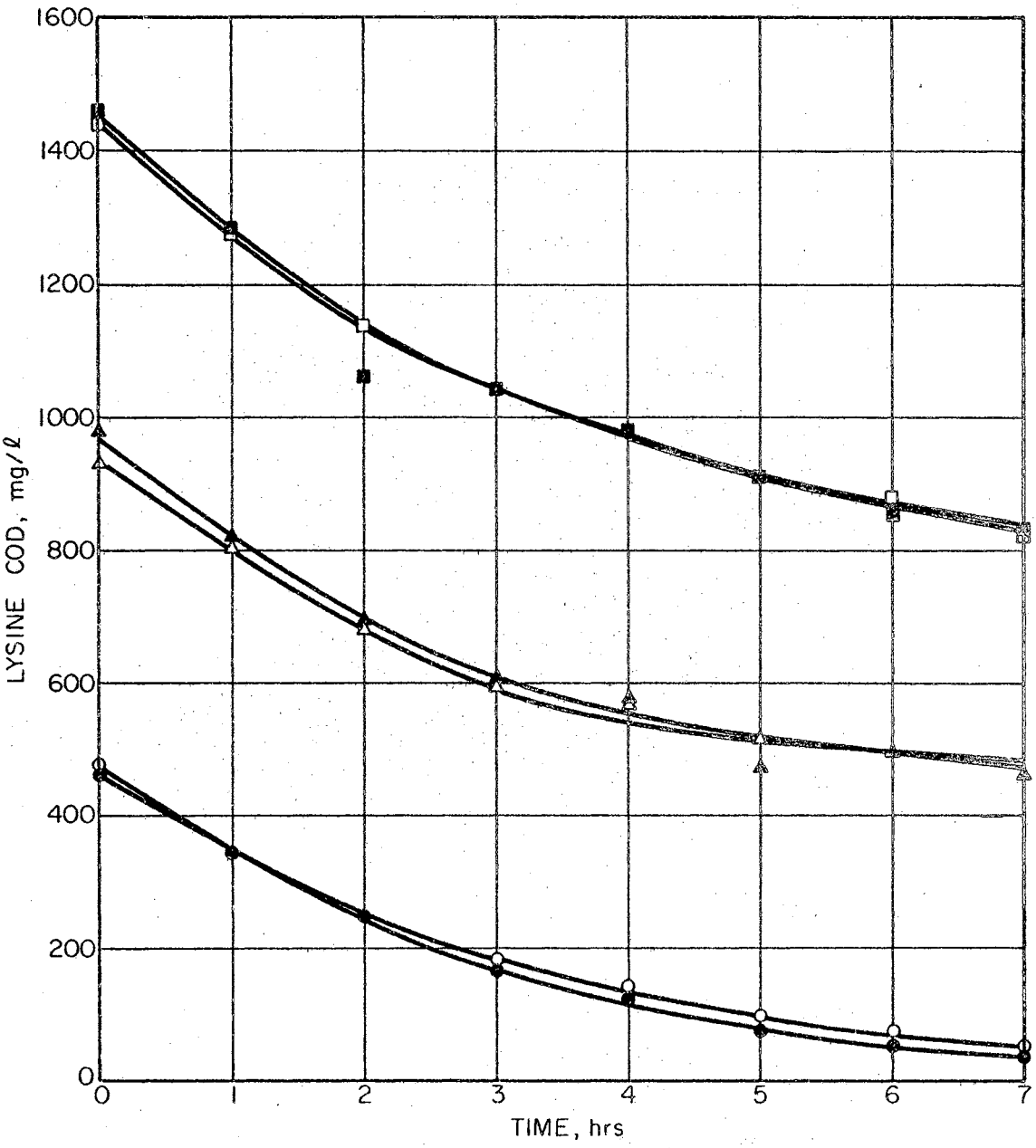

1600
1400
1200
1000
800
600
400
200
0
LYSINE COD, mg/ℓ
0
1
2
3
4
5
6
7
TIME, hrs

Figure 19. Effect of Ribose on the Removal Rate (R) and Enzymatic Capability (EC) for Lysine in a Culture Grown on Lysine (Expt. 45) (Lysine COD = 1000 mg/l. Ribose COD = 50 mg/l. A is the slope of the R versus solids plots; S is the slope of the EC versus solids plots.

○ = Lysine removal rate, R, in the control reactor.

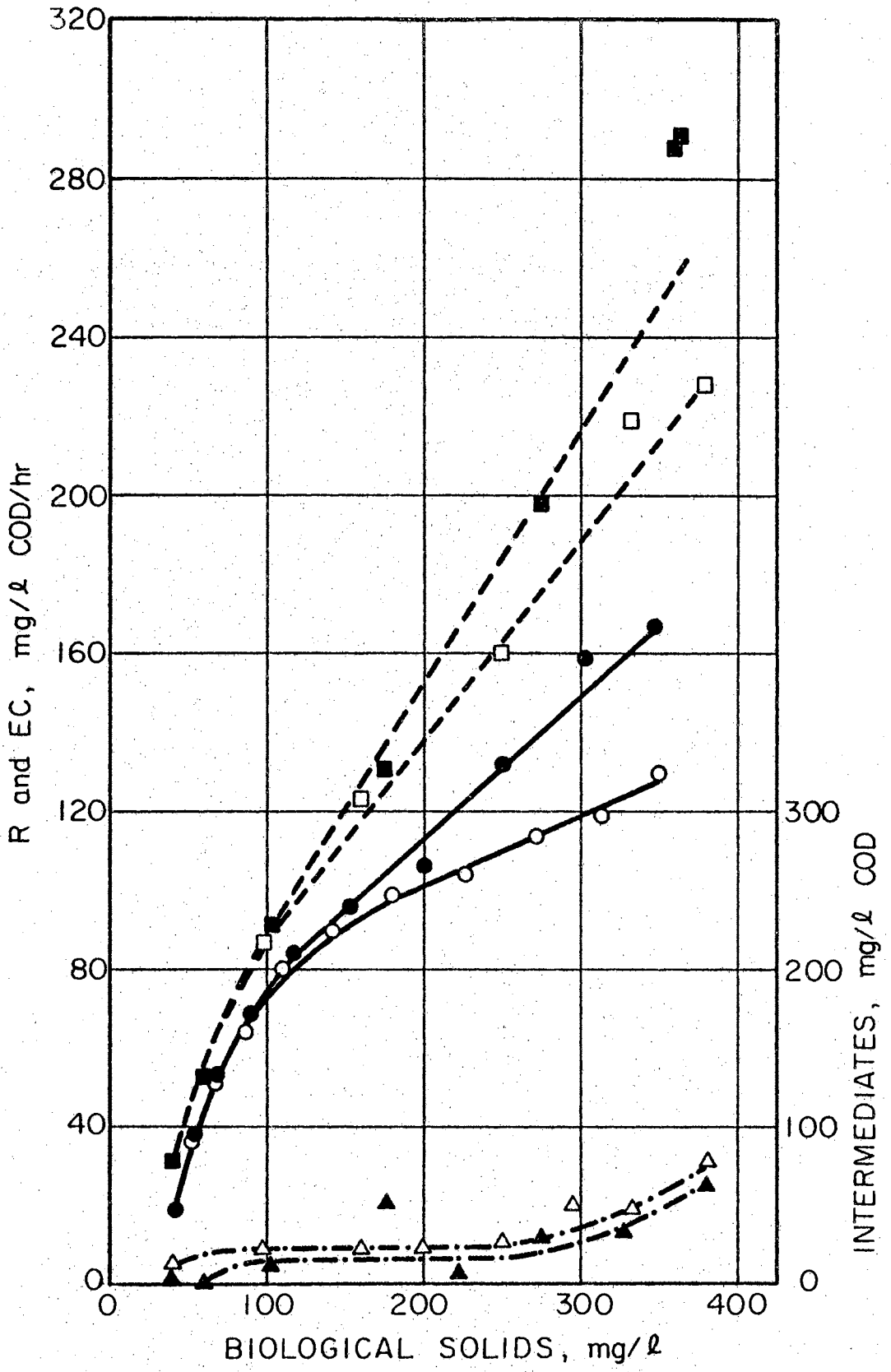
□ = Lysine enzymatic capability, EC, in control reactor.

△ = Exogenous intermediates in the control reactor.

● = Lysine removal rate, R, in the mixed reactor.

■ = Lysine enzymatic capability, EC, in the mixed reactor.

▲ = Exogenous intermediates in the mixed reactor.)


320
280
240
200
160
120
80
40
0
R and EC, mg/ℓ COD/hr
0
100
200
300
400
BIOLOGICAL SOLIDS, mg/ℓ
300
200
100
0
INTERMEDIATES, mg/ℓ COD

of lysine degrading enzymes, possibly by serving as a precursor for RNA synthesis, thereby improving the efficiency of the cells.

C. Continuous Flow Experiments

All of the experiments at the six-hour detention time were run first, then the flow rates were changed and the experiments at a twelve-hour detention time were run. However, in order to facilitate comparisons of the responses, the results will be grouped by shock compound: glucose, fructose, then ribose.

1. Effects of a Shock Load of 1000 mg/l Glucose (COD) Upon a Carbon-Limited Reactor (Reactor A)

a. Six-Hour Detention Time ($D = 0.167\ hr.^{-1}$). The results of this experiment are shown in Figure 20, which is a time plot of the response in the reactor when the influent was changed from 1000 mg/l lysine COD to 1000 mg/l lysine COD plus 1000 mg/l glucose COD. Zero time is the time at which the feed was changed, while negative time is prior to the shock. Little glucose utilization occurred during the first one and one-half hours (the concentration is approximately equal to the theoretical dilute-in value), but after that period, degradation of the carbohydrate started and was accompanied by a rapid accumulation of metabolic intermediates. The

Figure 20. Effect of a Glucose Shock Load on a Carbon-Limited Reactor (Reactor A) Growing on Lysine at a Six-Hour Detention Time (D = 0.167)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

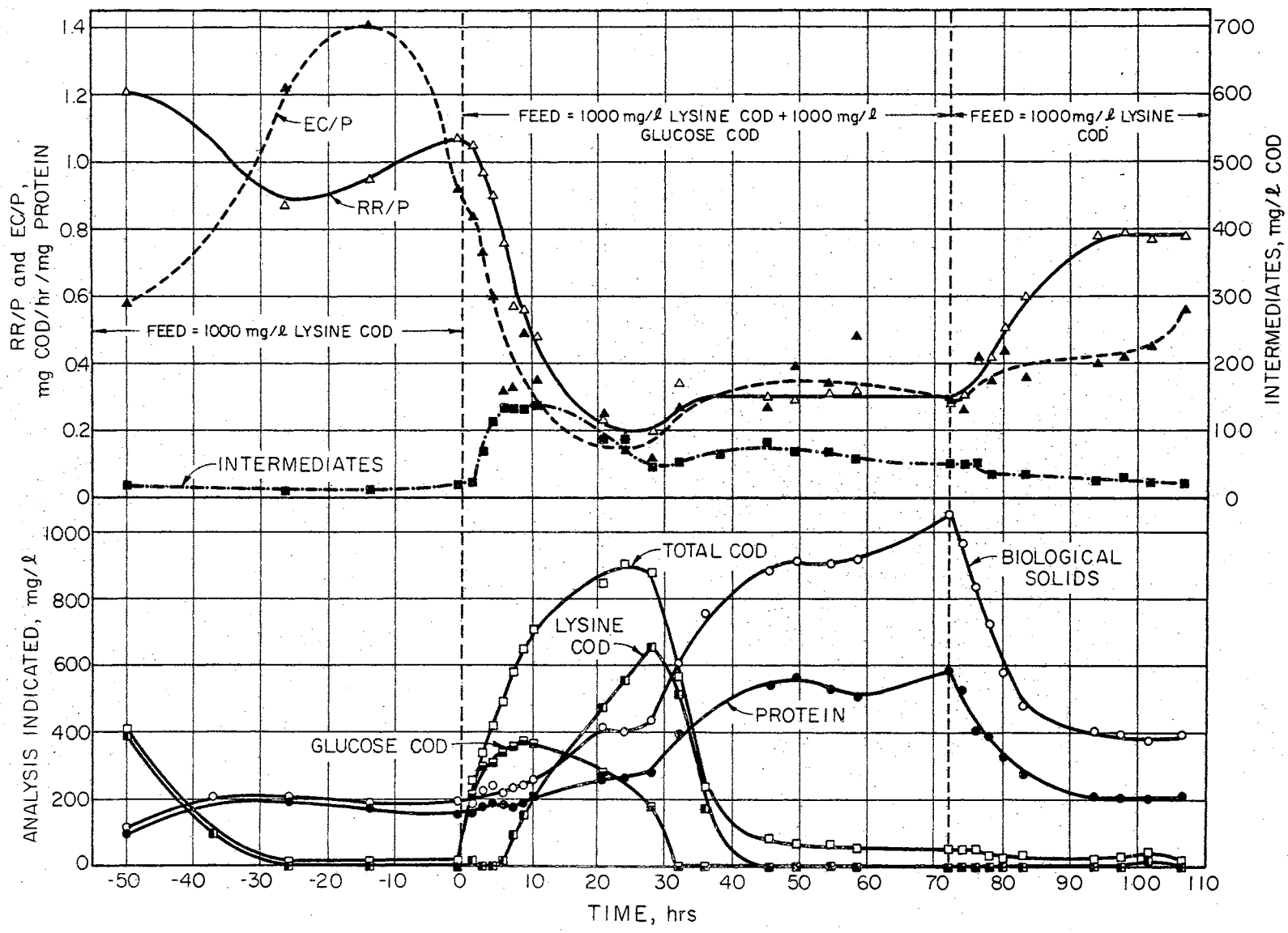
◧ = Lysine COD;

⬓ = Glucose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P).


RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
EC/P
RR/P
INTERMEDIATES
FEED = 1000 mg/ℓ LYSINE COD
FEED = 1000 mg/ℓ LYSINE COD + 1000 mg/ℓ GLUCOSE COD
FEED = 1000 mg/ℓ LYSINE COD
ANALYSIS INDICATED, mg/ℓ
TOTAL COD
BIOLOGICAL SOLIDS
LYSINE COD
PROTEIN
GLUCOSE COD
TIME, hrs

intermediates continued to increase in the medium for the first six hours, and repression of synthesis of lysine degrading enzymes occurred, so that by the end of that period the enzymatic capability and the specific removal rate were so low that lysine had started to build up in the reactor. It should be noted that up to this point, no significant change in protein or biological solids concentration had occurred. The enzymatic capability continued to decrease during the first 28 hours, allowing lysine to build up in the medium until its concentration was 65% of the influent value. At that time the combination of high lysine concentration and low intermediate concentration (it had been reduced to 50 mg/l) allowed the repression to be diminished, and EC/P began to increase, reaching a new value of approximately 0.30. When this occurred there was another increase in growth rate within the reactor because the solids started increasing very rapidly. Forty-five hours after administering the shock, all lysine had again been removed from the reactor and it was approaching a new steady state.

At the new steady state, there had been a change in the physiological characteristics of the cells because the protein was a more normal 55% of the cell mass instead of the abnormally high value of 85% which had been measured prior to the shock. Also, the cell yield had improved because the combination of 1000 mg/l lysine COD plus 1000 mg/l glucose COD supported 1000 mg/l solids whereas before

the shock the 1000 mg/l of lysine COD had supported only 200 mg/l of cells. These two occurrences were observed in several of the experiments. Prior to the shock the enzymatic capability with respect to protein (EC/P) had been erratic (probably associated with the unexplained disruption of the steady state that had occurred). At the new steady state, EC/P was 0.30, or 35% of the value immediately preceding the shock. During the transient, when the repression of the lysine degrading enzyme system was severe, the EC/P reached a minimum value of 0.15.

After the shock was removed (i.e., after the feed was changed to 1000 mg/l lysine), a washout of the excess solids occurred until a new steady state was reached. The EC/P of the culture did not increase as rapidly as did the specific substrate removal rate, and it is not certain how this could happen. It could mean either that a large amount of lysine was being used for protein synthesis, or that the enzymatic capability test was not actually measuring the full enzyme level in the cells.

A second experiment was performed with glucose but it was only run to the point of maximum lysine build-up. The response was the same to that point.

b. Twelve-Hour Detention Time ($D = 0.083\ hr.^{-1}$). At this flow rate, as shown in Figure 21, the response to the glucose shock was less severe. Glucose utilization was slight for the first three hours, but once it started there was a decrease in EC/P, from 0.67 to 0.38. No lag

Figure 21. Effect of a Glucose Shock Load on a Carbon-Limited Reactor (Reactor A) Growing on Lysine at a Twelve-Hour Detention Time (D = 0.083)

○ = Biological Solids concentration;

● = Protein concentration;

□ = total COD;

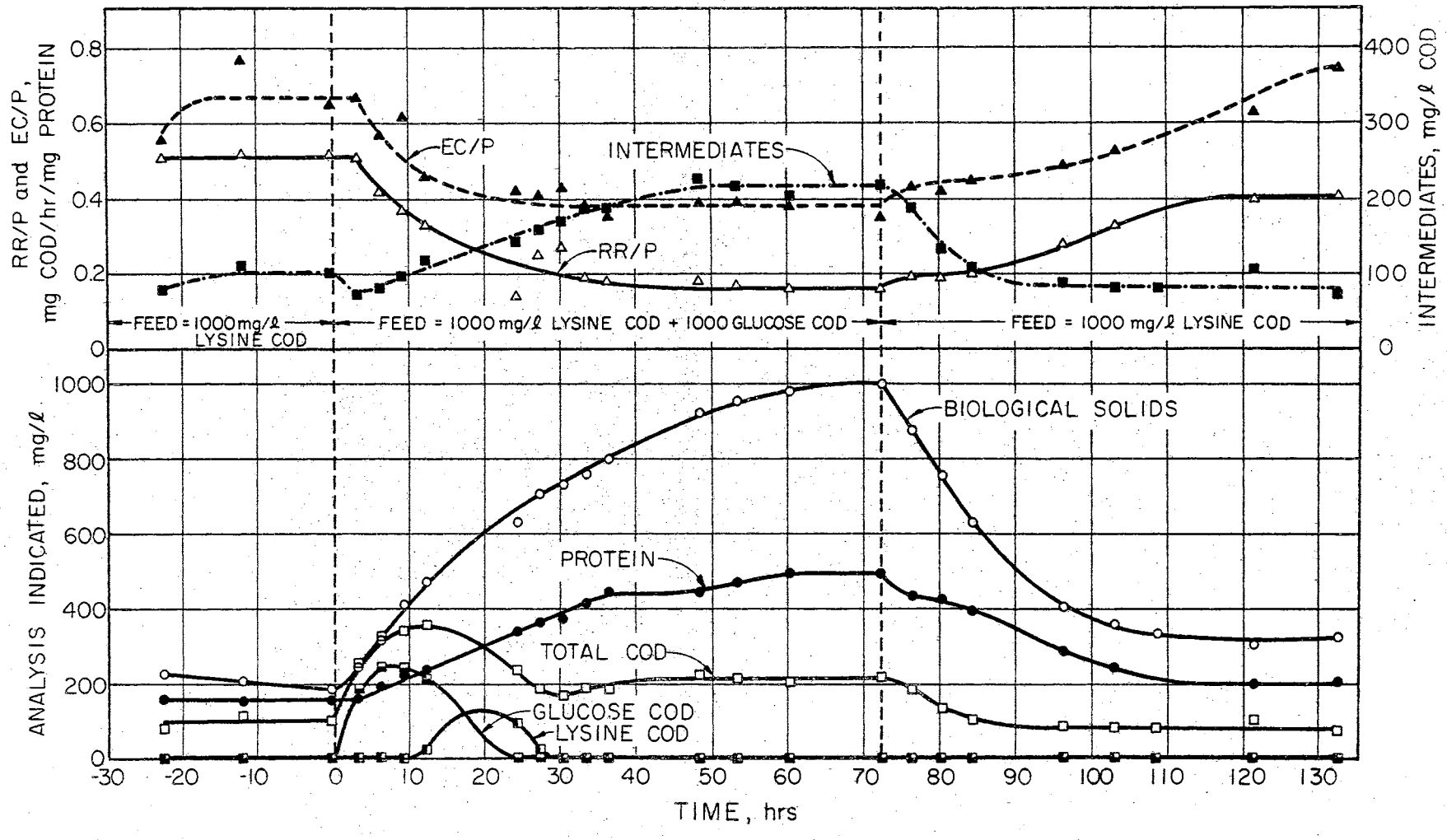
◧ = Lysine COD;

◒ = Glucose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)


RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
EC/P
INTERMEDIATES
RR/P
FEED = 1000 mg/ℓ LYSINE COD
FEED = 1000 mg/ℓ LYSINE COD + 1000 GLUCOSE COD
FEED = 1000 mg/ℓ LYSINE COD
ANALYSIS INDICATED, mg/ℓ
BIOLOGICAL SOLIDS
PROTEIN
TOTAL COD
GLUCOSE COD
LYSINE COD
TIME, hrs

was experienced before the solids and protein started to increase (as found at D = 0.167) so that despite the lowered capability (57% of the preshock value) there was only a small increase in the lysine content of the effluent. It is possible that the intermediates were of a different nature than those encountered in the previous experiment because they continued to build up until they reached a fairly steady concentration of about 200 mg/l during the last 24 hours, although the repression was not as severe. Since these intermediates were not removed, it is possible that they were refractory in nature and did not include the metabolite repressor or lead to the repressor. This build-up was observed in all experiments on this reactor at this flow rate (see Figures 25 and 29).

As in the previous experiment (D = 0.167 $hr.^{-1}$), there was a physiological change in the culture resulting in a decrease in the per cent protein to a more "normal" value, and an increase in cell yield. At this flow rate, the repression was not as severe because EC/P did not "overshoot", but stabilized directly at the new "steady state" concentration. After the shock was removed, EC/P returned to a value slightly higher than that prior to the shock.

2. Effects of a Shock Load of 1000 mg/l Glucose (COD) Upon a Magnesium-Limited Reactor (Reactor B)

It would be reasonable to expect that when a nutrient other than the organic substrate is limiting in a reactor,

the response to an increase in organic loading cannot be an increase in protein synthesis because the limiting nutrient has not been changed. If more organic substrate is added to the influent, the effluent COD will increase because the total rate of COD utilization must stay approximately the same. If the second (added) compound is capable of causing metabolite repression of enzymes for degradation of the first compound, it will cause an additional leakage of the first compound in the effluent.

a. Six-Hour Detention Time ($D = 0.167\ hr.^{-1}$). As shown in Figure 22, when glucose was introduced into the reactor, the concentration followed the theoretical dilute-in curve for the first hour and one-half, indicating no utilization, and, thus, there was no change in the enzymatic capability or lysine removal rate of the culture. However, as soon as glucose utilization started, severe repression of the synthesis of lysine degrading enzymes occurred because the enzymatic capability conformed to a theoretical wash-out curve for three hours. After 20 hours, repression was less severe because a new steady state was finally reached in which EC/P was 46% of the unrepressed value. In the new steady state, the 1000 mg/l of glucose COD added to the medium replaced only 750 mg/l of the 1500 mg/l lysine COD being used by the culture prior to the shock so that it is possible that if more glucose had been provided the repression would have been more severe. There was no increase in protein synthesis,

Figure 22. Effect of a Glucose Shock Load on a Magnesium-Limited Reactor (Reactor B) Growing on Lysine at a Six-Hour Detention Time ($D = 0.167\ hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

◧ = Lysine COD;

◒ = Glucose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)

RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
ANALYSIS INDICATED, mg/ℓ
TIME, hrs
RR/P
EC/P
INTERMEDIATES
FEED = 2000 mg/ℓ LYSINE COD +1000 mg/ℓ GLUCOSE COD
FEED = 2000 mg/ℓ LYSINE COD
FEED = 2000 mg/ℓ LYSINE COD
TOTAL COD
LYSINE COD
BIOLOGICAL SOLIDS
PROTEIN
GLUCOSE COD

confirming the biosynthetically restricted nature of the medium, but there was an increase in cell mass, possibly due to an increase in stored carbohydrate material. Unlike the cells in the carbon-limited system, which were 85% protein, the cells in this reactor were 56% protein and were encapsulated with a slimy capsule that was visible with India ink staining. The latter characteristic was common to all of the magnesium-limited experiments. After the glucose was removed from the feed, the enzymatic capability started to increase immediately, indicating a release from repression, and returned to approximately the pre-shock value, as did the other parameters. The intermediates in the reactor were higher under non-shock conditions (60 mg/l) than they had been in the carbon-limited system under similar conditions (20 mg/l), but this would be expected because of the biosynthetic restriction.

As a check, another experiment was run at this flow rate and gave similar results for the first 28 hours, after which the shock was removed.

b. Twelve-Hour Detention Time ($D = 0.083\ hr.^{-1}$).

Because of the greatly increased yield at slower growth rates when magnesium is limiting, it was necessary to decrease the magnesium concentration in the medium to maintain approximately the same solids level (82) (83). However, as shown in Figure 23, the final concentration of cells in the reactor was lower than at the other detention time. Prior to the shock EC/P was 0.60 but was reduced to

Figure 23. Effect of a Glucose Shock Load on a Magnesium-Limited Reactor (Reactor B) Growing on Lysine at a Twelve-Hour Detention Time ($D = 0.083\ hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

◧ = Lysine COD;

⬓ = Glucose COD;

▩ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)

RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
EC/P
RR/P
INTERMEDIATES
FEED=2000 mg/ℓ LYSINE COD
FEED = 2000 mg/ℓ LYSINE COD + 1000 mg/ℓ GLUCOSE COD
FEED = 2000 mg/ℓ LYSINE COD
ANALYSIS INDICATED, mg/ℓ
TOTAL COD
LYSINE COD
BIOLOGICAL SOLIDS
PROTEIN
GLUCOSE COD
TIME, hrs

approximately 0.15, or 25% of its former value, due to the metabolism of glucose. As would be anticipated by the theory of metabolite repression, the repression observed here was greater than that found at the faster flow rate in the same type system (35) (84). In a biosynthetically restricted system, the slower the flow rate, the more restriction there is upon anabolism. When a compound like glucose, which can be degraded readily, is added to the medium, it will contribute intermediates faster than biosynthesis can use them, and the slower the flow rate the greater the difference between the rate of utilization and the rate of growth, and the greater the repression. Other than the quantiative differences, the responses of the two magnesium-limited systems were similar: a lag in glucose utilization and a decrease in EC/P which occurred only after glucose utilization and intermediate build-up had begun. More lysine was replaced this time, 900 mg/l COD instead of 750, but after release from the shock all system parameters returned to their pre-shock levels.

