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Phosphorylation of free galactose by lactic streptococci was mediated by an adenosine triphosphate (ATP) dependent kinase which was repressed by glucose. The phosphoenolypyruvate (PEP) phosphotransferase system (PTS) was not involved in transport of galactose. The conversion of free galactose to glucose was also demonstrated. A key Leloir pathway enzyme, uridine diphosphogalactose-4-epimerase, was found present with at least 0.66 units/mg protein in cells grown in milk or broth containing galactose or lactose. A lower level of the enzyme (0.14 units/mg protein) was found in glucose-grown cells.

A modification of the standard technique for the assay of uridine diphosphogalactose-4-epimerase was introduced. Undialyzed crude extracts of lactic streptococcal cells could be used for the assay but only after incorporation of 0.2 M semicarbazide in the glycine buffer.

In cell dry weight yield studies, it was shown that glucose was the best carbohydrate for growth, and galactose the poorest. It was,

however, very difficult to show differences in growth rates of lactic streptococci using five carbohydrates. Also, in this group of organisms diauxic growth on a mixture of glucose and galactose could not be shown. Efforts were made to determine if lactic streptococci containing β-D-phosphogalactoside galactohydrolase (β-Pgal) could utilize glucose and galactose simultaneously. Results indicated that galactose-induced cells of Streptococcus diacetilactis 18-16 could catabolize both sugars at the same time. In Streptococcus lactis 7962, however, an organism which possesses β-galactosidase (β-gal), growth curves employing both glucose and galactose revealed two distinct slopes with no lag between the time of glucose exhaustion and the time when galactose was utilized.

In differential respirometry studies involving washed cells of S. diacetilactis 18-16, it was shown that less CO_2 (< 170μl/100 mg cell dry wt/hr.) was evolved from galactose by glucose-induced cells, compared to that (916 μl/100 mg cell dry wt/hr.) from glucose. The inability of glucose-induced cells to catabolize galactose and lactose to the same degree as glucose was apparently due to classical catabolite repression. Lactose-induced cells evolved CO_2 equally well from lactose or glucose, but the evolution of CO_2 from galactose and a mixture of glucose and galactose was somewhat repressed. Galactose-induced cells required more oxygen (> 1500 μl/100 mg cell dry wt/hr.) than did glucose or lactose-induced cells (< 850 μl/100 mg cell dry

wt/hr.).

Paper, thin layer, and column chromatographic techniques were employed in a search for galactose-6-phosphate from several wild type lactic streptococci. The compound was never detected nor was it possible to isolate it from a reaction mixture of o-nitrophenyl, β-D, galactopyranoside-6-phosphate (ONPG-6-P) and crude cellular extracts. A search for a mutant which transported and phosphorylated lactose but was defective in cleaving the phosphorylated derivative was undertaken. Of more than 60 lactose negative mutants examined, no such mutant was found. All mutants examined lacked any detectable β-P-gal and also the ability to transport and phosphorylate lactose.

In metabolism studies involving galactose-6-PO_4, it was found that whole cells of lactic streptococci could not utilize the compound as a carbon source. Possible routes for the metabolism of this compound were hypothesized, and experiments were designed to test each route. The only pathway which appeared to function in its catabolism was the pentose pathway. However, it was later found that the galactose-6-PO_4 used in these studies was contaminated with at least 0.2 percent glucose-6-PO_4 and observed activity was believed due to the contaminant.

Involvement of the PTS of transport in sugar utilization was investigated using ^{14}C-labeled substrates and toluene-acetone-treated cells. Substrates and phosphorylated products were separated by

ion-exchange chromatography and quantitated by liquid scintillation counting. The PTS was involved in the utilization of glucose, lactose, and mannose. In controlled experiments it was determined that ATP also acted as a phosphate donor for glucose, lactose and mannose. ATP was the exclusive phosphate donor for galactose. Two transport systems appeared to act for glucose, lactose and mannose.

In a survey conducted on representatives of the four major groups of streptococci, it was shown that all lactic streptococci examined cleaved ONPG-6-P except S. lactis 7962, which cleaved O-nitrophenyl β-D-galactopyranoside (ONPG). This survey also showed that all members of the viridans streptococci examined possessed β-gal and not β-Pgal activity.

Carbohydrate Metabolism in Lactic Streptococci with Special Reference to Galactose

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DEDICATION

This thesis is dedicated to my closest friends.

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INTRODUCTION

The lactic streptococci, as a group, are fastidious bacteria which ferment a variety of carbohydrates and produce L-lactic acid as a major end product. Since this acid possesses inhibitory properties toward many microorganisms, the lactic streptococci have been exploited by man for centuries in the manufacture and preservation of food products. Of special concern are dairy products in which the fermentable raw product is milk with lactose as its principle carbohydrate. Lactose is a disaccharide in which galactose is bonded to glucose through a β-1, 4-glycosidic linkage. The enzyme which cleaves this linkage is called β-galactosidase (β-gal). This enzyme has received much research attention. It has been purified and characterized from many bacteria, including one lactic streptococcus, Streptococcus lactis 7962 (Citti et al., 1965). When cleavage of lactose occurs through the action of this enzyme, one molecule of glucose and one molecule of galactose is formed. The metabolic fate of the glucose moiety from lactose has been well documented (Nandan, 1967); however, the fate of the galactose portion is not known. The lactic streptococci can metabolize free galactose and many other hexoses, but the metabolic route for free galactose has not been

detailed. One objective of the present research was to determine the pathway of free galactose metabolism in the lactic streptococci. It has been assumed, because of the single report of Shahani (1960), that the metabolic pathway was that of Leloir (1951). A public health concern over galactose metabolism also is noteworthy and arises from the fact that certain humans are unable to convert galactose from milk into glucose, and then metabolize it.

It has been noted that growth of the lactic streptococci is somewhat inhibited in galactose, as compared to other sugars such as glucose, mannose, lactose and maltose. Consequently, it was decided that another objective of this research would be to compare the growth of a typical lactic streptococcus on various carbohydrates from the standpoints of doubling time and cell dry weight yields. Further efforts to note responses of the organisms on various carbohydrates would be devised using differential respirometry, in which CO_2 evolved from carbohydrates and oxygen absorbed would be measured. It was hoped that some insight into the induction for utilization of various sugars might be obtained using this technique.

Research workers in this laboratory were perplexed in the early 1960's to find that out of some 40 lactic streptococci surveyed for the ability to cleave O-nitrophenyl-β-D-galactopyranoside (ONPG), a chromogenic substrate for β-galactosidase, only one strain, S. lactis 7962, possessed this enzyme (Citti et al., 1965). McKay et al. (1969),

however, gave understanding to this problem when they showed that the phosphoenolpyruvate phosphotransferase system (PTS) of Roseman was involved in lactose metabolism by the organisms. Essentially, it was shown that lactose uptake in strains other than S. lactis 7962 was sensitive to fluoride, and this fluoride inhibition was reversed by phosphoenolpyruvate (PEP). It was therefore understood why all the other lactic streptococci would not cleave ONPG; since lactose was transported by the PTS, it was phosphorylated during transport and a new enzyme called β, D-phospho galactoside galactohydrolase (β-P-gal) was the cleavage enzyme. A new chromogenic substrate was needed for activity measurements, and this new substrate was O-nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-6-P).

It has been shown that the position of phosphorylation on the lactose molecule is the 6 carbon of the galactose moiety (Hengstenberg et al., 1968; Kundig et al., 1964). When cleavage of lactose phosphate occurs, a molecule of glucose and a molecule of galactose-6-PO_4 is formed. The metabolic fate of the galactose-6-PO_4 was unknown. Another objective of this research, therefore, was to isolate galactose-6-PO_4 from a lactic streptococcus and determine the route of its metabolism. The isolation of this compound from the organisms was recognized as a difficult problem, and a mutant defective in β-P-gal, but normal for transport of lactose, was needed. Another aim of this research was to find such a mutant.

In order to study the metabolism of galactose-6-PO_4, it was necessary to hypothesize the possible routes by which the compound could be catabolized and devise experiments to test each route. When the PTS was first implicated in carbohydrate metabolism in the lactic streptococci, the only carbohydrate studied was lactose. Another goal of this research project was to elucidate which carbohydrates were transported by the PTS. In order to accomplish this, a system in which phosphorylation of the sugar could be observed using PEP as a phosphate donor and radioactive sugars as recipients was employed. Simultaneous controls were run using adenosine triphosphate (ATP) as the phosphate donor.

No understanding had been obtained to the time of this research undertaking on the distribution of β-gal and β-P-gal in the streptococci. Therefore, representatives of each of the four major groups of streptococci were chosen and activity on both ONPG and ONPG-6-P was determined. Finally, the API system of taxonomy for lactic acid bacteria was run on chosen representatives of streptococci in an effort to check the relatedness of S. lactis 7962 to other major groups of streptococci.

In summary the objectives of this research were to:

1. Determine the pathway of free galactose metabolism in the lactic streptococci.

2. Compare growth of a typical lactic streptococcus using various carbohydrates.
3. Isolate galactose-6-phosphate from a lactic streptococcus and determine its route of metabolism.
4. Find a mutant lacking β-P-gal but normal for lactose transport.
5. Show which growth sugars in the lactic streptococci are transported by the PTS.
6. Study the distribution of β-gal and β-P-gal in streptococci.

REVIEW OF LITERATURE

Sugar Transport

The transport of carbohydrates by living organisms has been a subject of research for a number of years, and it was recognized early that a rate limiting step in metabolism of a compound often was the entrance of that compound into the cell. It is necessary to understand the mechanisms by which organisms transport raw materials in order to maximize the usefulness of these organisms to the fermentation industry. For example, stimulation of transport as a method of speeding up the metabolism of a starter culture organism could mean outstanding economic gains for industry.

In early studies on transport, the substrate was assumed to pass across the membrane and become chemically transformed. This process was known as group translocation (Harold, 1972). Also, at one time it was thought that sugar transport across the mammalian intestine was linked to phosphorylation (Harold, 1972); however, this hypothesis likewise did not persist and was replaced by other hypotheses, among which are the theories of facilitated diffusion and active transport. It is now believed by most microbiologists that uptake requires the interaction of substrate with a highly specific membrane component which translocates it across the membrane in a catalytic manner (Cohen and Monod, 1957).

In 1965, a component called the "M" protein was isolated from E. coli and was shown to be involved in lactose transport in that organism (Fox and Kennedy, 1965). A model for β-galactoside transport was proposed which was consistent with the earlier models of Kepes and Cohen (1962) and Koch (1964). These workers suggested that a molecule of lactose in the medium combined with the "M" protein on the external surface of the cell membrane. The complex then moved to the inner surface where it dissociated. Therefore, in the presence of β-gal a continuous reaction with lactose resulted in an influx of the sugar. The overall process was called "facilitated diffusion" and did not require energy. In mutants of E. coli it has been shown that lactose can be transported by this process without being phosphorylated (Pardee, 1968).