3. Effects of a Shock Load of 1000 mg/l Fructose (COD) Upon a Carbon-Limited Reactor (Reactor A)

a. Six-Hour Detention Time (D = 0.167 $hr.^{-1}$). The response of the carbon-limited system to a fructose shock load (Figure 24) was similar to, but less severe than, its response to glucose. Approximately one and one-half hours

Figure 24. Effect of a Fructose Shock Load on a Carbon-Limited Reactor (Reactor A) Growing on Lysine at a Six-Hour Detention Time ($D = 0.167\ hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

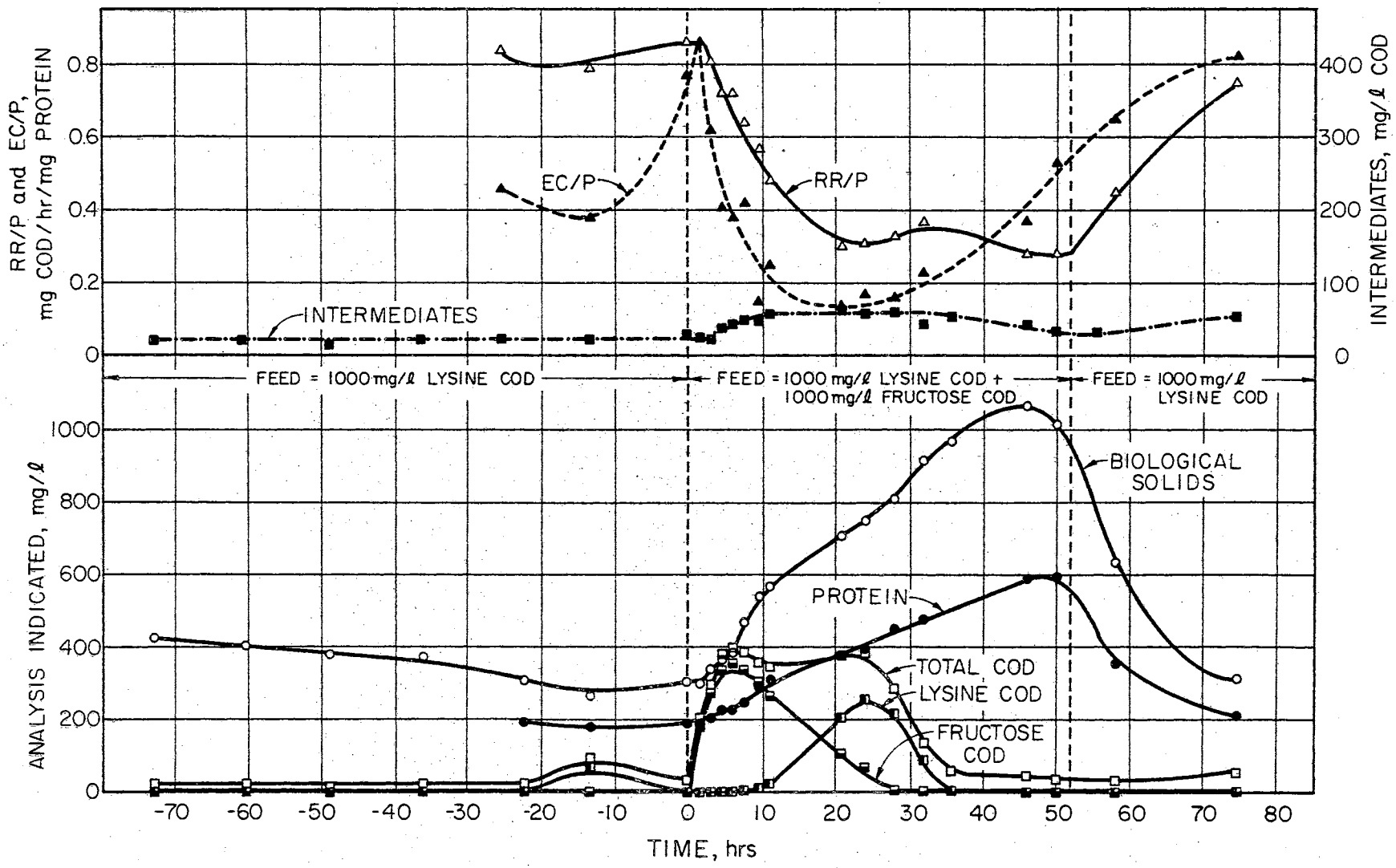
◧ = Lysine COD;

⬓ = Fructose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)


RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
EC/P
RR/P
INTERMEDIATES
FEED = 1000 mg/ℓ LYSINE COD
FEED = 1000 mg/ℓ LYSINE COD + 1000 mg/ℓ FRUCTOSE COD
FEED = 1000 mg/ℓ LYSINE COD
ANALYSIS INDICATED, mg/ℓ
BIOLOGICAL SOLIDS
PROTEIN
TOTAL COD
LYSINE COD
FRUCTOSE COD
TIME, hrs

were required for the initiation of fructose utilization and the highest level of fructose in the medium was 350 mg/l compared to 370 mg/l for glucose. Repression of synthesis of lysine degrading enzymes occurred in spite of the fact that the concentration of intermediates did not increase greatly. It should be noted that the enzymatic capability of the culture was increasing (probably related to the slight leakage of lysine 13 hours before the shock) when the shock was applied, but the trend was reversed by the repression, which appears to have been almost complete. This decrease in enzymatic capability caused a temporary leakage of lysine into the effluent, but it was not as great as the leakage when glucose was the shock compound because the biological solids concentration was already increasing. The enzymatic capability was smallest during the period of maximum leakage of lysine, but it then started increasing and had returned to 0.53, or 62% of the pre-shock value, just before removal of the shock. The enzymatic capability and removal rate plots diverged, but they both show the same trend. After the shock was removed, the biological solids, protein, and enzymatic capability all returned to their pre-shock values.

b. Twelve-Hour Detention Time ($D = 0.083\ hr.^{-1}$).

There was a dual character to the response to a shock load of fructose at this flow rate. During the first 36 hours after the application of the shock (Figure 25), the response anticipated from examination of the previous

Figure 25. Effect of a Fructose Shock Load on a Carbon-Limited Reactor (Reactor A) Growing on Lysine at a Twelve-Hour Detention Time (D = 0.083 $hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

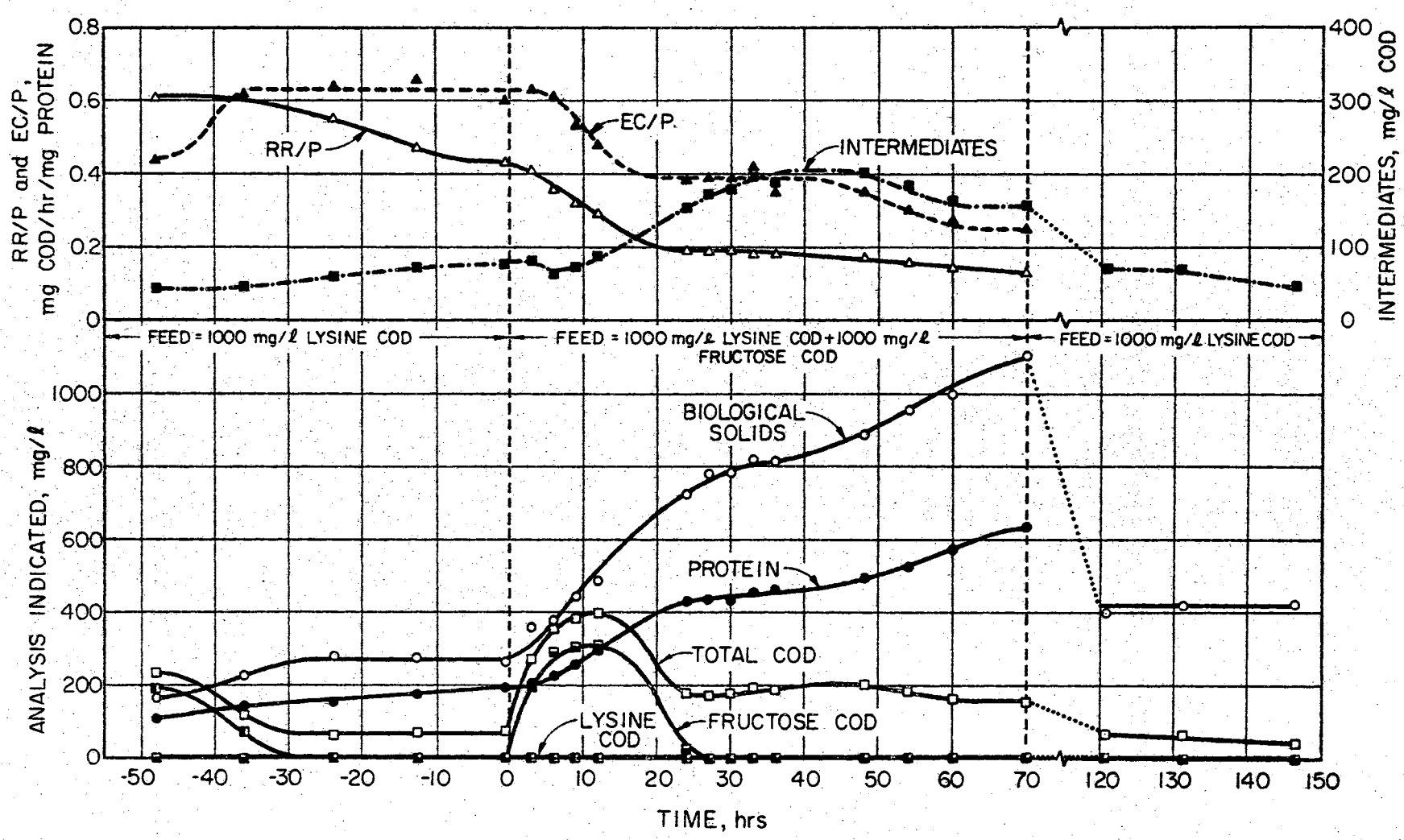
◧ = Lysine COD;

⬓ = Fructose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)


RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
ANALYSIS INDICATED, mg/ℓ
TIME, hrs
RR/P
EC/P
INTERMEDIATES
FEED = 1000 mg/ℓ LYSINE COD
FEED = 1000 mg/ℓ LYSINE COD + 1000 mg/ℓ FRUCTOSE COD
FEED = 1000 mg/ℓ LYSINE COD
BIOLOGICAL SOLIDS
PROTEIN
TOTAL COD
LYSINE COD
FRUCTOSE COD

results occurred. Fructose built up gradually in the system, but was eventually removed as a new apparent steady state was reached. As expected, this resulted in a repression of synthesis of lysine degrading enzymes as shown by the decrease of enzymatic capability from a pre-shock value of 0.63 to a new value of 0.39 (62%). At this time, the concentration of intermediates was high, but the level of metabolite repressor must not have been excessive, because the repression was mild. The second portion of the response started sometime after 36 hours and resulted in another rise in protein and solids, although very little change occurred in the total effluent COD. Accompanying the rise in cell mass and protein was a concomitant decrease in enzymatic capability to 0.25, or only 40% of the pre-shock value. Although it cannot be proven since replica plating was not done in this series of experiments, it appears that a predominance shift occurred in the culture after 36 hours because the solids concentration increased without any change in COD (indicating a change in yield) and did not return to the old steady state value after the shock was removed; instead it stabilized at 420 mg/l, 155% of the pre-shock value (again indicating a change in yield). During the shock, the total enzymatic capability (EC/P x P) at the first steady state was approximately equal to the value at the second steady state (168 versus 159) implying that the second decrease in EC/P could have been caused solely by the increase in

yield of the solids. Thus, for comparisons with the other experiments the first value (0.39) was used.

4. Effects of a Shock Load of 1000 mg/l Fructose (COD) Upon a Magnesium-Limited Reactor (Reactor B)

a. Six-Hour Detention Time ($D = 0.167\ hr.^{-1}$). The response of the magnesium-limited reactor to the fructose shock load is shown in Figure 26. The most obvious difference between this experiment and those on glucose is that at this flow rate fructose was being added faster than the cells could remove it, causing the level in the reactor to build up to 520 mg/l. Since the feed concentration was 933 mg/l, the amount removed was 413 mg/l which was enough to lower the enzymatic capability of the cells for lysine and displace 400 mg/l lysine COD, as shown by the lysine plot. Initially, a severe repression of synthesis occurred, as shown by the decrease in EC/P (although the protein concentration stayed constant), followed by a lesser repression allowing synthesis at a reduced rate. When the new steady state was reached, EC/P was 0.49, or 58% of the pre-shock value. The intermediates did not increase appreciably until the repression mechanism was operative, but once they increased they stayed relatively high. After fructose was removed from the feed, the repression was relieved and the capability returned to its pre-shock level.

Figure 26. Effect of a Fructose Shock Load on a Magnesium-Limited Reactor (Reactor B) Growing on Lysine at a Six-Hour Detention Time ($D = 0.167\ hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

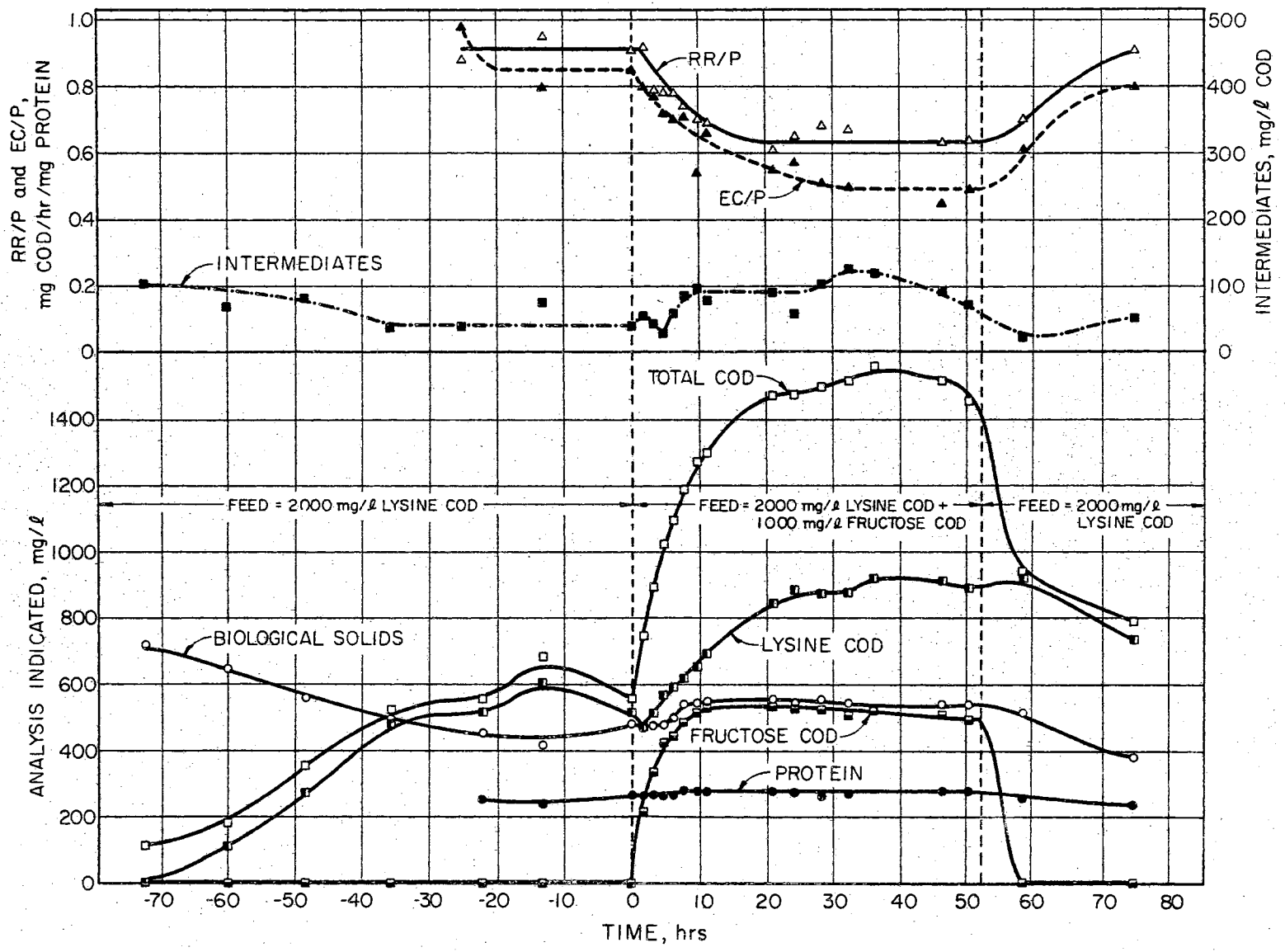
◧ = Lysine COD;

⬓ = Fructose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)


RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
ANALYSIS INDICATED, mg/ℓ
TIME, hrs
RR/P
EC/P
INTERMEDIATES
TOTAL COD
BIOLOGICAL SOLIDS
LYSINE COD
FRUCTOSE COD
PROTEIN
FEED = 2000 mg/ℓ LYSINE COD
FEED = 2000 mg/ℓ LYSINE COD + 1000 mg/ℓ FRUCTOSE COD
FEED = 2000 mg/ℓ LYSINE COD

b. Twelve-Hour Detention Time ($D = 0.083\ hr.^{-1}$).

Figure 27 shows the response of this system to fructose. The pattern was similar to that observed at the higher flow rate; as fructose utilization began, the enzymatic capability of the cells for degrading lysine decreased. The reactor appears to have gone through two stages of fructose utilization, with a slight decrease in fructose concentration after 36 hours. As pointed out previously, the solids concentration in the reactor at this flow rate was not as great as at the six-hour detention time, and this probably explains why the fructose concentration in the effluent was not lower. As is expected under magnesium-limited conditions, there was no increase in protein, but the enzymatic capability of cells did decrease, yielding a value of 0.40, or 56% of the pre-shock value. It is interesting to note that few intermediates were detectable in the medium indicating that it is not necessary for the intermediates to be excreted for metabolite repression to occur.

5. Effects of a Shock Load of 1000 mg/l Ribose (COD) Upon a Carbon-Limited Reactor (Reactor A)

a. Six-Hour Detention Time ($D = 0.167\ hr.^{-1}$). In the batch experiments, it was found that the maximum specific growth rate on ribose was 0.04; therefore, when it was used as a shock substrate (Figure 28), the cells could not utilize ribose fast enough to remove all

Figure 27. Effect of a Fructose Shock Load on a Magnesium-Limited Reactor (Reactor B) Growing on Lysine at a Twelve-Hour Detention Time ($D = 0.083$ hr.$^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

◧ = Lysine COD;

▬ = Fructose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)

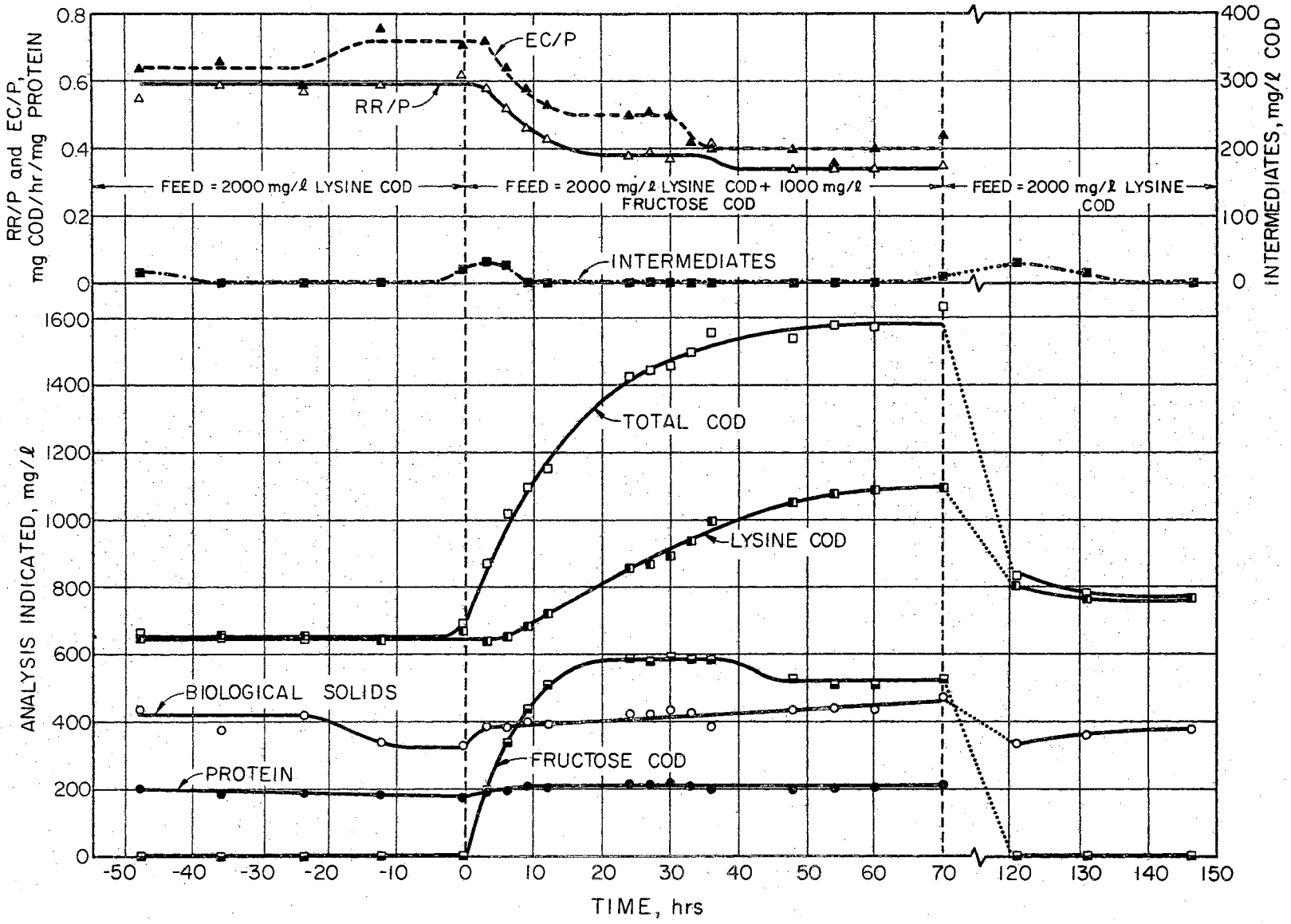

RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
ANALYSIS INDICATED, mg/ℓ
TIME, hrs
EC/P
RR/P
INTERMEDIATES
FEED = 2000 mg/ℓ LYSINE COD
FEED = 2000 mg/ℓ LYSINE COD + 1000 mg/ℓ FRUCTOSE COD
FEED = 2000 mg/ℓ LYSINE COD
TOTAL COD
LYSINE COD
BIOLOGICAL SOLIDS
PROTEIN
FRUCTOSE COD

Figure 28. Effect of a Ribose Shock Load on a Carbon-Limited Reactor (Reactor A) Growing on Lysine at a Six-Hour Detention Time ($D = 0.167\ hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

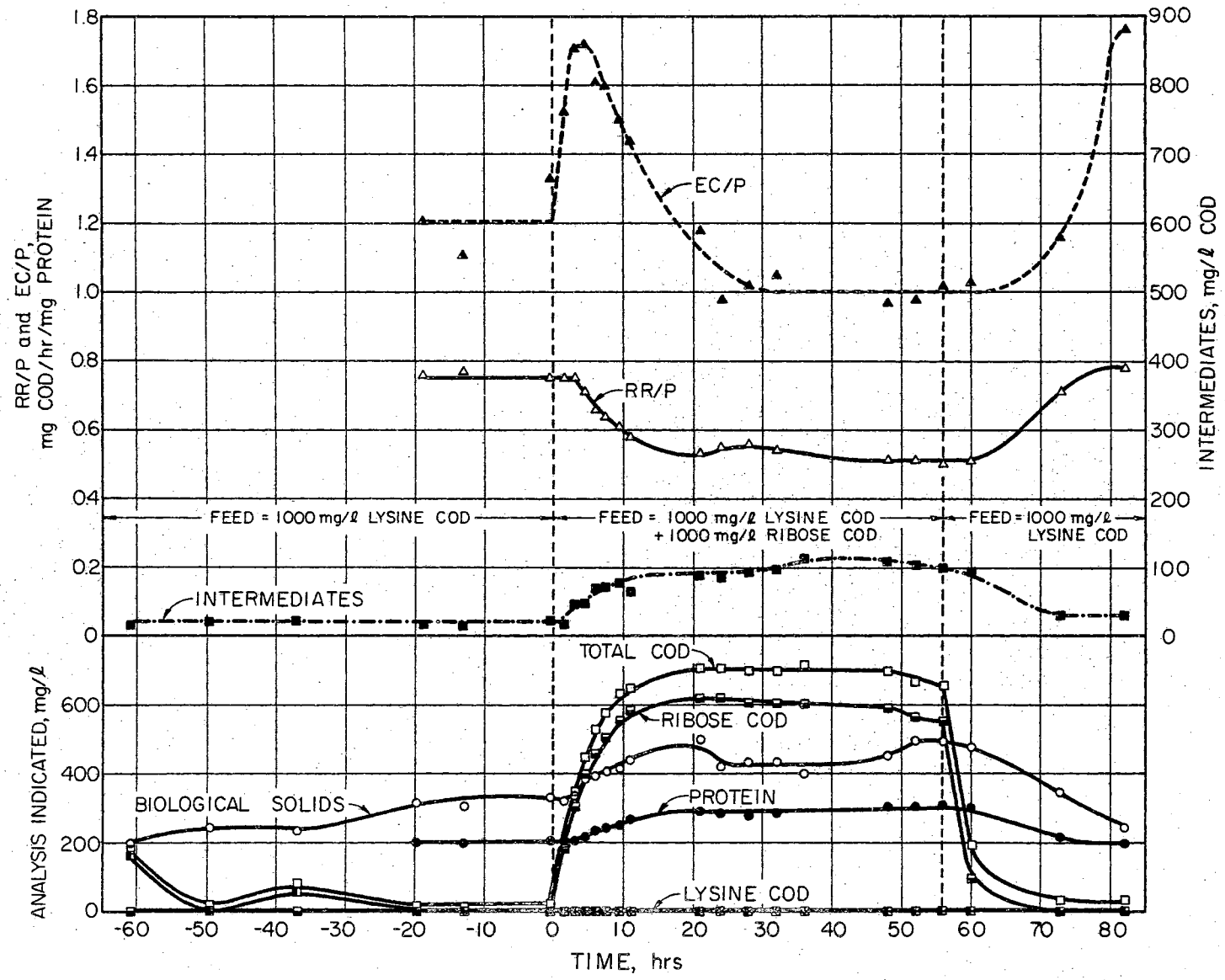
◧ = Lysine COD;

⬓ = Ribose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)


RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
ANALYSIS INDICATED, mg/ℓ
TIME, hrs
EC/P
RR/P
INTERMEDIATES
TOTAL COD
RIBOSE COD
PROTEIN
BIOLOGICAL SOLIDS
LYSINE COD
FEED = 1000 mg/ℓ LYSINE COD
FEED = 1000 mg/ℓ LYSINE COD + 1000 mg/ℓ RIBOSE COD
FEED = 1000 mg/ℓ LYSINE COD

of it from solution and the concentration built up to 600 mg/l; i.e., approximately 300 mg/l was used. The response observed in Figure 28 can be explained in light of the batch experiments and the theories of catabolite repression (35). Immediately after ribose was introduced into the system, the enzymatic capability of the cells increased (as shown in the previous batch experiments), but as the slow ribose utilization started, the enzymatic capability began to decrease due to mild repression. The new steady state value of enzymatic capability with respect to protein was 83% of the pre-shock value which was adequate for removing all of the lysine supplied to the reactor so that no leakage to the effluent occurred. After the shock was removed, the protein level returned to the pre-shock value but the total solids decreased slightly. The actual rate of removal of lysine returned to its original level but EC/P increased greatly; however, since there is only one sample at the high level it is possible that it is in error.

b. Twelve-Hour Detention Time (D = 0.083 $hr.^{-1}$).