Facilitated diffusion of galactosides has been shown to be rapid when the energy supply is limited by the presence of inhibitors (Pardee, 1968). However, another process which requires energy has been shown to result in accumulation of lactose in E. coli against a concentration gradient; it is called "active transport."

Scarborough et al. (1968) proposed a model for the interaction of an energy source, such as ATP, with a carrier protein. They suggested that the "M" protein could exist in two different states -- a functional M state in high affinity combination with the substrate and the low affinity state called Mi; a continuous expenditure of energy

would increase the affinity of the protein for the substrate (changing Mi back to M). How this is done is not exactly known, but it is believed to be due to some conformational change in the protein itself. Also, there is an oscillation of the M and Mi states of the protein in the membrane, with ATP postulated as the energy source necessary for the changes in state of the M protein.

Recently, several workers have provided more evidence which supports the involvement of ATP in activity of transport permeases. Wong et al. (1970) proposed that high affinity carriers react with substrate molecules external to the cell membrane, followed by translocation to the inner face of the membrane; these steps do not require energy. At the inner face, energy coupling leads to a marked reduction in affinity resulting in release of the substrate from the carrier. According to these authors, the low-affinity carriers then return to the outer face of the membrane, where the high affinity state is restored by a process which does not require energy.

Kashket and Wilson (1972) have also shown that in Streptococcus lactis ATCC 7962, energy is coupled to transport of Thiomethyl-β-galactoside (TMG) in much the same way as in E. coli. The substrate is not phosphorylated during transport and enters as the free sugar. Agents that uncouple oxidative phosphorylation abolished active transport but not carrier-facilitated entry of TMG.

In a recent paper Koch (1971) retracted the concept of "facilitated diffusion" which he first proposed in 1964 and concluded that permease-mediated transport was dependent on energy and that energy coupling to transport was obligatory. Klein and Boyer (1972) recently showed that an "activated" membrane state was necessary for the active transport of certain amino acids, carbohydrates, and cations by E. coli. The activated state could be generated by either oxidative energy or phosphate bond energy. ATP and PEP, however, were not suggested as energy sources. The authors indicated, however, that glucose-6-phosphate and other intracellular phosphate compounds may be involved in phosphorylation by an unknown mechanism.

One of the most widely studied and best understood transport systems in microorganisms was first reported by Kundig et al. (1964) and is often referred to as the Roseman system. Most workers, however, call it the PEP system of transport since it was first designated the phosphoenolpyruvate phosphotransferese system; recently it has been called by another abbreviation the PTS (Simoni et al., 1973b). This system has received considerable attention in recent reviews (Kaback, 1970A; Kaback, 1970B; Roseman, 1969), so only an abbreviated scheme will be presented here.

In the series of reactions shown below, both transport and the first step in sugar dissimilation (phosphorylation) is accounted for:

(1) $PEP + HPr \underset{\leftarrow}{\xrightarrow{\text{Enz I, } Mg^{++}}} HPr\sim P + Pyruvate$

(2) $HPr\sim P + Sugar \xrightarrow{\text{Enz II, Factor III}} Sugar - P + HPr$

Sum: $PEP + Sugar \xrightarrow[HPr,\ Mg^{+++}]{\text{I, II, III}} Sugar - P + Pyruvate$

In the first reaction the small protein, HPr, which is heat stable, is phosphorylated by PEP. The phosphate is linked to a histidine residue on the molecule. Enzyme I, which is constitutive and inducible, catalyzes the first step. The second reaction, which is more complex than the first, is the transfer of the phosphate group from the HPr to any number of sugars. Enzyme II and Factor III are involved in this transfer. Enzyme II is membrane-bound and inducible. Factor III is part of the soluble fraction of the cell and is also inducible. It should be pointed out that of the sugars transported by the PTS, all share a common Enzyme I; however, each sugar possesses its own Enzyme II and Factor III. The sugars metabolized by the PTS in E. coli are glucose, α-methylglucose, mannose, fructose, mannitol, and β-glucosides (Harold, 1972). Recently, Simoni et al. (1973b) have shown that in lactose transport in Staphylococcus aureus, the phosphoryl group is transferred successively from PEP to Enzyme I, to HPr, to Factor III and finally to lactose.

A number of research workers have reported the occurrence of this transport system in a variety of microorganisms, so the system is widely but not universally distributed. McKey et al. (1969) showed

that this system was involved in lactose utilization by the lactic streptococci, but involvement in metabolism of other sugars was not studied.

In a recent paper, Molskness et al. (1973) reported on the characteristics of the cleavage enzyme for lactose-PO_4 in the lactic streptococci. The enzyme was studied in milk-grown organisms which differed widely in both proteolytic and acid-producing abilities. It was shown that differences in proteolytic ability did not influence synthesis of the enzyme; also, the rate of lactic acid production was independent of the amount of enzyme present in the cells. The only inducer of the enzyme was galactose, either as the free hexose or combined with glucose in lactose. No gratuitous inducers were reported. Premi et al. (1972) reported on research done on the lactose-hydrolyzing enzymes in lactobacilli. β-gal and β-P-gal activities were observed in all of 13 Lactobacillus species studied except L. casei and L. buchneri. Only the later enzyme was detected in nine strains of L. casei. The β-gal from L. thermophilus and the β-P-gal from L. casei were purified and characterized; they were similar in amino acid composition and had molecular weights of 5.4×10^5 and 1.3×10^5, respectively. The optimal temperature and pH for β-gal were 55°C and 6.2, whereas for β-P-gal they were 37°C and 5.0.

McKay et al. (1970) presented further evidence that in lactic streptococci lactose was utilized through the PTS. Complementation

experiments were done using extracts of S. lactis and extracts of mutants of S. aureus lacking each component of the PTS. It was shown that a wild type S. lactis would restore ONPG hydrolysis in all the staphylococcal mutants lacking any one component of the PTS; however, none of the S. lactis lactose-negative mutants were able to complement the S. aureus mutants lacking Factor III and Enzyme II. It therefore was shown that the PTS of S. lactis was identical to that of S. aureus, but the researchers were unable to explain why only two classes of mutants were obtainable in S. lactis. Nor were they able to explain why these mutations always appeared together in S. lactis.

Recently, McKay et al. (1972) reported on studies in which the lactose-fermenting ability in lactic streptococci was lost by acriflavin treatment and continuous culture. It was suggested that the ability to ferment lactose might reside on extrachromosomal genetic material which was acquired by transfer from another genus of bacteria by the interaction of a phage. Recently Cirillo (1973) has shown complementation between E. coli and mycoplasma; wild-type mycoplasma was used to complement lactose negative mutants of E. coli and the cytoplasmic and membrane fractions complemented each other with essentially the same specific activity. Complementation between mycoplasma and E. coli fractions were non-reciprocal; mycoplasma cytoplasm complemented E. coli membranes with nearly the same specific activity as E. coli cytoplasm, but E. coli cytoplasm

complemented fresh mycoplasma membranes with only low specific activity.

In a survey of a number of bacteria, Romano et al. (1970) found that representative genera of facultative anaerobes possessed a PTS for glucose transport. The system appeared to be absent in those genera which were strictly aerobic. However, it was noted that Bacillus subtilis and Achromobacter parvulus, which are aerobic in their metabolism, appear to be exceptions. Also, in a recent report, Sobel et al. (1973) showed a functional PTS in fructose transport in Arthrobacter pyridinolis, another aerobic organism. Romano and coworkers suggested that under conditions in which energy is limited, the PTS allows for conservation of ATP, since the product of the transport event is a phosphorylated sugar which can enter catabolic and anabolic pathways directly.

Kaback (1968) reported on the involvement of the PTS in sugar transport using isolated membrane vesicles of E. coli. He developed a novel system in which to study transport in the isolated vesicles and showed that the vesicles specifically required PEP. There was also a stoichiometric relation between ^{32}P loss from PEP and the appearance of ^{32}P in α-methylglucoside-P. The sugars shown to be taken up and phosphorylated by glucose-grown cells were α- and β-methylglucoside, glucose, 2-deoxyglucose, fructose, galactose and 3-o-methylglucose. It was assumed that the sugars were

phosphorylated in position 6 but this was shown only with glucose. By applying double labeling, he showed that internal glucose-6-phosphate was almost entirely derived from ^{3}H-glucose added externally; ^{14}C-glucose already present in the vesicles was not phosphorylated. In this respect, Kaback provided the best evidence to date that translocation of glucose occurs by vectorial phosphorylation.

By further work with the isolated vesicles, Barnes and Kaback (1970) came up with a new mechanism for coupling energy to transport. They showed that the transport of galactosides by isolated membrane preparations from E. coli was markedly stimulated by the conversion of D-lactate to pyruvate. The addition of D-lactate to the membrane preparations produced a 19-fold increase in the initial rate of uptake and a 10-fold stimulation of the steady-state level of intramembranal lactose or TMG. The site of energy coupling of respiration to transport was placed between D-lactate dehydrogenase and cytochrome b. Kerwar et al. (1972) came to the same conclusion with respect to galactose uptake by the vesicles.

Barnes and Kaback (1970) have suggested the existence of a specialized transport chain connecting D-lactate with cytochrome b. The carrier for β-galactosides is either a member of this chain or closely linked to its redox carriers. The carrier can exist in two states, differing in orientation and affinity for the substrate. The oxidized form, S-S, has high affinity for β-galactosides at the

external surface. When the disulfide bond is reduced, reorientation occurs, and the substrate is translocated to the inner surface and at the same time the affinity of the carrier for the substrate is lowered. Dissociation of the carrier and substrate occurs, and the substrate accumulates inside the vesicle. Oxidation of the S-H bonds of the carrier returns it to its high-affinity state (Harold, 1972).

The Staphylococcus Aureus System

The PTS of S. aureus is perhaps the best understood and much work has been done to characterize all its components. McKay et al. (1971), in a review on lactose utilization, summarized the earlier work done on this system in S. aureus. The characterization of a pleiotropic mutation in S. aureus was detailed by Egan and Morse (1965). The carbohydrate-negative (car^-) mutant was unable to ferment eight different carbohydrates, and the genetic defect was shown to be a single gene effect; also it appeared that the defect involved transport of the carbohydrates. The authors at first suggested the lack of a common carrier responsible for the transport of carbohydrates into the cell. In studies on the transport of carbohydrates by S. aureus, it was found by Egan and Morse (1966) that a mutant defective in β-gal did not accumulate lactose, but rather a derivative of lactose. This derivative was characterized by Hengstenberg et al. (1967, 1968) and determined to be a phosphate compound with the

phosphoryl group residing on the galactose moiety of lactose. It was suggested that this derivative was the result of the compound being transported by the PTS. Hengstenberg and Morse (1968) synthesized ONPG-6-PO_4 and showed that it was a substrate for staphylococcal "β-galactosidase," and when cleavage of lactose-PO_4 occurred, a molecule of glucose and galactose-6-PO_4 was formed.