For the first 36 hours after the shock was applied (Figure 29), the response was as expected from the previous experiments and from theory. There was an initial increase in EC/P followed by a slight decrease to a value that was 89% of the pre-shock value. Up to this point, ribose removal was similar to that in the last experiment, but after 36 hours the rate of utilization of ribose

Figure 29. Effect of a Ribose Shock Load on a Carbon-Limited Reactor (Reactor A) Growing on Lysine at a Twelve-Hour Detention Time (D = 0.083 $hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

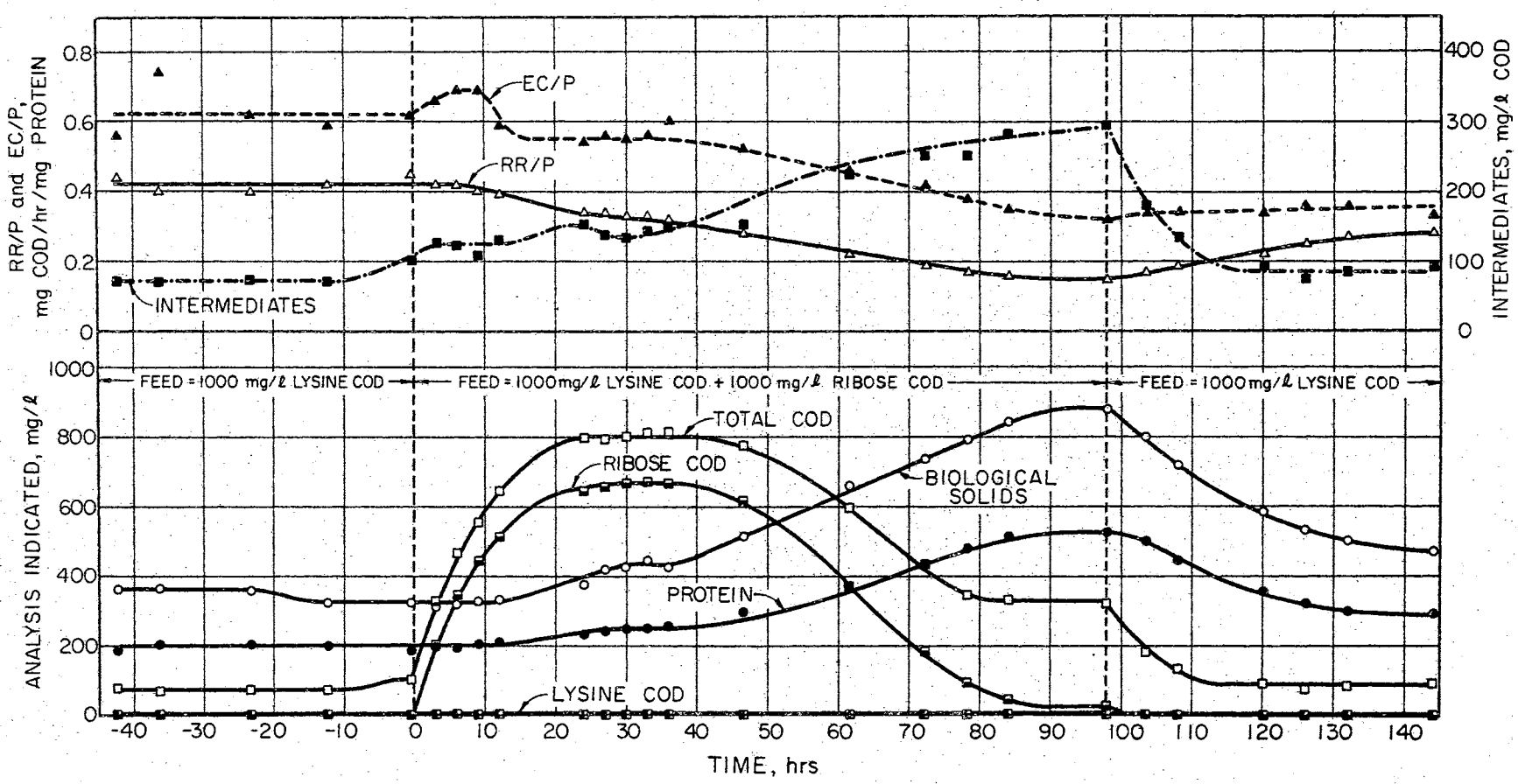
◧ = Lysine COD;

▬ = Ribose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)


RR/P and EC/P,
mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
EC/P
RR/P
INTERMEDIATES
FEED = 1000 mg/ℓ LYSINE COD
FEED = 1000 mg/ℓ LYSINE COD + 1000 mg/ℓ RIBOSE COD
FEED = 1000 mg/ℓ LYSINE COD
ANALYSIS INDICATED, mg/ℓ
TOTAL COD
RIBOSE COD
BIOLOGICAL SOLIDS
PROTEIN
LYSINE COD
TIME, hrs

increased until it was removed from solution, with the production of many intermediates and a further decrease in EC/P. From this it would appear that ribose had exerted a severe effect upon lysine enzymatic capability, but attention must also be given to the response of the system once the ribose was removed since, when the steady state was re-established, the solids and protein concentrations were higher than they had been prior to the shock, implying a change in the culture. Further evidence for a change is given by the enzymatic capability which was considerably smaller after the shock was removed than it was before the shock was applied. It should also be noted that the EC/P value immediately prior to removal of the shock (98 hrs) was 89% of the steady state value after the shock (126 hrs); the EC/P at 36 hours is also 89% of the capability prior to the shock (-0.5 hrs). This, together with the findings from the batch experiments that growth on ribose would shift the population, is taken as evidence that an actual shift did occur. In addition to the above, there were observable morphological changes in the culture after 36 hours, although, unfortunately, no replica plating was performed. Thus, the effect of ribose on the enzymatic capability of the culture for lysine removal is less than at the previous flow rate causing only a slight decrease in EC/P (89% of the pre-shock value).

6. Effects of a Shock Load of 1000 mg/l Ribose (COD) Upon a Magnesium-Limited Reactor (Reactor B)

a. Six-Hour Detention Time (D = 0.167 $hr.^{-1}$). As shown in Figure 30, the steady state with respect to COD and solids was not well established prior to the shock, although EC/P was fairly stable. Upon the introduction of ribose, EC/P for lysine increased slightly causing the lysine to be completely removed from solution. After the small amount of ribose removal started, the intermediates in the system increased, followed by a decrease in EC/P for lysine to a value that was 96% of the pre-shock value. The enzymatic capability increased again just before the shock was removed, but a decrease in the protein concentration occurred at the same time so it is difficult to conclude what caused the effect. In general, the response is consistent with the other data. Only 150 mg/l ribose were removed.

b. Twelve-Hour Detention Time (D = 0.083 $hr.^{-1}$). The results at this flow rate, shown in Figure 31, agree quite well with the results at the previous flow rate. After the introduction of ribose into the reactor, the EC/P value rose, but then as ribose utilization began the enzymatic capability decreased to 85% of the pre-shock level, allowing an increase in the lysine concentration in the effluent. After 61 hours, there was another increase in EC/P and a concurrent decrease in lysine concentration.

Figure 30. Effect of a Ribose Shock Load on a Magnesium-Limited Reactor (Reactor B) Growing on Lysine at a Six-Hour Detention Time (D = 0.167 $hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

◧ = Lysine COD;

◨ = Ribose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein, (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)

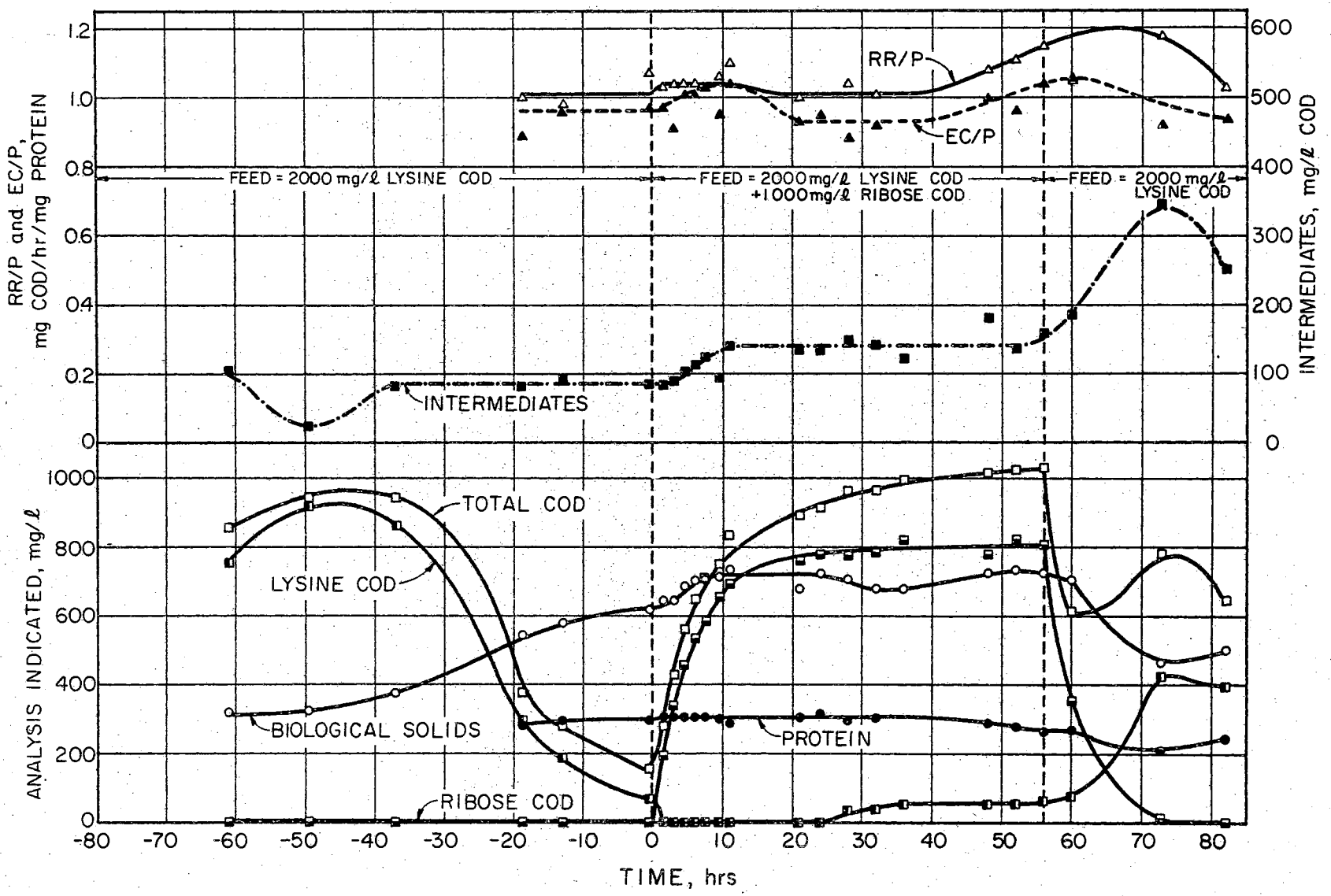

RR/P and EC/P,
mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
ANALYSIS INDICATED, mg/ℓ
TIME, hrs
RR/P
EC/P
FEED = 2000 mg/ℓ LYSINE COD
FEED = 2000 mg/ℓ LYSINE COD
+1000 mg/ℓ RIBOSE COD
FEED = 2000 mg/ℓ
LYSINE COD
INTERMEDIATES
TOTAL COD
LYSINE COD
BIOLOGICAL SOLIDS
PROTEIN
RIBOSE COD

Figure 31. Effect of a Ribose Shock Load on a Magnesium-Limited Reactor (Reactor B) Growing on Lysine at a Twelve Hour Detention Time (D = 0.083 $hr.^{-1}$)

○ = Biological solids concentration;

● = Protein concentration;

□ = Total COD;