The metabolism of glucose is known to involve the glycolytic pathway. In a paper by Simoni and Roseman (1973) it was suggested that galactose-6-PO_4 is metabolized in S. aureus by conversion to galactonic acid-6-phosphate and further metabolized through the familiar Entner-Doudoroff-Wood pathway. Recently, however, Bissett and Anderson (1973) provided data to the contrary. Their results showed that galactose-6-phosphate is metabolized in S. aureus through a previously unreported pathway. The compound is first isomerized to tagatose-6-phosphate, which is phosphorylated with ATP. The resulting tagatose 1,6-diphosphate is cleaved to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The isomerase, kinase, and aldolase that catalyze these reactions are inducible and are distinct from the corresponding enzymes of glucose-6-phosphate metabolism.

It is thought by some that galactose-6-phosphate is the true inducer for β-P-gal in S. aureus because an eight-fold higher level of the enzyme has been obtained from induction using this compound

as compared to galactose (Morse et al., 1968). Also in a gal⁻lac⁻ mutant which could not be induced for β-P-gal by galactose, it was found that galactose-6-phosphate induced high levels of the enzyme. In a car⁻ mutant unable to phosphorylate galactose, Simoni et al. (1968) found that gal-6-phosphate induced Enzyme II and Factor III in this mutant and galactose showed no induction ability.

In a recent series of papers from Roseman's laboratory, the PTS of S. aureus has been further characterized. The system was isolated and described by Simoni et al. (1973b) as consisting of four protein components: Enzyme I; two phosphocarrier proteins, HPr and Factor III^{lac}, and Enzyme II^{lac}. This system catalyzed the transfer of a phosphoryl group from PEP to β-galactosides yielding the corresponding galactoside 6-phosphate esters. Only one of the proteins, HPr, was purified to homogeneity. S. aureus HPr has approximately the same molecular weight as E. coli HPr, but the two proteins differ with respect to amino acid composition. The HPr from S. aureus contains one histidine and two tyrosine residues whereas HPr from E. coli has two histidine residues; also, tyrosine is absent.

In a companion paper, Hayes et al. (1973) reports the purification of staphylococcal factor III^{lac} to homogeneity. The molecular weight was reported as 35,000. A large amount of alpha helical structure (about 60 percent) was reported present, and it was determined to consist of three identical subunits. And in a third paper the

reaction catalyzed by the four component PTS of S. aureus was studied by Simoni et al. (1973a). Phosphorylated Enzyme I appeared to be an intermediate in the initial reaction, that is, the transfer of a phosphoryl group from PEP to HPr, a reaction catalyzed by Enzyme I. Only one phosphoryl group was transferred to each HPr molecule. The subsequent reversible phosphoryl transfer from phospho-HPr to the sugar-specific phosphocarrier protein Factor III^{lac} was entirely self-catalyzed. This novel reaction, shown with homogeneous HPr and Factor III^{lac}, did not require any additional protein component and each protein appeared to be a substrate for the other. The final step, formation of galactoside-6-phosphate, was catalyzed by the membrane-bound Enzyme II^{lac}. This reaction did not proceed by a "ping-pong" mechanism, but a ternary complex was formed by the two substrates, phospho-Factor III^{lac} and lactose, and Enzyme II^{lac}.

In a final paper in the series, Simoni and Roseman (1973) gave evidence for the physiological role of the PTS in the utilization of lactose by S. aureus. The sugar is taken up by group translocation, i.e., the sugar is phosphorylated while it traverses the cell membrane. Mutants defective in proteins of the PTS provided evidence to support this interpretation. Mutants lacking Enzyme I were unable to ferment a variety of sugars. Mutants defective in Enzyme II^{lac} or Factor III^{lac} were incapable of transporting TMG. Since the mutants were derived from a constitutive strain, the inability of this last class of mutants

to transport lactose or its analogues cannot be explained by lack of induction of the lac operon.

The inducible parent gave a class of mutants lacking all three proteins of the lac operon but never could a mutant lacking only Factor III^{lac} be isolated. Contrastingly, the constitutive parent yielded mutants as above, but also mutants lacking only Factor III^{lac} could also be isolated. This difference in behavior of the inducible and constitutive strains led the authors to speculate that Factor III^{lac} may play a role in regulating transcription or translation, or both, of the lac operon in S. aureus.

The Galactose Operon

When E. coli or Salmonella are grown on D-galactose as the sole carbon source, the synthesis of three enzymes (Kalckar et al., 1959) and an uptake system for galactose, galactose permease (Horecker et al., 1960) are induced. These enzymes enable the cell to concentrate galactose and then metabolize it through the Leloir pathway. These enzymes are:

1. Galactose permease
2. Galactose kinase
3. Galactose-1-P, uridyl transferase
4. UDP-gal-4-epimerase

and the result of the activities of these three enzymes is the conversion

of galactose to galactose-1-phosphate which can be further metabolized (Ames and Martin, 1964).

Mutants of E. coli, unable to use galactose as a carbon source, have been isolated, mapped, and tested for complementation (Morse et al., 1956); the genes for kinase (K), transferase (t), and epimerase (e) have been shown to be in a cluster on the chromosome between a gene for adenine biosynthesis and the prophages 82 and λ (lambda).

ad K t e (82) (λ)

This cluster is the galactose operon and a similar cluster has been mapped in Salmonella (Fukasawa and Nikaido, 1961).

The kinase, transferase, and epimerase have a constant ratio over a 10- to 25-fold range of specific activities in E. coli (Buttin, 1963b) and the enzymes have been shown to exhibit simultaneous rather than sequential induction. Buttin also has shown that the gal operon can be induced in E. coli by 6-deoxygalactose. Additionally, a mutation in the gene for one of the earlier enzymes does not prevent the induction of later enzymes.

Galactokinase mutants show induced levels of the transferase and epimerase in the absence of added inducer (Jordan et al., 1962). These authors have attributed this phenomenon to internal induction, presumably caused by the accumulation of galactose normally removed by small amounts of kinase. Buttin (1963a) has shown that the

permease is also induced in these mutants. A mutation (0^c) in the epimerase gene causes constitutive levels of the three enzymes in the operon, but not of the permease (Buttin, 1963b). When a heterozygote containing this 0^c mutation is studied, only the structural genes on the same chromosome as the mutation are affected. Therefore, the epimerase end of the operon appears to be the end on which the operator is located in E. coli.

Transport and Metabolism of Galactose

The transport of D-galactose was described in two mutant strains of E. coli K_{12} lacking galactokinase by Anraku (1967). The cells showed a 50 percent reduction in the capacity for galactose uptake and in the rate of exit as a consequence of osmotic shock. After shock treatment, a factor was released into shock fluid which formed a complex with galactose in vitro. The material was heat labile and non-dialyzable and behaved like a protein. More of the material appeared when cells highly induced for galactose transport were used. Interestingly, the reduced uptake of galactose in shocked cells could be restored by incubating them with shock fluid. In further work Anraku (1968) isolated two proteins from the shock fluid; one protein bound D-galactose, and the other bound leucine. Of a number of carbohydrates tested, only galactose and glucose were bound. A number of related sugars, including mannose, fucose,

lactose, galactosamine, galactose-1- and galactose-6-phosphate did not interfere with the binding of galactose.

Boos (1969) showed the release of two galactose-binding components from the cell envelope of E. coli. Galactose and β-glycerol-galactoside are the most efficiently transported substrates of the permease. Glucose, β-glycerolgalactoside, β-methylgalactoside, and D-fucose inhibited the galactose uptake by the β-methylgalactoside permease in the same decreasing order as they inhibited the galactose binding activity of the galactose binding protein.

Wilkins (1970) recently showed that glucose-grown cells of Streptococcus faecalis would accumulate but not metabolize D-galactose. He stated that uptake involved a carrier-mediated process which showed a low affinity for galactose, but which could accumulate galactose against a concentration gradient. Concurrent fermentation of arginine increased the initial rate of uptake and maximal intracellular concentration. The addition of phosphoenolpyruvate to the uptake medium increased the accumulation of free galactose but did not alter the amount bound. ATP, added extracellularly, inhibited both processes.

Galactose metabolism has received considerable research attention in recent years since more information concerning its biochemical pathways and reactions has been needed for public health reasons. Most of the advances in this area of metabolism can be

attributed directly to the significant observations of Leloir (1951) and Kalckar (1953, 1957). At least five enzymatic reactions are important in the galactose-glucose interconversion (Isselbacher, 1959).

1. Galactokinase converts galactose to galactose-1-phosphate and requires ATP. The reaction is not reversible.

   Galactose + ATP $\longrightarrow$ Galactose-1-P + ADP

2. Galactose-1-phosphate uridyl transferase catalyzes the conversion of gal-1-P to glucose-1-P, and requires the cofactor, uridine diphosphoglucose.

   Gal-1-P + UDP-glucose $\rightleftharpoons$ UDP-gal + glu-1-P

   In this reaction gal-1-P is transferred to the uridine nucleotide to yield uridine diphosphogalactose and glu-1-P which is derived from UDP-glucose. UDP-galactose is involved in synthesis of such galactose-containing compounds as galactolipids, mucopolysaccharides, and lactose.

3. UDP-Galactose-4-epimerase directly interconverts the two nucleotides, UDP-gal and UDP-glu. This enzyme was formerly called "galactowaldenase," but when nicotinamide adenine dinucleotide (NAD) was shown to be required, it was renamed as an epimerase. It is believed that in the reaction, two hydrogens are removed from the fourth carbon of galactose and then added back but on the opposite direction so that the glucose

epimer is formed.

The reaction is reversible.

UDP-gal $\underset{\text{NAD}}{\rightleftharpoons}$ UDP-glu

4. UDP-glucose pyrophosphorylase is not directly involved with the galactose-glucose interconversion but is a means of synthesizing UDP-glucose.

UTP + glu-1-P $\rightleftharpoons$ UDP-glu + PP

The reaction is reversible and requires uridine triphosphate (UTP); pyrophosphate (PP) is formed.

5. UDP-galactose pyrophosphorylase was recently shown to be present in mammalian liver (Isselbacker, 1957).

UTP + gal-1-P $\rightleftharpoons$ UDP-gal + PP

The reaction is reversible and is of significance in people who possess the congenital disease galactosemia, a condition in which the second enzyme of the pathway is missing. This enzyme allows galactosemic individuals to metabolize galactose.

The biochemical and genetic aspects of galactose metabolism in bacteria has recently been reviewed by Fukasawa (1964), but very little work has been done in this area concerning lactic acid bacteria. Marth (1962) reported that in lactose metabolism, the molecule is first hydrolyzed into glucose and galactose. He reported the glucose to be metabolized through the well-known glycolytic pathway. Galactose was reported to first be converted into glucose and then utilized.

Fukuyama and O'Kane (1962) showed the pathway by which galactose-1-^{14}C was fermented by Streptococcus faecalis. Lactic acid was shown to be about 50 percent of the end product when the fermentation occurred. The distribution of the radioactivity suggested that the fermentation followed the glycolytic pathway. It has been assumed from the single report of Shahani (1960) that galactose metabolism in the lactic streptococci follows the well-known Leloir pathway; however, precise experimental data have not been reported.

Galactose-6-Phosphate Metabolism

As has been mentioned earlier in this review, it is now known that most [one single exception is known (McKay et al., 1969)] of the lactic streptococci utilize lactose through the PTS. Lactose becomes phosphorylated as it traverses the membrane, and the position of phosphorylation is presumed to be the sixth carbon of the galactose moiety since this is what occurs in S. aureus (Hengstenberg et al., 1968).

When cleavage of lactose-phosphate occurs, a molecule of glucose and a molecule of galactose-6-phosphate should be produced. The metabolic fate of the galactose-6-phosphate is unknown. Recently, however, Simoni and Roseman (1973) suggested that the compound may be converted by S. aureus to galactose-1-phosphate by a mutase and then metabolized through the Leloir pathway. Another

suggestion was that it be converted to 6-phosphogalactonic acid and then enter the Entner-Doudoroff pathway. Enouye and Hsia (1964) reported that glucose-6-phosphate dehydrogenase which was purified from either yeast or human erythrocytes converted galactose-6-phosphate to 6-phosphogalactonic acid in the presence of NADP. Presence of 6-phosphogalactonic acid was tested for by the oxidation in the presence of NADP and 6-phosphogluconate dehydrogenase. The change is absorbance at 340 nm as a function of time was compared with that observed using 6-phosphogluconate as substrate. The rate of the reaction with 6-phosphogalactonic was 65 percent the rate when 6-phosphogluconate was the substrate.

There are other reports in the literature to substantiate the fact that glucose-6-phosphate dehydrogenase recognizes several substrates. Greiling and Kisters (1965) showed that galactose-6-phosphate, 2-deoxyglucose-6-phosphate, and glucosamine-6-phosphate were all substrates for glucose-6-phosphate dehydrogenase. The reactions were not due to impurities in substrate or enzyme. Also, Egyud and Whelan (1963) showed that yeast glucose-6-phosphate dehydrogenase oxidized the 6-phosphates of 2-deoxyglucose, D-galactose and D-altrose.

It has also been recently shown that hexose-6-phosphate dehydrogenase from rat liver will reduce NAD^+ or $NADP^+$ in the presence of galactose-6-phosphate or glucose-6-phosphate

(Srivastava and Beutler, 1969). The reaction product of galactose-6-phosphate and the enzyme was isolated by Dowex 1-chloride column chromatography. It readily formed lactone on boiling with 1 N HCl and was identified as 6-phosphogalactonic acid by paper chromatography. But, when liver fractions were incubated with 6-phosphogalactonic acid-1-^{14}C, only minimal amounts of $^{14}CO_2$ were formed. The authors suggested that the metabolism of 6-phosphogalactonic acid required research attention.

In order to investigate the metabolism of galactose-6-phosphate in the lactic streptococci, four possible schemes for the metabolism of the compound were hypothesized. These are shown in Figure 1. Scheme 1 would only involve one enzyme, an epimerase to convert gal-6-P into glu-6-P. Schemes 2 and 3 would both involve the enzymes of the Leloir pathway; Scheme 3, however, would not require galactokinase. Scheme 4 would involve the dehydrogenation of galactose-6-P to 6-phosphogalactonic acid which could go through the pentose cycle or the Entner-Doudoroff pathway.

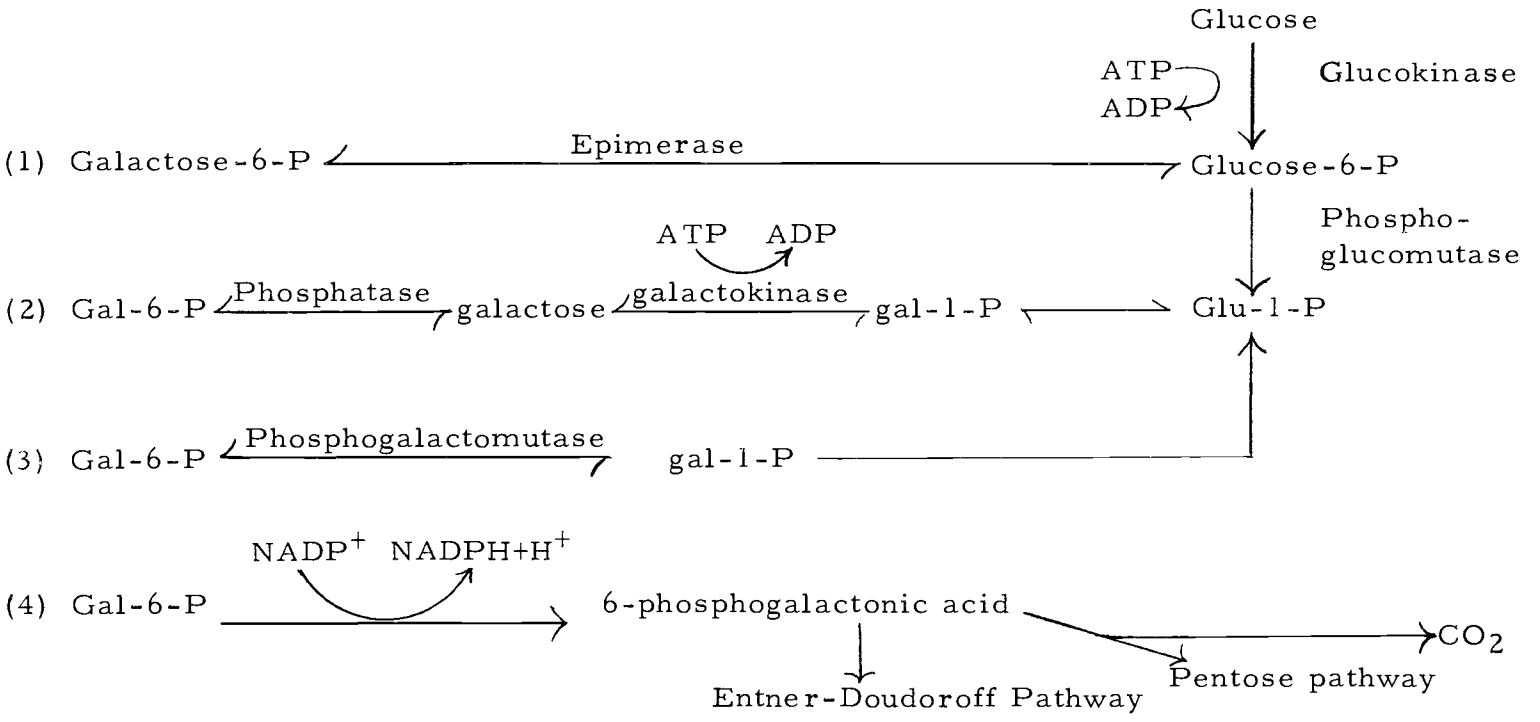


Figure 1. Possible routes for the metabolism of galactose-6-phosphate.

MATERIALS AND METHODS

Microorganisms

Single-strain organisms were obtained from the stock culture collection maintained by the Department of Microbiology, Oregon State University. The strains of lactic streptococci used in this study were S. lactis C2, C10, and 7962, S. cremoris HP and S. diacetilactis 18-16. The physiological characteristics of these bacteria were determined by Sandine, Elliker, and Hays (1962). Streptococcus bovis was a gift of Dr. M. Wolin, University of Illinois. Streptococcus Group B Strain Lo was a gift of Dr. N. Svartz, Stockholm, Sweden.

Reserve stock cultures of the organisms were maintained in 10 ml of sterile 11 percent nonfat milk. The cultures were started by a one percent inoculum into 10 ml of sterile milk, incubated at 30 C for 12-14 hours and placed in freezer at -20 C until ready for use. Routine tests performed on organisms taken from frozen reserve stocks included Gram stains for typical morphology, arginine hydrolysis in Niven's broth and litmus milk reaction.

The procedure followed for the King's test was to add 1 ml of King's reagent A (40% KOH) to 2 ml of an active milk culture in a test tube. One ml of King's reagent B (alpha-naphthol) was then added to the tube, and the tube incubated at 30 C for 30 minutes. The

tube was shaken periodically. A red to lilac colored reaction was indicative of a positive test for the C4 compounds diacetyl, acetylmethylcarbinol and 2,3-butylene glycol.

The procedure for the test for arginine hydrolysis was to grow the organisms for 24 hours at 30 C in Niven's arginine broth (Nivens, Smiley and Sherman, 1942). After this time, one drop of culture and one drop of Nessler's reagent were mixed on a spot plate. A yellowish orange to brown color, indicating the presence of NH_3, was a positive test for arginine hydrolysis.

Strains also were examined for biochemical characteristics using the API test system. The test sets, which consisted of sterile prepared media in plastic impress forms, were obtained from Analytab Products, 516 Mineola Ave., Carle Place, N. Y.

Chemicals

D-Galactose (1-^{14}C), specific activity 59 mCi/mmol and 3 mCi/mmol and D-Glucose (U-^{14}C), specific activity 3.0 mCi/mmol, were obtained from Amersham/Searle Corp., Arlington Heights, Illinois. Lactose-^{14}C (D-galactose-U-^{14}C), specific activity 11.4 mCi/mmol, was obtained from New England Nuclear, Boston, Massachusetts. D-Mannose (U-^{14}C), specific activity 180 mCi/mmol, was a gift from Dr. Ed Thompson, Oregon State University. Maltose (U-^{14}C), specific activity 7.0 mCi/mmol, was obtained from

Calbiochem, Los Angeles. Glucostat and Galactostat reagents were purchased from Worthington Biochemical Corp., Freehold, New Jersey. The remainder of the chemicals used were obtained from Sigma Chemical Co., St. Louis, Missouri, or were laboratory-grade chemicals.

Media and Growth Conditions

Before experimental use, cultures were transferred at least three times in a broth medium of the following composition in grams: desired carbohydrate, 10.0; Difco Tryptone, 10.0; Difco yeast extract, 1.0; gelatin, 2.5; sodium acetate, 1.5; ascorbic acid, 0.5; and distilled water to one liter (Elliker, Anderson, and Hannesson, 1956). The pH of this medium was adjusted to 7.0 prior to autoclaving at 121 C for 15 min.

Disappearance of Galactose

Cells of S. lactis C2 were grown for six hours in one liter of galactose broth and harvested in the Servall refrigerated centrifuge at 6000 x g. Initial inoculum was one percent and cells were harvested in late log phase. The cells obtained were washed two times in 0.05 M sodium phosphate buffer at pH 7.0 and then suspended in 40 ml of the same buffer; 2 ml of a 1:9 (v:v) mixture of toluene:acetone were then added to the cells and the mixture shaken vigorously for five minutes

at 25 C. These toluene-acetone treated cells were used as a crude extract with which to study the disappearance of free galactose. At 0 time, 5 ml of the cell mixture were added to each of the following solutions, each of which had an optical density (O. D.) of 0.45 at 420 nm:

Galactose (168 μg/ml)

Galactose (168 μg/ml) + .03 M potassium fluoride (KF)

Galactose (168 μg/ml) + .03 M KF + .01 M PEP

Galactose (168 μg/ml) + .03 M KF + .01 M ATP

Final volume of each solution was 20 ml. Four samples were removed at five-minute intervals and centrifuged at 12,000 x g to remove the cells. The supernatant was assayed for free galactose using the Galactostat assay (Worthington Laboratories, Freehold, New Jersey).