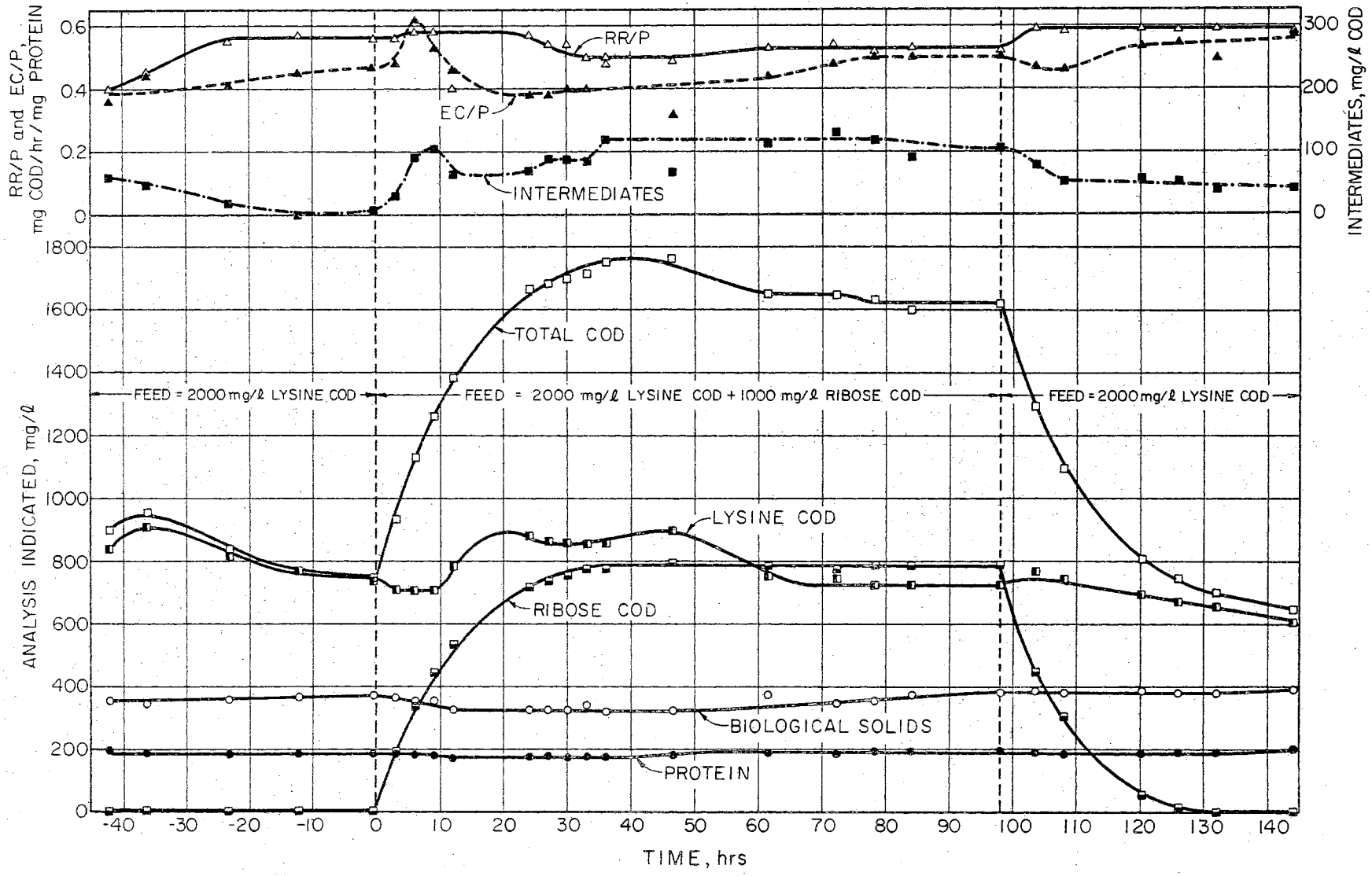
◧ = Lysine COD;

◨ = Ribose COD;

■ = Metabolic intermediate COD;

△ = Lysine removal rate per unit of protein (RR/P);

▲ = Lysine enzymatic capability per unit of protein (EC/P)


RR/P and EC/P, mg COD/hr/mg PROTEIN
INTERMEDIATES, mg/ℓ COD
ANALYSIS INDICATED, mg/ℓ
TIME, hrs
RR/P
EC/P
INTERMEDIATES
TOTAL COD
LYSINE COD
RIBOSE COD
BIOLOGICAL SOLIDS
PROTEIN
FEED = 2000 mg/ℓ LYSINE COD
FEED = 2000 mg/ℓ LYSINE COD + 1000 mg/ℓ RIBOSE COD
FEED = 2000 mg/ℓ LYSINE COD

This new EC/P value was higher than the pre-shock value, but was only 88% of the value attained after the ribose shock was removed. Since natural populations reach only "dynamic" steady states, it is possible that this was a "normal" change, or alternatively it could have been a population shift caused by the ribose. The latter does not seem as likely in this case as it did in the carbon-limited system because no morphological changes were evident in the culture, and there were no appreciable differences between the protein and solids concentrations before and after the shock.

7. Effect of Flow Rate on the Level of Repression in Continuous Flow Reactors

As pointed out in Chapter III, Boddy et al. (65) reported greater repression of amidase synthesis in carbon-limited continuous flow reactors at higher flow rates. They postulated that the greater production of metabolic intermediates at the higher flow rates caused the greater repression. Conversely, in nitrogen-limited chemostats Mandelstam (36) showed that at lower flow rates the repression of β-galactosidase was greater. Thus, it appears that the type of limitation placed on a continuous flow reactor governs its response to shock loads at different flow rates. In Table VI, the average degrees of repression by the various compounds at the different flow rates under the two growth conditions are compared. This table

TABLE VI

EFFECT OF FLOW RATE ON THE LEVEL OF REPRESSION IN CONTINUOUS FLOW REACTORS

Shock	D	Pre-shock EC/P	Average during shock EC/P	% of Pre-shock
Carbon-limited reactor				
Glucose	0.167	0.85	0.30	35
Glucose	0.083	0.67	0.38	57
Fructose	0.167	0.85	0.53	62
Fructose	0.083	0.62	0.39	62*
Ribose	0.167	1.21	1.00	83
Ribose	0.083	0.62	0.55	89*
Magnesium-limited reactor				
Glucose	0.167	0.97	0.45	46
Glucose	0.083	0.60	0.15	25
Fructose	0.167	0.85	0.49	58
Fructose	0.083	0.72	0.40	56
Ribose	0.167	0.96	0.92	96
Ribose	0.083	0.47	0.40	85*

*See text

shows that for glucose, which is rapidly degradable, the data agree with the findings reported above. For fructose and ribose, which were more difficult to degrade and showed less repression under batch growth conditions, the difference in flow rates made little difference in the degree of repression, although the trends were the same as with glucose.

Based on the general concepts of metabolite repression presented in Chapter II, and on the postulations of Boddy, et al. (65), the following rationale appears to be logical. The organisms in a carbon-limited reactor can respond to an increase in influent COD by increasing their growth rate (up to μ_m) during the transient state. The faster the flow rate, the smaller is the difference between the rate of supply of the excess carbon source and the maximum possible growth rate, thus the greater the concentration of the metabolic pools. Conversely, the slower the flow rate, the lower is the level of the metabolic pools since the growth rate is unrestricted. At the new steady state, there will be a relationship between the flow rate and concentration of the metabolic pools, with the lower flow rate allowing less internal metabolites since the system is carbon-limited. Thus, if the pools contain the metabolite repressor, the slower the flow rate, the lower the level of the repressor and the less the severity of the repression. In a biosynthetically restricted medium (e.g., magnesium-limited), the system cannot respond to an

increase in influent COD by increasing the growth rate because the rate limiting substance has not changed. In this system, the lower the flow rate, the lower is the rate of biosynthesis; thus, if an easily degraded carbon source is added, the lower the flow rate, the greater the disparity between the rates of catabolism and anabolism and the higher the level of metabolic pools, leading to more severe repression.

8. Relationship Between the Type of Growth Limitation and the Control of Lysine-Degrading Enzyme Synthesis at Steady State

Although not of primary importance to the objectives of this work, the following comments are presented in conjunction with the data obtained with the continuous flow reactors. In a carbon-limited reactor growing on a single substrate, the steady state substrate concentration is low, and, therefore, the rates of activity of enzymes acting on that substrate are probably not maximal, but are governed by the substrate concentration. Most of the metabolic control would be exercised at the level of enzyme activity so that the actual amount of enzyme present in the cells should be greater than that expressed by the substrate removal rate, and EC/P should be greater than RR/P. However, in a biosynthetically restricted reactor (i.e., one not limited by carbon source), such as the magnesium-limited one, the substrate is in excess, and,

thus, according to Michaelis-Menten kinetics, all enzymes acting directly on the substrate would be operating near maximal rate. In such a case, the level of enzyme should be approximately equal to that expressed by the actual removal rate and EC/P should be approximately equal to RR/P. Table VII shows a comparison of the EC/P and RR/P values for the various reactors. In the carbon-limited reactor, EC/P was significantly greater than RR/P in four of the six experiments, while in the magnesium-limited reactor this was true in only one case (Figure 27). Therefore, enzyme synthesis in the magnesium-limited system appears to be under more strict control than it is in the carbon-limited system, which agrees with the theory of metabolite repression since the internal level of intermediates in the former should be greater than that in the latter. This is also in accord with the assertion by Neidhardt (85) that the metabolism of any carbon and energy source which is degraded by repressible enzymes must be responsive to the over-all biosynthetic rate of the cell.

TABLE VII

COMPARISONS OF ENZYMATIC CAPABILITY AND ACTUAL REMOVAL RATES BEFORE THE SHOCKS WERE APPLIED

Figure No.	EC/P	RR/P
Carbon-limited reactor		
20	0.85	1.06
21	0.67	0.51
24	0.85	0.85
25	0.62	0.43
28	1.21	0.75
29	0.62	0.42
Magnesium-limited reactor		
22	0.97	0.90
23	0.60	0.61
26	0.85	0.91
27	0.72	0.59
30	0.96	1.01
31	0.47	0.56

CHAPTER VI

DISCUSSION

Any basic engineering study, such as the continuing investigation of metabolic control mechanisms in the Bio-environmental Engineering Laboratories at Oklahoma State University, must be founded on principles obtained in simple systems; thus, the over-all protocol included combinations of all classes of compounds. Amino acids serve as the basic components of protein, and as such are present in all cells. Because of their biosynthetic importance it would seem likely that cells have developed mechanisms to conserve them and prevent their degradation unnecessarily; therefore, a study of the control mechanisms involved in amino acid degradation, and the ubiquity of those controls, is of significance for both basic and applied science.

Although amino acids may not form a large portion of the organic matter in waste water, they are present. Kahn and Wayman (86), for example, identified 13 different free amino acids in raw domestic sewage with a total concentration of 115 mg/l. Amino acids are readily removed from sewage but the significance of the control mechanisms involved in their utilization may increase if the wet

oxidation (Zimmerman) process of sludge disposal becomes more widespread (87). Teletzke et al. (88) have shown that the oxidation process hydrolyzes polypeptides, releasing free amino acids in the filtrate. Prior to oxidation, a slurry contained about 42 mg/l (as N) of free amino acids, but afterwards the filtrate contained 1400 mg/l (as N). Certain types of wastes, such as packing house wastes, are high in protein and, thus, could contribute high concentrations of amino acids to treatment plants. Therefore, although this study was performed on amino acids because of the importance of the basic information available, it has practical ramifications as well.

A. Preliminary Experiments

As would be anticipated by some of the literature cited in Chapter II, the removal of several of the amino acids was slowed by the presence of glucose in the medium. When Jacoby (89) studied the effect of glucose on the adaptation of Pseudomonas fluorescens to amino acids, he found that the carbohydrate adversely affected induction to many of them, including all of those screened in these preliminary experiments, except cysteine and threonine, upon which his organisms would not grow at all. In the natural microbial population used in these studies, however, glucose significantly retarded the removal of only five of the amino acids. The difference probably lies in the fact that these preliminary experiments were

designed to measure an effect upon amino acid degradation by cells already acclimated, while Jacoby was studying the effect upon acclimation. There was one other result in the preliminary experiments that was contrary to published literature and that was the failure of glucose to affect histidine utilization as had been reported by Magasanik (35). When the cells were acclimated to histidine, they lost the capacity to use glucose rapidly and this was probably the cause of the discrepancy. However, Cowen (90) found that glucose had no effect upon utilization of histidine by Pseudomonas aeruginosa strain PA-1.

As pointed out in the results section, lysine was chosen on the basis of the preliminary experiments. One of the assertions of the theory of metabolite repression is that the repressing compound and the repressed compound share a common metabolic intermediate (35). The pathway of lysine degradation in bacteria is not certain, but recent work indicates that lysine first undergoes oxidative decarboxylation to δ-aminovaleramide which is deamidated to yield δ-aminovaleric acid (91). The latter compound undergoes transamination to give glutaric semialdehyde (92), which in turn yields glutaric acid. Glutaric acid is then converted to acetyl-CoA via glutaryl-CoA (93) (94). If indeed glucose and lysine do share a common intermediate, the first would be acetyl-CoA. No samples were taken for the identification of intermediates, in either the preliminary or the lysine

experiments, but if acetyl-CoA were involved with the control exerted by glucose, these results might be correlated with the findings of Dobrogosz on the involvement of energy relationships and pyruvate metabolism in metabolite repression (47) (48) (49).

B. Batch Experiments

Even though the bacterial population employed in these experiments was selected for its ability to use lysine as a carbon source, the lysine degradative system was subject to metabolic control. The culture was started from sewage, and underwent natural selection, limited only by the abilities of the species to use lysine. Although the changes that occurred in the population during the latter stages of the study interfered with the quantitative interpretation of the results, the qualitative findings were consistent with the earlier experiments with regard to the current theories of metabolite repression. The validity of comparisons between experiments on fructose and ribose and those on glucose is substantiated by the fact that the glucose experiment shown in Figure 13 was performed on day 191 with seed removed from a slant and grown up for use in much the same manner as for Expt. 49 on fructose.