Conversion of Galactose to Glucose

Cells of S. lactis C2 were grown in one liter of galactose broth for five hours and harvested in the usual manner. The initial inoculum was one percent and cells grew until late log phase. Treatment of the cells with toluene-acetone was as described above. Five ml of the resulting cell suspension was brought to a 40-ml final volume (O. D. of 0.35) in 0.05 M sodium phosphate buffer pH 7.0 with 800 μg/ml added galactose, 0.3 M KF and 0.1 M ATP. This mixture was incubated at 37 C. Eight samples (5 ml each) were removed every

four minutes and quickly frozen in acetone-dry ice. To assay the carbohydrate intermediates, the samples were thawed and centrifuged at 12,000 x g. The supernatants were assayed for glucose, glucose-1-P, and glucose-6-P using the Glucostat assay and for galactose, galactose-1-P, and galactose-6-P using the Galactostat assay. To determine the glucose-1-P or galactose-1-P concentrations, the increase in free glucose or galactose was measured after ten minutes of hydrolysis of the sample in 1N HCl at 100 C. This technique measures only the sugar-1-P, for it was determined that none of the sugar-6-P was hydrolyzed by this time. To measure the glucose-6-P or galactose-6-P concentration, the further increase in free glucose or galactose was determined on the acid hydrolysed sample after an additional hydrolysis with alkaline phosphatase at pH 8.5 for one hour at 37 C.

Chromatography of the Sugar Phosphates

Descending paper chromatography was done under a fume hood. The paper was first dipped in 0.1 M ethylenediamine tetraacetic acid (EDTA) at pH 7.0 and dried before use. The solvent used was ethanol: methylethyl ketone: 0.5 M morpholinium borate buffer; 70:20:30 (v:v:v) (Carminatti and Passeron, 1965). Sugars and sugar-6-phosphates were detected by p-anisidine spray which was formulated as follows:

2 grams of p-anisidine plus 6 grams of trichloroacetic acid in a final volume of 200 ml of 70 percent ethyl alcohol. The sugar-1-phosphates were detected by first drying the paper and then dipping in 1.0 percent ammonium molybdate solution containing 11 ml of 3N HCl, 2 ml of 70 percent perchloric acid and made to a final volume of 100 ml with acetone.

Inhibition of Galactose Uptake by 6-Deoxygalactose

Galactose uptake by whole cells of S. lactis C2 was measured in the presence of different concentrations of fucose (6-deoxygalactose). A 3.0×10^{-5} M solution of galactose (containing 2.2×10^{-2} μCi galactose-1-^{14}C/ml) was prepared in 0.05 M sodium phosphate buffer at pH 7.0. Four equal aliquots were taken and fucose was added to provide concentrations of 0.1 M, 0.01 M, 0.001 M, and 0.00001 M. At 0 time, whole cells of S. lactis C2 grown on galactose were introduced into the solutions to an O.D. of 0.28. At two-minute intervals, samples of 1 ml were removed, quickly filtered through Millipore filters (H.A.-0.45 μ diameter) and the filters washed with 10 ml of the ice-cold phosphate buffer. The filters were transferred to scintillation vials and dried overnight at 45 C. The next morning, 10 ml of toluene-based scintillation fluid [PPO-2, 5-diphenyloxazole 5g; POPOP 1, 4-bis-2-(5-phenyloxazolyl)-benzene 0.1 g and toluene to 1 liter] were added to the vial and the radioactivity of the samples

determined by liquid scintillation counting.

Galactose Uptake

Competition by Carbohydrates

Competing sugars were prepared in a concentration of 40 μ M and 0.1 ml of this solution was added with 0.9 ml of 0.1 M sodium phosphate buffer, pH 7.0 in a test tube. Galactose-1-^{14}C was diluted to contain 0.2 μCi/ml and one ml was added to the tubes containing the competing sugars. The final concentration of the competing sugars was 2 mM. At time 0, S. lactis C2 cells were added in a final amount of approximately 0.1 mg/ml cell dry weight. Five minutes were allowed for the cells to assimilate the galactose at 25°C. After this time 1-ml quantities of the suspension were quickly filtered through Millipore filters (H.A. 0.45 μ pore size) and washed with 5 ml of cold phosphate buffer. The filter was placed in a dry clean scintillation vial and dried overnight at 45 C. The next morning, 10 ml of toluene-base scintillation was added to each vial and the radioactivity determined by liquid scintillation.

Effect of Energy Sources

These experiments were performed in the same manner as the experiments involving competition by carbohydrates. The energy sources were all in final concentration of 20 mM.

Effect of Metabolic Inhibitors

These experiments were performed in a manner identical to the experiments involving competition and effect of energy sources.

Sugar Phosphorylation Studies

Radioactive substrates were prepared (0.1 μCi/ml) in 0.05 M phosphate buffer, pH 7.0, which contained KF (0.03 M), Mg^{++} (0.01 M) and ATP (0.01 M) or PEP (0.01M). At 0 time, toluene-acetone treated cells of S. lactis C2 were added, and every two minutes, samples (2 ml) were removed and immediately frozen. To check for phosphorylation, the samples were thawed and 0.5 ml of each was placed on a Bio-Rad AG1-X2 (formate form) anion-exchange column (4.0 by 0.4 cm). Unphosphorylated sugars were washed from the column with 5 ml of water. Sugar phosphates were eluted from the column with 5 ml of 0.5 M ammonium formate in 0.2 M formic acid. Aliquots (0.1 ml) of the eluted compounds were placed in 10 ml of Bray's fluid and counted by liquid scintillation. In experiments to evaluate the column procedure, about 70 percent of the total radioactivity applied to the column bed was recovered in the eluate, which approximated the counting efficiency of the liquid scintillation spectrmeter.

Simultaneous Use of Glucose and Galactose by Galactose-Adapted Cells of Streptococcus diacetilactis 18-16

S. diacetilactis was used for these experiments because it releases CO_2 from glucose (Busse, 1963). Assuming the absence of an unknown metabolic pathway, the detection of the radioactive gas produced from galactose-1-^{14}C would provide evidence for the conversion of galactose to glucose; also determination of the specific activity of radioactive CO_2 with time under appropriate experimental conditions would indicate whether or not galactose and glucose were used simultaneously. Media were formulated in sodium phosphate buffer, pH 7.0, as follows in 30 ml-capacity serum bottles:

33 mM galactose + 0.2 μCi galactose-1-^{14}C

33 mM glucose + 0.2 μCi glucose-U-^{14}C

33 mM galactose + 0.2 μCi galactose-1-^{14}C + 33 mM glucose

33 mM glucose + 0.2 μCi glucose-U-^{14}C + 33 mM galactose

Specific activity of each radioactively labeled substrate was 3 mCi/mmole.

The media were inoculated with washed cells adapted and grown in galactose for 12 hours. The optical densities at 420 nm of a 1:100 dilution of the inoculum varied from 0.28 to 0.32. Approximate cell dry weight values for the inoculum varied from 22.4 mg/ml to 24.2 mg/ml. Incubation of the vials was performed under static conditions at 30 C for 30 minutes. The serum bottles were fitted with a small

cup to hold filter paper on which to later trap CO_2. In order to collect the CO_2, the pH was adjusted to 2.0 by adding 6N HCl. The volume of HCl added was determined using a duplicate vial not used in the experiment, but containing the same materials as the experimental vials. Twenty minutes was allowed for the CO_2 to be evolved from the medium into the vial; at this time 0.2 ml of phenylethylamine was injected onto the filter paper through the cap of the vial. One hour was allowed for absorption of CO_2 to the phenylethylamine. The filter paper was then placed in 10 ml of Bray's scintillation fluid and counted in a Packard liquid scintillation spectrometer. Under identical conditions, the volume of CO_2 produced was determined using a Gilson differential respirometer.

Galactose-6-Phosphate Dehydrogenase Assay

The procedure followed was that of Srivastava and Beutler (1969). Cells were grown for 18 hours in 1 percent lactose broth at 30 C, harvested as usual and washed twice in 0.1 M phosphate buffer, pH 7.0. A crude extract of the cells was prepared by treatment of 30 ml of the cell suspension with a Raytheon sonic oscillator at maximum settings. The cell debris was removed by centrifugation at 10,000 x g, and the supernatant was used as the crude extract. Galactose-6-phosphate was prepared as a 0.1 M solution. NAD^+ or $NADP^+$ were prepared in final concentration of 0.001 M. In the assay procedure,

0.1 ml amounts of enzyme, gal-6-phosphate, and NAD^+ or $NADP^+$ were pipetted into glass cuvettes with a 1 cm light path. The final volume was brought up to 3 ml with phosphate buffer. Absorbance was read at 340 nm using a Gilford DU Recording Spectrophotometer.

Galactose Oxidase Assay

Cells of S. diacetilactis 18-16 were grown for 12 hours in 1 percent galactose broth, harvested and washed by the usual way. A crude enzyme preparation was prepared by sonic oscillation. The chromagen was prepared by dissolving 5 mg of 0-dianisidine in 0.5 ml of methanol. 30 ml of water was added and then 5 ml of 0.1 M phosphate buffer was added with 5 ml of 1 percent horseradish peroxidase. Final volume was brought up to 50 ml with water. Substrates used were prepared as 10 percent solutions by weight: volume. In the reaction mixture were placed 1 ml of the chromagen-peroxidase solution, 0.05 ml of substrate and 0.05 ml of enzyme. The mixture was incubated for ten minutes at 30 C and the absorbance read on a Spectronic-20 at 420 nm. One unit of activity was defined as the amount of enzyme which caused an absorbance change of 1.0 in ten minutes under the test conditions of Wood (1966).

Lactose Dehydrogenase Assay

The procedure of Nishizuka and Hoyaishi (1962) was followed. In the complete system each of the following were used:

1 ml tris buffer (pH 6.6)

1 ml lactose (10 μMoles/ml)

0.5 ml 2,6-dichlorophenolindophenol (0.25 μMoles/ml)

1 ml crude enzyme preparation

Reduction of the dye was measured at 590 nm on the Spectronic-20 over a ten-minute period. A control in the reaction was maintained in which all components were used except lactose.

Uridine Diphosphogalactose-4-Epimerase Assay

The method supplied by Sigma Chemical Company, St. Louis, Missouri, and published by Wilson and Hogness (1966) was used with one modification. This was the incorporation of 0.2 M semicarbazide HCl (4.4 grams semicarbazide HCl per 200 ml of 1.0 M glycine buffer, pH 8.8) in the standard glycine buffer. Measurement of reduced NAD (NADH) was accomplished using a Gilford DU recording spectrophotometer with a wavelength setting of 340 nm. Experiments were performed at room temperature. The procedure was to pipette successively the following reagents into a glass cuvette with 1 cm light path:

0.30 ml of 1.0 M glycine buffer + 0.2 M semicarbazide

0.06 ml of 0.05 M NAD^+

0.06 ml of 0.005 M UDP galactose

2.38 ml of H_2O

0.1 ml of UDP glucose dehydrogenase with approximately one unit/ml

The cuvette was mixed and allowed to equilibrate at room temperature for two to three minutes. 0.1 ml of cold UDP gal-4-epimerase or crude cell-free extract was added and mixed well. The OD_{340} was read and recorded using a Gilford DU recording spectrophotometer. Activity was calculated on the initial linear rate. Enzyme activity was calculated according to the method supplied by Sigma Chemical Company, St. Louis, Missouri.

$$\text{Units/mg UDP gal} = \frac{\Delta OD_{340}/\text{minute} \times 3}{12.44 \times \text{mg UDP gal-4-epimerase in reaction}}$$

A unit was defined as the amount of enzyme needed to convert 1.0 μ Mole of UDP galactose to UDP glucose per minute at pH 8.8 and room temperature. Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

Inorganic Phosphate Assay

Reagents:

a. Ascorbic acid 10 percent

b. Ammonium molybdate (0.42%) in 1N H_2SO_4

c. Mix one part of a with six parts of b; this solution is stable in ice bath for only one day.

Procedure:

The procedure was that described by Ames (1965). Add 2.1 ml of reagent c to 0.9 ml of the solution to be tested. Place in 37 C water bath for one hour and read the absorbance at 820 nm; color is stable for several hours. A standard curve can be obtained by plotting concentration of phosphate versus OD_{820} nm.

Assay for Galactose-1-Phosphate Uridyl Transferase

The procedure as outlined by Sigma technical bulletin UV-600, Sigma Chemical Company, St. Louis, Missouri, was followed:

1. 1.2 ml glycine buffer, 1.0 M pH 8.7, mix with 0.3 ml of UDP glucose (7 μMoles/ml)
2. Two test tubes prepared as follows:
   a. 0.3 ml of sol. 1 + .15 ml H_2O
   b. 0.3 ml of sol. 1 + .15 ml gal-1-phosphate (8 μMoles/ml)

   Tubes were placed in 37 C water bath and at 0 time, 0.3 ml

of crude extract of galactose-grown cells of S. lactis C2 was added and incubation proceeded for 15 minutes.

3. Add 1.5 ml of 0.15 M NaCl solution to each tube and place in boiling H_2O bath for two minutes. Place in ice bath for five minutes and centrifuge to obtain a clear supernatant.

4. To determine residual UDP glucose, pipette 0.6 ml of the supernatant from each tube into two cuvettes and add:

   0.45 ml of the glycine buffer

   0.30 ml NAD^+ solution (13 μMoles/ml)

   1.35 ml H_2O.

   Mix and equilibrate for two minutes. Immediately before test, add 0.3 ml of UDP glucose dehydrogenase solution (800 units/ml). Mix and read change in O.D. at 340 nm using a Gilford DU recording spectrophotometer.

It can be expected that the transferase enzyme is present if one sees a greater reaction in cuvette a than in cuvette b.

Differential Respirometry

The volumes of CO_2 and O_2 were measured by employing a Gilson Medical Electronics (GME) Respirometer. The temperature setting was 30 C. The method of Warburg as stated by Umbreit, Burris, and Stauffer (1964) was used for measurement of gases. Each flask contained 3.2 ml of solution (final volume). This consisted

of 2.5 ml of resting cell suspension in 0.1 M sodium phosphate buffer, pH 7.0, and 0.5 ml of the substrate which was 33 mM final concentration. In the flask which was to be used for measuring oxygen uptake, the center well contained 0.2 ml of a 20 percent KOH solution. The KOH solution was replaced by water in the flasks measuring both oxygen uptake and CO_2 evolution. When all components were introduced into the flasks, the flasks were lowered into the water bath and allowed to equilibrate to that temperature for 15 minutes. After equilibration the reaction was initiated by tipping the flask to mix the substrate with cells. Data were plotted as microliters (µl) of CO_2 evolution or O_2 uptake versus time. Endogenous control values of the cells without any substrate were also measured.

Growth Curves

All growth curves in this study were obtained from data using a Klett-Summerson Photoelectric colorimeter with the purple filter #42 having a light transmission between 400-450 nm. Side arm flasks were prepared with the various components except carbohydrates. The flask and contents were sterilized, cooled, and the carbohydrate was added as a filter-sterilized solution to a concentration of 1 percent usually. Inocula for the flasks were taken from suspensions of cells which had been washed two times in sterile 0.1 M sodium phosphate buffer and suspended to an O.D. of 0.4 at 420 nm. Five ml of this

cell suspension was the usual inoculum. Temperature of incubation was 30 C.

Mutagenesis

Ultraviolet Irradiation

Cells were grown up overnight in lactose broth; they were centrifuged and washed twice in physiological saline. The cells were suspended to about 6 x 10^7 cells/ml and 10 ml of the suspension was placed in a sterile petri dish. Irradiation was performed for 10, 20, 30, 40 and 60 seconds at a distance of one foot with a General Electric Germicidal lamp. Appropriate dilutions were plated on the lactose indicator agar of Morse and Alire (1958) as modified by Wagner (1965).

Ethylmethanesulfonate (EMS) Treatment

Cells were grown overnight and diluted in physiological saline to just visible turbidity. EMS was added to a final concentration of 3 percent and allowed to remain with cells for 25 minutes at 32 C. Appropriate dilutions were plated on lactose indicator agar.

Acriflavin Treatment

Acriflavin was prepared in lactose broth to a final concentration of 6 μg/ml. The media were autoclaved and cooled to room temperature. Two loopfuls of an overnight culture of S. lactis C2 were placed

into 2-ml amounts of the acriflavin-lactose broth. Cells were allowed to grow overnight. Appropriate dilutions were plated on glucose, galactose, and lactose indicator agar.

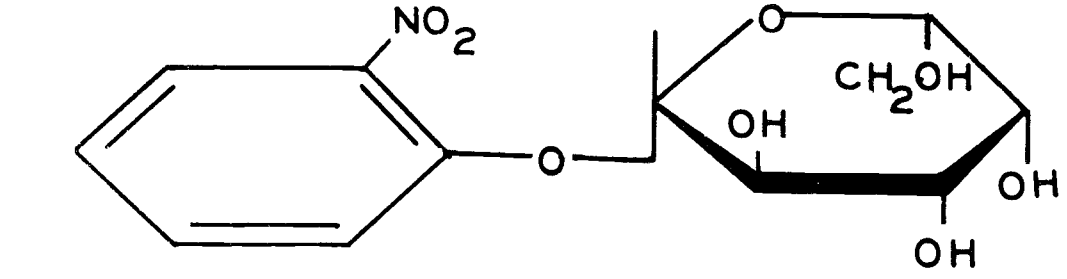
RESULTS

Assay for Enzymes of Lactose Hydrolysis

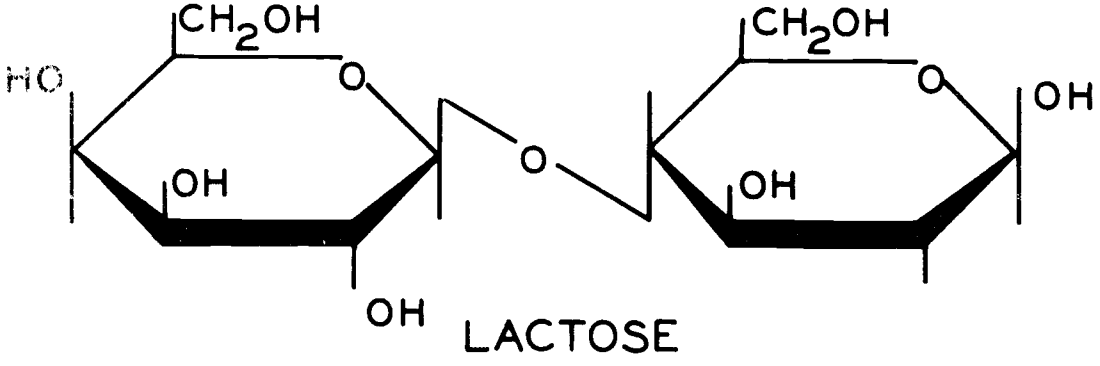
One rapid screening method for studying PTS involvement in lactose utilization is the use of the chromogenic substrate, ONPG-6-P. Structure of this compound as well as the unphosphorylated form, ONPG, and lactose are shown in Figure 2. Hydrolysis of the phosphorylated form exclusively, or at much greater rate than the unphosphorylated form, would be a criterion for implicating the PTS in the utilization of lactose.

When enzyme assays were performed using both of these substrates with a variety of streptococci, interesting results were obtained (Table 1). Most streptococci possessed a greater amount of β-P-gal than β-gal. The only lactic Streptococcus found to possess more β-gal than β-P-gal was Streptococcus lactis ATCC 7962. It was interesting to note that all of the viridans streptococci examined possessed a greater amount of β-gal activity. Of the viridans organisms examined, S. bovis possessed the greatest specific activity of β-gal (>305) while S. thermophilus possessed the least (>111). It should be pointed out that S. bovis showed significant β-P-gal activity.

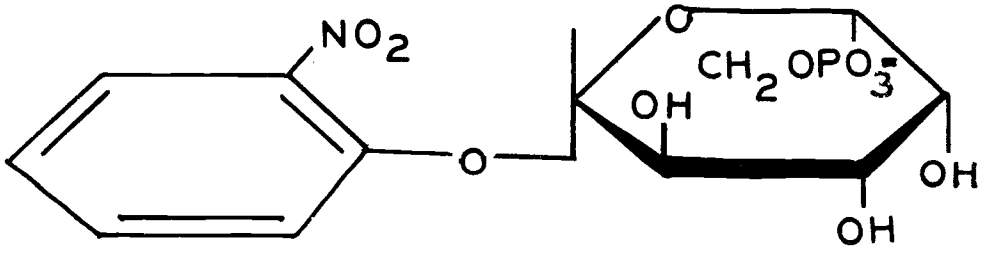
Non-specific phosphatase assays were performed under the identical assay conditions using p-nitrophenylphosphate as the substrate and crude enzyme preparations of representative organisms.



ONPG



LACTOSE



ONPG-6-PO_4

Figure 2. Structures of ONPG, lactose, and ONPG-6 phosphate.

Table 1. Specific activity of β-galactosidase (β-gal) and β,D-phosphogalactoside galactohydrolase (β-P-gal) in various streptococci.

Organism	Specific Activity[a]	
	β-gal	β-P-gal
S. agalactiae ATCC 6638	0	210.5
S. bovis ATCC 15351	305.8	90
S. cremoris Hp[b]	< 1.0	263
S. diacetilactis 18-16	< 1.0	120
S. faecalis	0	236
S. lactis C2[b]	< 1.0	367
S. lactis 7962[b]	259	14
S. salivarius ATCC 9222	122.2	33
S. thermophilus C-3	111.5	19
Streptococcus Lo (Group B)	280	80
S. challis	0	158.3
S. durans ATCC 11576	0	83.3
S. uberis OSC	0	115.4

[a]Specific Activity is reported as nanomoles ONP formed/mg protein/min at 37 C.