The results have shown that glucose repressed the formation of the inducible lysine degradative system, in an <u>en masse</u> response, and that the metabolic intermediates

of glucose degradation also exerted the effect. Pre-induction offered a small degree of protection against the repression, but removal of ammonia nitrogen from the system did not overcome it. Inhibition of activity of pre-formed enzymes played only a minor role in the control of the system. The response of the system to the different carbohydrates was a function of the growth rates on, and production of intermediates from, those carbohydrates. Glucose supported relatively rapid growth (μ_m = 0.45 hr^{-1}) with the production of many intermediates and, consequently, it had a rather severe effect upon the production of lysine degrading enzymes. Fructose, on the other hand, allowed the cells to grow with a μ_m of only 0.17 hr^{-1} with the excretion of few intermediates and its effect was rather mild, with $A_{mixture}/A_{control}$ being about 0.84. Inhibition played no part in its effect, which was due entirely to a reduction in the differential rate of synthesis. Ribose supported growth very slowly, with a μ_m of 0.04 hr^{-1}. Part of the population apparently could use ribose only poorly or not at all, and the major utilization in the culture was apparently for macromolecule synthesis, thus increasing the efficiency of the cells and allowing a faster rate of synthesis of lysine degrading enzymes. These findings agree with the basic concepts of metabolite repression which state that the severity of the effect is related to the rate at which a substrate is utilized (35). It has been shown in basic studies on

this phenomenon that glucose is dissimilated much more rapidly than the synthetic machinery of the cells can use the intermediates produced, and, thus, glucose produces a severe effect.

Monod (34) stated that the difference between constitutive and inducible enzymes should be considered a quantitative rather than a qualitative one. Most inducible enzyme systems show a slight, but significant, activity in uninduced cells which is enhanced greatly by the presence of the substrate. This was the case for the lysine degrading enzyme system. Jacoby (89) also observed inducibility while studying the oxidizing capability of Pseudomonas fluorescens for lysine and several other amino acids.

The major evidence for a repression effect in the work reported here has come from a whole cell assay, referred to as the enzymatic capability test. Gale (79) pointed out several years ago that whole cell assays are limited by: 1) the activities of other enzymes within the cell, 2) permeability of the cell membrane, and 3) differences between the external and internal environment. However, the task of the environmental or biological engineer, and other scientists concerned with the response of natural or mixed microbial populations to changes in the chemical (or physical) environment (or indeed the effects which the microbial population can exert on the chemical or physical environment), is one which often requires

dealing with whole cells. Although the above limitations of whole cell assays are quite valid and should be considered in evaluation of results obtained using whole cells (and perhaps especially with mixtures of species), it is important that they not impede the progress of research dedicated to assessing for natural microbial populations the utility and real effects of the basic findings delineated in "unnatural" systems. Mandelstam (95) reported that there appears to be relatively free passage of lysine into gram negative bacteria, while Taylor (96) stated that gram negative bacteria do not concentrate lysine greatly within the cell. Thus, it is possible that for the naturally selected population employed in the present research, which was predominantly gram negative, the last two limitations are minor compared to the first.

There are three primary assertions which are basic to the hypothesis of metabolite repression (35) (37) (97). The first is that the formation of a catabolic enzyme is controlled by the intracellular level of some particular metabolite which is an intermediate or an ultimate product of the action of the enzyme. The second is that glucose inhibits the formation of many inducible enzymes because it gives rise to those metabolites. The third is that the dissimilation of glucose occurs faster than the synthetic capacities of the cell can utilize the metabolities formed, leading to high intracellular levels of intermediary metabolites. In the experiments presented here, it was

observed that high levels of metabolic intermediates were excreted into the medium by cells growing on glucose. The importance of these intermediates in metabolic repression was attested to by the fact that an increase in the rate of formation of enzymatic capability did not occur until the level of these intermediates in the medium had been reduced.

In studies on the β-galactosidase system in E. coli, Nakada and Magasanik (39) showed that a product of glucose metabolism, not glucose, was responsible for the effect of glucose upon the system. They stated that the concentration of metabolites can determine the rate of m-RNA synthesis. Since the rate of decay of m-RNA is independent of the level of inducer or metabolite repressor, the equilibrium amount of m-RNA depends only on its rate of synthesis. They also said that the differential rate of synthesis of β-galactosidase is determined by the proportion of the m-RNA specific for β-galactosidase in the total cellular m-RNA. Thus, the rate of β-galactosidase synthesis is governed by the level of metabolites within the system. Additional evidence for this hypothesis has been added by the discovery of the CR gene, which is similar to the regulator gene, except that it appears to be involved with the expression of metabolite repression (43).

In the lac system, Loomis and Magasanik (44) found that pre-induction gave partial protection against metabolite repression by glucose. They attributed the

partial protection to an increased internal level of lactose in the pre-induced cells due to increased permease activity. Since there appears to be relatively free passage of lysine into gram-negative bacteria, the small degree of protection offered by pre-induction in the present study is probably not due to a permease effect (95). However, it does seem possible that the greater repression which was exhibited by cultures pre-grown on glucose could be due to an enhanced ability for glucose utilization. There was a lag in the glucose control reactor when the seed culture was grown on lysine alone, but there was none when the seed culture was grown on a mixture of glucose and lysine, or on glucose alone. Some inducible enzymes are known to be involved in glucose degradation (35) (80).

The lysine system did not respond to the removal of ammonia nitrogen in the same manner as did the histidine system reported by Neidhardt and Magasanik (81). The difference can probably be explained by the normal extent of metabolite repression in the two systems. The histidine system was very severely repressed by glucose, with a complete shut-off of histidase synthesis. But when the cells were placed in a system with no ammonia nitrogen and had to use histidine as a nitrogen source, they produced histidase at forty per cent of the unrepressed level. On the other hand, in the system studied here, there was not a complete shut-off of synthesis of lysine degrading enzymatic capability in the presence of glucose. If this

normally repressed level could meet the nitrogen requirements of the cells in the absence of ammonia, then there would be nothing to cause a further derepression. Apparently this is what happened.

In reports published by Gaudy and his coworkers (15) (16), evidence was presented for the inhibition of activity of sorbitol degrading enzymes by glucose since substrate removal was sequential under non-proliferating conditions in which glucose could only affect pre-formed enzyme activity. Stumm-Zollinger (61) found evidence for the inhibition of activity of the galactose utilizing enzymes in a heterogeneous culture. Recently Zwaig and Lin (32) and Tsay (33) observed catabolic inhibition in pure cultures. Tsay discussed two possible explanations for the rapid inhibition observed in her studies. One was feedback inhibition due to an accumulated intermediate and the other was competition for a common permease.

In the study presented herein, little evidence of inhibition of lysine degrading enzymes was found with any of the carbohydrates. As pointed out previously, no permease appears to be involved in lysine transport so the lack of an inhibition mechanism was probably due to the absence of a feedback inhibition mechanism for lysine degradation. It is possible that this is a general characteristic of amino acid degradative systems since amino acids often must serve as nitrogen sources for the cell. Tsay (33) found only a slight inhibition of histidine

utilization by glucose. If feedback inhibition prevented the breakdown of the carbon skeleton of the amino acid after the nitrogen was removed, there would be a further accumulation of metabolic intermediates, perhaps leading to a greater inhibition. It would appear to be to the advantage of the cell not to inhibit the activity of amino acid degradative enzymes, because when a carbohydrate (or some other easily degradable compound) is placed into the medium, the continued activity of those enzymes could aid in the use of the amino acid as a supplemental nitrogen source by removing the "waste" carbon skeletons. This explanation does not preclude the continuing function of the repression mechanism since it would reduce the total level of enzymes to that required by the cell and prevent the unnecessary degradation of the amino acids.

The comparative results with the different carbohydrates presented here for a natural population agree with the observations of investigators in the basic field. For example, in his early work on diauxie, Monod (34) found that for Bacillus subtilis both glucose and fructose were "A" compounds; that is, that they did not produce diauxic growth when used together, but when either was used with a compound of the "B" type, the "A" compound was used first, causing diauxie. However, in E. coli, H glucose was a typical "A" compound but fructose was "A" only in the sense that it did not give diauxie when mixed with glucose.

Fructose also did not give diauxie when mixed with "B" compounds.

In an investigation which led to his work on metabolite repression, Magasanik (98) studied the growth of a mutant of Aerobacter aerogenes unable to produce histidine. When the cells were placed in media containing 20 γ/ml of histidine plus a carbon source it was found that the carbon sources fell into two groups, those that allowed a high percentage of the growth obtained under unrestricted conditions (i.e., with unlimited histidine), and those that allowed only a small amount of growth. The first group supported growth at a rapid rate in the parent strain and prevented the synthesis of histidase much more severely than did the second group, so that when the mutant was placed with compounds of the first group, histidine was used only for protein synthesis (instead of being destroyed by histidase) and more growth occurred. Glucose was in the first group and allowed growth equal to 109% of unrestricted growth, but fructose and ribose were in the second group allowing only 20% and 4% growth, respectively. Magasanik noted that it appeared significant that the compounds supporting the most rapid growth also were the most potent inhibitors of histidase synthesis. This was later to be confirmed when he and Neidhardt (99) showed that glucose and several other compounds repressed the synthesis of histidase and that

the effect of each compound was related to the rate at which it supported growth.

Mandelstam (36) studied the production of β-galactosidase by a culture of E. coli that had a mutation in the regulator gene making it constitutive for the enzyme. Glucose, fructose, and several other compounds still exerted metabolite repression upon the synthesis of the enzyme and the slower the rate at which the carbohydrate supported growth, the less severe the repression. On glucose, the doubling time was 48 minutes and only 200 units of enzyme/mg of cells were formed, while on fructose the doubling time was 67 minutes and the enzyme production was 613 units/mg. Cohn and Horibata (100) found that glucose had a severe effect upon β-galactosidase synthesis, but that fructose and ribose had only minor effects when the inducer and carbohydrate were added at the same time to uninduced cultures.

In studies on the induced formation of tryptophanase in E. coli, Raunio (101) found that various compounds affect the production of indole (used as a measure of tryptophanase). Glucose allowed synthesis of 2% of the unrepressed amount, while fructose allowed 29% and ribose 59%.

Kirkland and Durham (102) reported that ribose was not oxidized and did not support growth in P. fluorescens, but it did shorten the lag period required for the synthesis of protocatechuate oxygenase. The ribose appeared

to be used as a "specific" carbon source for the synthesis of RNA.

In general, it could be concluded from the above pure culture studies that fructose would exert a lesser effect than glucose because it supports growth at a slower rate, and that ribose would exert an even smaller effect. The results reported here for fructose agree quite well with conclusions drawn from pure culture studies. It is possible that, if a culture had been obtained which could grow on ribose, the pentose would have shown a minor effect upon the lysine degrading enzyme synthesis of the system. However, replica plating indicated that if that were done, the response would no longer be en masse, so this possibility was not pursued.

In these studies, if only substrate removal curves or growth curves had been considered, it might have been erroneously concluded that the carbohydrates exerted no effect upon the lysine degrading system because classic "diauxie" or sequential substrate removal was not exhibited. Concurrent removal alone is not sufficient evidence against repression, since repression can be merely a decrease in the rate of synthesis of an enzyme (or system) in relation to the rate of formation of other proteins. Only by considering the change in enzyme content or removal rate (the latter case requires additional evidence that the effect is not due to inhibition) in relation to the change in concentration of cells in the reactor can

the existence of metabolite repression be established in a growing system. As pointed out in Chapter III, it is not sufficient to consider the rate of bacterial growth as an approximate measure of the rate of enzyme formation as proposed by Stumm-Zollinger (61). Researchers in the applied area must consider the rate of synthesis in relation to the rate of synthesis of total protein just as the biochemists must. As Melvin Cohn (78) said in 1957:

> The course of the synthesis of a cellular constituent as a function of time is difficult to interpret in terms of the action of specific factors because it depends simultaneously on the nonspecific metabolic factors. As a first approximation, these metabolic factors are eliminated when, instead of considering time-rate of synthesis (dE/dt) of a given enzyme protein (E), we consider the rate of synthesis relative to the total rate of protein synthesis (dE/dx) where x represents the total mass of protein. We have simply substituted 'physiological time' (dx) for 'absolute time' (dt).

Comments are in order concerning the applicability of studies with restricted populations to generalizations about the field of industrial waste water treatment, since it may be held by some workers in this field that the results of investigations in which the mixed or heterogeneous populations were subjected to specific selective pressures (e.g., selection from a sewage seed of species which could grow on lysine) are not usefully applicable to the solution of waste water treatment problems or to the understanding of such systems. To be sure, the synthetic medium used in these studies did restrict the number of species present. Indeed, the object was to study

repression effects for those organisms best suited to the use of the particular substrate. Also, the method of maintaining the seed culture was chosen to keep the population "young", with a high metabolic activity toward lysine by always keeping it under growth conditions. Although the substrate, being a single amino acid, did restrict the population, it is certainly no more restrictive than the many highly complex organic compounds found in industrial waste streams. It is to this end that the work was directed: a better understanding of the basic control mechanisms operative in a population which was by natural selection best suited for the degradation of a particular compound.

While discussing the merits of the use of restricted versus truly heterogeneous populations, it should be pointed out that the latter can lead to false conclusions in the study of control mechanisms. Consider an unselected population in which 90% of the cells can use glucose, but not lysine, for growth and 10% can use lysine and in which glucose has no affect on lysine utilization. If the enzymatic capability were measured for the removal of lysine in a system with both substrates and in one with only lysine, the plot of enzymatic capability versus solids for the mixed unit would indicate a much lower rate of synthesis of lysine degrading enzymes with respect to cell mass than that in the lysine control because 90% of the cells in the mixed unit would have been formed from

glucose and would have no lysine degrading enzymes. Thus, the result could indicate metabolite repression even though it was a simple case of two populations developing independently. Therefore, it is important to ensure that the response measured is an en masse response, and is not due to a shift in the population. For the results reported here, replica plating was used to ensure that the response measured was not due to a shift in the population during the course of an experiment, but was due to an en masse response.