[b]Data of T. A. Molskness

It was found that the activity on this substrate under these enzyme assay conditions was negligible.

In order to examine the similarity of S. lactis 7962 to a variety of streptococci, the API tests for identification of lactic acid bacteria were performed on a variety of streptococci. The results are shown in Table 2. From the standpoint of carbohydrate fermentation, S. lactis 7962 showed similarity to the other lactic streptococci examined; however, this organism fermented amydalin, aesculin, salicin, and cellobiose. No other lactic streptococcus was found to ferment these carbohydrates. Arginine hydrolysis was much more rapid in S. lactis 7962 than in any other strain examined.

From the results in Table 2 it can be seen that S. lactis 7962 was the only lactic streptococcus examined which had the ability to cleave ONPG, the artificial chromogenic substrate for lactose. This is evidence that this strain possesses a typical β-gal and apparently this is the only strain of lactic streptococci which possesses this characteristic.

Whole cell agglutination studies were performed on the lactic streptococci listed in Table 2 as well as S. faecalis using group N antiserum supplied by Difco Laboratories. In these studies it was noted that certain streptococci were not suitable for cell agglutination studies due to the fact that the cells after being washed twice in physiological saline had a tendency to clump (autoagglutinate) in the

Table 2. Characteristics of selected streptococci as determined using the API test system for characterizing the lactic acid bacteria.[a]

Fermentation of:	S. lactis 7962	S. lactis C10	S. cremoris HP	S. diacetilactis 18-16	S. faecalis	S. bovis	S. salivarius	S. agalactiae	Group B LO	S. thermophilus
Glycerol		+		+		+			+	
Erythritol										
d(-) arabinose						+	−	+	+	+
l(+) arabinose						+	+	+	+	+
Ribose						+	+			+
d(+) xylose							+			
l(-) xylose			+				+	+		+
adonitol										
methyl xyloside							+	+		
galactose	+	+	+	+	+	+		+	+	
glucose	+	+	+	+	−	+		+	+	+
d(-) levulose (fructose)	+	+	+	+	+	+		+	+	
d(+) mannose	+	+	+	+	+	+		+	+	
l(-) sorbose										
rhamnose										
dulcitol										+
meso-inositol										
mannitol										
sorbitol										
methyl-d-mannoside		+	+	+		+		+	+	
methyl-d-glucoside	+	+	+	+	+	+	+	+	+	
n-acetyl glucosamine	+	−	+	+	+	+			+	
amydalin	+					+			+	
arbutine iron citrate						+				
aesculin iron citrate	+					+				
salicin	+					+				
d(+) cellobiose	+					+				
maltose	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+
d(+) Melibiose										
sucrose								+	+	
d(+) trehalose						+				
Inuline										
d(+) melizitose										
d(+) raffinose						−		+		
dextrine							−		+	
Amylose										
Starch										
Glycogen										
Arginine	−	+		+	+	+		+		
Gas from glucose		+	+	+	+			+		+
Teepol . 4%		+	+	+						
Teepol . 6%		+		+						
NaCl 4%										
NaCl 6%										
NaCl 10%										
ONPG	+					+	+		+	+
Vogues-Proskaur				+		+				

[a]The tests were performed according to directions in the API leaflet; + designates a positive reaction by 48 hours and blanks indicate a negative reaction.

saline control. Notable examples of this were seen with Leuconostoc citrovorum and S. lactis C10. All strains studied, even S. faecalis, were agglutinated in group N antiserum.

Enzyme assays also were performed on the various streptococci (Table 1) using toluene-acetone treated cells. Under these conditions, certain of the bacteria examined would give no activity on either substrate. A notable example of this was provided by Streptococcus durans. Consequently, it was felt that this organism possibly metabolized lactose by another route such as the converstion of lactose to lactobionate as reported by Vakil and Shahani (1969). It should also be pointed out that S. durans (Table 1) showed a much lower activity of either enzyme in cell free extracts than most of the other organisms examined.

Figure 3 depicts a ten-minute reduction of the dye, 2,6-dichlorophenolindophenol by a cell-free extract of S. durans. Lactose is included in the reaction mixture. The slope of the linear region of the line connecting the points was 9/15. As shown by Figure 4 the same slope (9/15) was obtained when the reaction was run without lactose in the mixture.

Growth Studies

The lactic streptococci can utilize a variety of carbohydrates as carbon sources (Sandine et al., 1962). Table 3 shows the results of experiments performed to determine the cell dry weight yield of

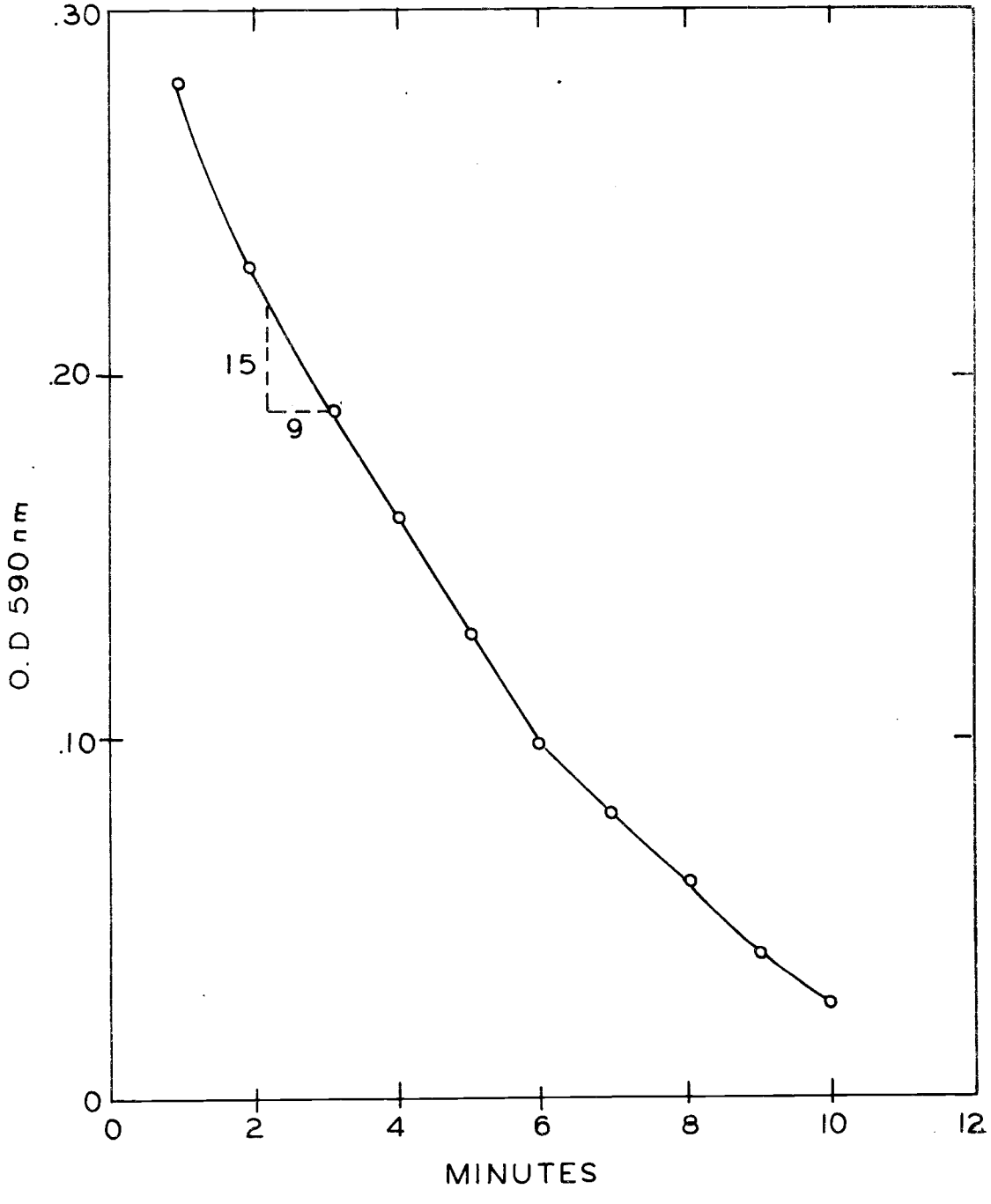


Figure 3. Reduction of 2, 6-dichlorophenolindophenol by a cell free extract of S. durans in a complete system with lactose.

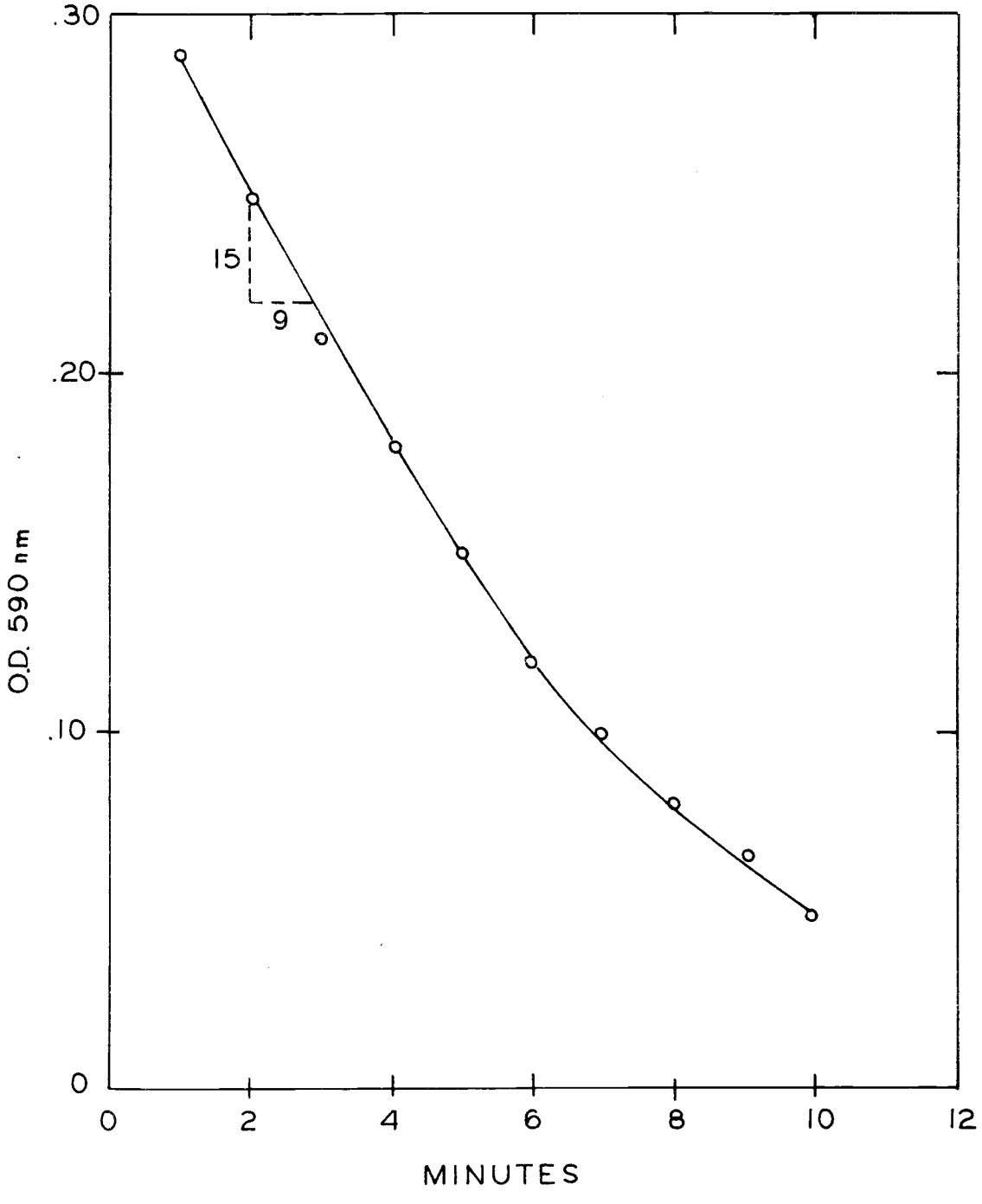


Figure 4. Reduction of 2, 6-dichlorophenolindophenol by a cell free extract of S. durans in a system without lactose.