C. Continuous Flow Experiments

1. Carbon-Limited Reactors

The results demonstrated that when a carbon-limited chemostat growing on lysine was subjected to a shock load of either glucose or fructose a significant level of metabolite repression occurred, resulting in leakage of lysine into the effluent during the transient period. The severity and duration of the response was a function of the flow rate. The addition of ribose to the system caused an increase in enzymatic capability until ribose utilization started, at which time a slight repression occurred. There appeared to be a shift in the population associated with ribose utilization at the slower flow rate but the degree of repression after the shift was the same as that before. All of these findings are in agreement with the results of the batch experiments. The occurrence

of metabolite repression in a continuous flow reactor is particularly significant to the waste water treatment field because continuous flow conditions bear more resemblance to actual waste treatment systems than do the batch conditions under which most repression experiments have been performed. The agreement of the results obtained with the two types of growth conditions is also evidence for the ubiquity of control mechanisms, since the selective pressures exerted by the two environments are different. Batch (or enrichment) cultures select for organisms tolerant of high concentrations of nutrients, while continuous cultures (growing in "externally" controlled chemostats) select for organisms having a high affinity for the limiting substrate (i.e., ones which are good scavengers) (82).

Previous studies in these laboratories demonstrated the disruption of both sorbitol and glycerol removal by the introduction of glucose into the feed of continuous flow reactors (64). In a natural population growing on sorbitol (1500 mg/l) at a four-hour detention time, the addition of 1500 mg/l of glucose caused the sorbitol in the effluent to increase from an undetectable amount to 200 mg/l in two hours. The total COD rose to 600 mg/l but the glucose in the effluent only reached 75 mg/l, indicating the production of many metabolic intermediates. When glucose was added to a similar reactor growing on 500 mg/l glycerol, there was an initial rapid build-up of

glucose, but after glucose utilization started, metabolic intermediates were produced and the glycerol concentration in the effluent rose to 225 mg/l. In the experiments presented in this report, the production of exogenous intermediates was also observed.

When Komolrit's studies on sorbitol removal were extended to other detention times, it was found that the severity of the response to a glucose shock load was a function of the detention time (103). When the feed to a reactor growing at a two-hour detention time (near the dilute out point) was changed from 1000 mg/l sorbitol to 1000 mg/l sorbitol plus 1000 mg/l glucose, the levels of both sorbitol and glucose in the effluent increased. The glucose was reduced after about eight hours, but the sorbitol level remained at over 1000 mg/l for almost twelve hours after the start of the shock. Evidently, at the early stage of the shock loading, the sorbitol metabolism was totally replaced by glucose metabolism, and did not start again until the glucose concentration had been reduced to a very low level. When a similar shock was applied to a reactor with a four-hour detention time, there was a slight increase in effluent COD, but no detectable build-up of glucose or sorbitol. When a sixteen-hour detention time was employed, the system was capable of receiving more severe shock loads without disrupting the metabolism of sorbitol.

In the continuous flow, pure culture experiments

discussed in Chapter III, repression was greater at higher growth rates, probably due to higher levels of metabolic pools. At low flow rates, the metabolites are probably used and the internal carbon pools depleted, but at the higher rates, Boddy et al. (65) have postulated that the pools are probably less depleted causing a shift in favor of repression. This relationship between the level of repression and the growth rate was observed in the experiments with the carbon-limited reactor reported herein. Since glucose was the strongest repressor-producing carbohydrate studied, the effect was much more severe with it. These results also add evidence for the importance of metabolic pools, since the level of metabolic intermediates in the effluent rose rapidly at the faster growth rate when glucose utilization started. Boddy et al. (65) noted that the metabolite repression of amidase by succinate appeared to be partially overcome after a period of time, and attributed the severe repression during the transient state to an imbalance of metabolites in the system. When the influent was changed from succinate to succinate plus acetamide, there was an increase in growth rate resulting in a high level of catabolite repressor and a low rate of amidase synthesis, but as the growth rate returned to its original level, there was probably a corresponding reduction in the concentration of intermediates, and, thus, a decrease in repression. In discussing the fact that the period between steady states is one in which an adjustment

in the concentration of metabolites occurs, they point out that metabolic rearrangements have been implicated in the transient repression of β-galactosidase synthesis in E. coli. This seems to imply that the effect observed is related to transient repression. Transient repression has been postulated to be linked with the rapid production of phosphorylated intermediates of glucose metabolism (53). When glucose was added to the medium, in batch experiments, phosphorylated intermediates were produced, causing a severe repression for two-thirds of a generation, after which the pool size was reduced and synthesis recovered to about 15% of the differential rate prior to the introduction of glucose. In a study of metabolite-promoted heat lability of β-galactosidase, Brewer and Moses (104) showed that the heat lability of the enzyme was increased by several metabolic intermediates. After investigating the effects of the compounds postulated by Prevost and Moses (53) to be involved with transient repression, they concluded that the specificity of the heat-labilizing effect was not great enough to support any conclusions relating it to transient repression. Tyler, Loomis, and Magasanik (55) have recently studied transient repression in the lac system and found it to be distinct from the mechanism of metabolite repression. Their conclusions were based on evidence that showed: 1) that transient repression did not involve the CR gene, 2) that the phosphorylated compounds were not metabolized further, and presumably did

not enter the common metabolite pool involved in metabolite repression, and 3) that transient repression appeared to be effective as soon as the new compound entered the cell, while, in contrast, a period of protein synthesis was required before metabolite repression due to glucose reduced the differential rate of enzyme synthesis. Since transient repression has only been demonstrated in E. coli, and since a lag period was required before the repression of lysine degrading enzymes was exerted, it does not appear likely that transient repression played a part in the results presented here.

In the glucose and fructose shock loads at the six-hour detention time, the initial repression was severe, so that the enzymatic capability of the culture dropped below the eventual steady state value. This overshoot was probably caused by high levels of metabolic intermediates produced during the period when the growth rate was high, and may be related to the phenomenon of growth rate hysteresis observed when a quantitative shock load is placed on a continuous flow reactor (105). Just as the growth rate cannot respond instantaneously to a change in substrate concentration, it is possible that a finite time period is required for the rate of enzyme synthesis to respond to changes in the level of internal intermediates. This overshoot was not observed at the lower flow rate.

2. Magnesium-Limited Reactors

Neidhardt (97) in discussing the assertions of the "feedback" hypothesis on the glucose effect pointed out that the theory predicts that a situation which slows down the anabolic processes without affecting the catabolic processes would allow carbon sources that do not normally repress to do so. He then cited evidence that the prediction had indeed been borne out (106). Mandelstam (36) also demonstrated that at slow growth rates in a nitrogen-limited chemostat, poorer carbon sources could exert a repression upon the synthesis of β-galactosidase. In the experiments presented here, magnesium-limitation allowed only slightly more repression with fructose and ribose than had been found under carbon-limited conditions. The reason that no more repression was exerted is probably related to the flow rates used. Neither fructose nor ribose was used very rapidly in batch, and neither was removed completely under the magnesium-limited conditions in continuous flow, although glucose was. If even slower growth rates had been used, then fructose and ribose possibly would have caused greater repression than they did.

In the magnesium-limited system, the theory of metabolite repression would also predict that the slower the growth rate the greater the repression because the greater would be the disparity between anabolism and catabolism.

This was found to be true, especially when glucose was the repressing compound.

The response of the magnesium-limited reactor to the glucose shock load was very similar to the response observed by Komolrit and Gaudy (64) when they shocked a sorbitol reactor (BOD:N = 20:1) with glucose so that the final BOD:N ratio was 40:1. This is presented as evidence for the argument in Chapter III that their system was probably growing as a nitrogen-limited reactor before the shock and, thus, reacted as a biosynthetically restricted reactor to the shock. In their reactor and in the ones presented here, the shocking compound replaced part of the original substrate as energy source for the cells. In the case of glucose, which was easily degraded, all of the glucose was used, with the displacement of an equal portion of lysine. Fructose and ribose were more difficult to degrade, so that they were not entirely removed, but the portion removed displaced an equal portion of the lysine. Although it would take much more study to prove the point, a system such as this might serve as a measure of the relative ease of degradation of compounds, and as a measure of the interactions involved.

Concerning the magnesium-limited reactor, there is one other item which should be mentioned, although only briefly, because the evidence is not sufficient to warrant further discussion. Neidhardt (85) stated that the basic premise of the theory of metabolite repression has the

further implication that the metabolism of any carbon and energy source which is degraded by repressible enzymes must be responsive to the over-all biosynthetic rate of the cell. If a system that is biosynthetically restricted has its growth rate lowered, continued rapid substrate degradation would lead to high intracellular levels of repressing metabolites; thus, an adjustment in metabolism must occur to lower the level of the metabolites. This adjustment could be brought about by: 1) a reduction in the rate of substrate degradation caused by a decreased enzyme level (for the particular compound), or 2) the expansion of metabolic routes to prevent the accumulation of excess metabolites. The first would lead to a tight quantitative coupling between specific substrate removal rate (RR/P) and growth rate, while the second would lead to the accumulation of nonrepressing by-products in the culture. If the values of RR/P (prior to shocks) from Figures 22, 26, and 30 (given in Table VII) are averaged, the value is 0.94 for D = 0.167 hr^{-1}. If the other three values for the magnesium-limited reactor (D = 0.083 hr^{-1}) are averaged, their value is 0.59. For 100% coupling between growth rate and substrate utilization, the RR/P value at zero growth rate would be zero and the slope of the RR/P versus growth rate (μ) line that passes through $\mu = 0$ and $\mu = \mu_m$ would represent 100% coupling. If the per cent coupling is estimated by dividing the coupling actually measured (i.e., the slope of the actual RR/P

versus μ line by the slope required for 100% coupling, the value found is 75%. Since Table VII does show an almost 1:1 relationship between EC/P and RR/P (i.e., all enzyme present is active at maximal rate) part of the control of catabolism is exerted by a lowered level of enzyme, while the rest of the control is by the second method, in this case by the production of the extracellular capsule that was evident in the magnesium-limited reactor at both flow rates, but was found in the carbon-limited reactor at only the lower one.

One would expect all enzyme present in a biosynthetically-restricted culture to be active at maximal rate (due to the high level of substrate) and, thus, any control exerted would have to be by repression. If the culture were carbon-limited, the level of metabolites would be lower (under non-shock conditions) and the enzyme level could be higher than the amount expressed by the actual substrate removal rate. Nevertheless, there was a change in EC/P with flow rate in the carbon-limited system also, indicating some coupling. Coupling of anabolism and catabolism is extremely important to the bioengineer, especially in carbon-limited systems, and is an area of great interest to the author, who intends to pursue it further.

CHAPTER VII

CONCLUSIONS

A. Batch Experiments

1. Metabolic control mechanisms were operative upon the enzyme system responsible for the selection of the species present in a natural microbial population. A change in the population occurred but did not affect the mechanisms. Since several different types of colonies were readily observed on agar plates made from the population, the mechanisms were probably not limited to a single species. This emphasizes the importance of control mechanisms in natural microbial systems.

2. The lysine degrading enzyme system of the population was inducible.

3. Glucose caused a decrease in the differential rate of activity of lysine degrading enzymes with respect to cell mass. Fructose caused a small decrease but ribose caused a slight increase. The responses were *en masse* and were not due to shifts in the population during the course of individual experiments.

4. Inhibition of pre-formed enzyme activity played only a minor role in the response to glucose, and had no effect on the responses to fructose or ribose.

5. The effects of glucose and fructose were due to metabolite repression, causing a decrease in the rate of formation of lysine degrading enzymatic capability. This effect was also manifested by excreted metabolic products of glucose degradation and was not relieved until the concentration of these products was reduced.

6. Pre-induction offered only a small degree of protection against repression.

7. The normal degree of derepression could meet the nitrogen requirements of the culture and, thus, removal of ammonia nitrogen from the system did not allow further derepression.

B. Continuous Flow Experiments

1. In both carbon- and magnesium-limited reactors, glucose and fructose caused a significant degree of repression of the synthesis of lysine degrading enzymes, resulting in a decrease in the enzymatic capability of the cells.

2. When ribose was initially placed into either type of reactor, it caused an increase in enzymatic capability, just as in the batch experiments. Once ribose degradation began, there was a slight repression.

3. At the higher flow rate under carbon-limited conditions, the decrease in enzymatic capability was more rapid than the increase in total biological solids so that the total capacity of the system for lysine removal was

decreased, resulting in the escape of lysine into the effluent. At the lower flow rate (and at both flow rates with ribose), the system was able to respond by increasing the biological solids rapidly enough to prevent a major release of lysine. In all of the carbon-limited experiments, the system eventually recovered to the extent that no more leakage of lysine occurred.

4. Under magnesium-limited conditions, the carbohydrates replaced lysine as carbon source and the degree of replacement was related to the ease with which the carbohydrate could serve as a carbon source. Under these conditions, the slower the flow rate, the greater the repression.

CHAPTER VIII

SUGGESTIONS FOR FUTURE WORK

1. The enzymatic capability test should be performed with a pure culture system so that enzyme assays can be done concurrently in order to establish the relationship between the capability and the actual level of enzyme.

2. Continuous flow experiments should be run to determine the relationship between growth rate and actual enzyme level (or enzymatic capability) for both carbon-limited and nutrient-limited systems.

3. Shock load experiments should be run over a broader range of flow rates, and with cell recycle to establish the relationship between growth rate, enzymatic capability, and metabolite repression. Various configurations should be used for the reactors, such as two-stage, etc.

4. Further study should be made of the possible use of a biosynthetically restricted system as a measure of the relative ease of degradation of compounds.

5. Experiments similar to those reported for pure cultures, in which a compound requiring an inducible system is added to a continuous flow reactor degrading a compound

capable of repression should be initiated for systems containing natural populations. This effect on induction should be studied at various flow rates as suggested for the repression system.

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