S. lactis C2 on five of the common carbon sources. The data are averages of triplicate determinations and are expressed as mg cell dry weight/ml of culture. As expected, glucose proved to be used most efficiently with a cell dry weight yield of 0.178 mg/ml. Galactose proved to be the poorest carbon source for growth, with a cell dry weight yield of 0.096 mg/ml. This result was also not surprising.

Table 3. Cell dry weight yields of S. lactis C2 grown on five different carbohydrates.[a]

Carbohydrate	Dry Wt. Yield (mg/ml)[b]
Glucose	0.178
Mannose	0.168
Lactose	0.159
Maltose	0.143
Galactose	0.096

[a]The dry weight yield is expressed as mg/ml and was obtained after six hours of growth in various carbohydrates at 30 C with a 1 percent initial inoculum of washed cells.

[b]Average of triplicate determinations; the range of values for Glucose was 0.174-0.182; Mannose, 0.158-0.178; Lactose, 0.131-0.178; Maltose, 0.136-0.148 and Galactose 0.093-0.099.

Figure 5 illustrates a typical growth response of S. lactis when induced in galactose and returned to three common growth carbohydrates, galactose, glucose, or lactose. A very similar growth response in each carbohydrate was noted with S. lactis. The lag in growth was most generally one-half to one hour in S. lactis. However, when the same experiment was performed with S. cremoris one striking thing was noted (Figure 6). The lag phase of growth was

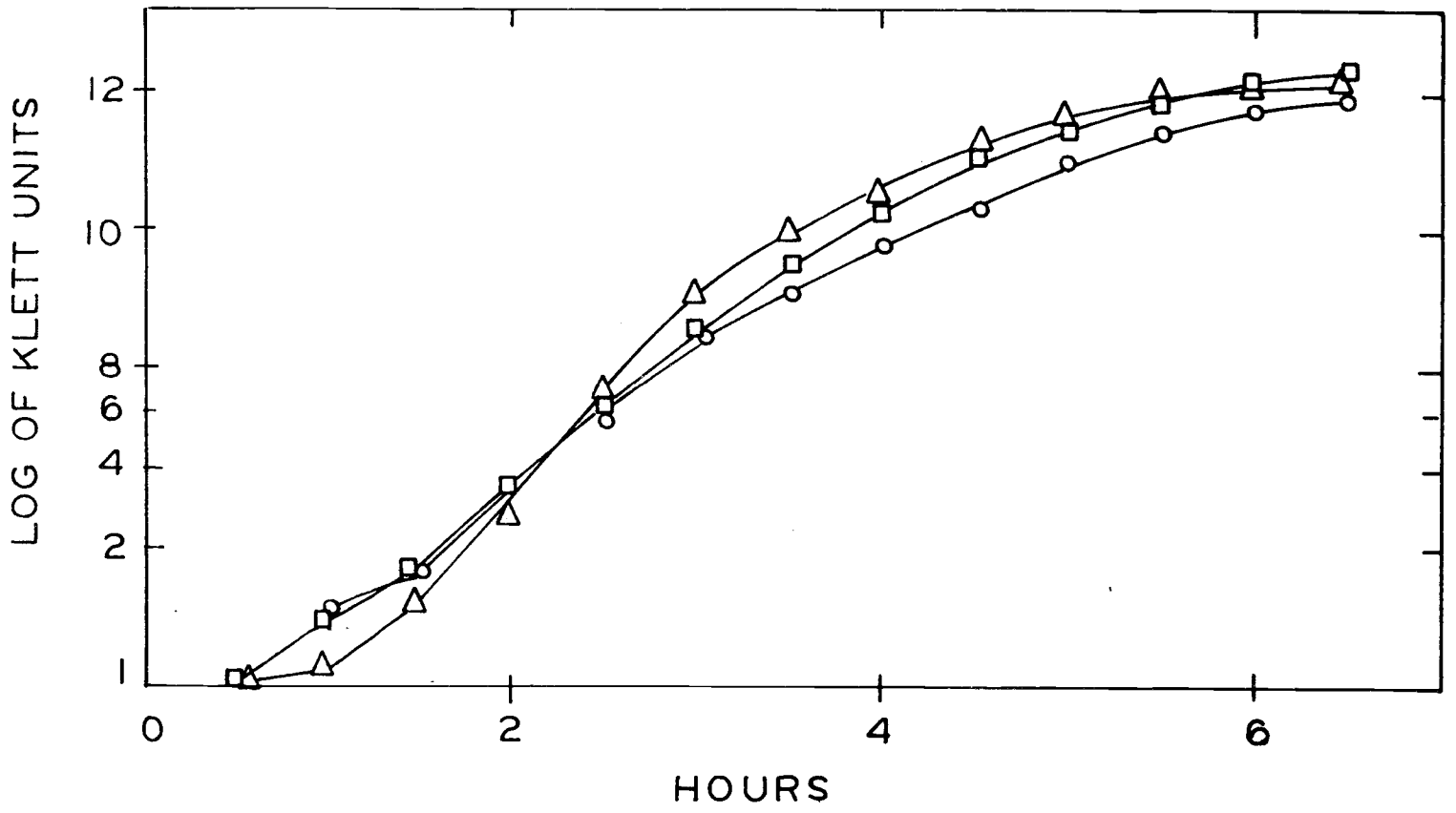


Figure 5. Growth curve of galactose-induced cells of S. lactis C2 at 30 C on glucose (Δ - Δ), galactose (o-o) or lactose (□ - □).

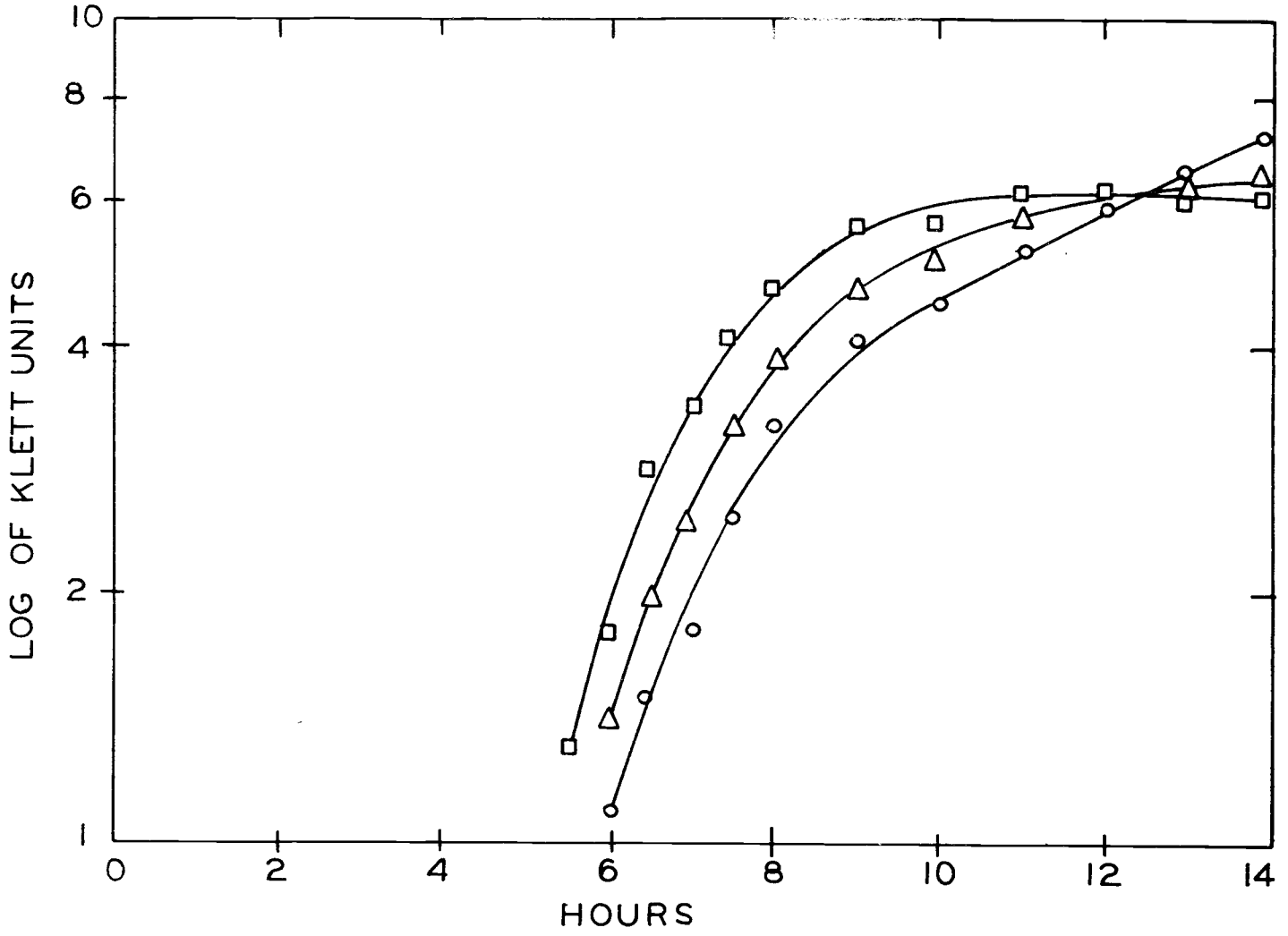


Figure 6. Growth curve of galactose-induced cells of S. cremoris 459 at 30 C on glucose (Δ - Δ), galactose (o-o) or lactose (□ -□).

greatly increased from approximately one hour in S. lactis to five or six hours in S. cremoris. It therefore was desirable to check lactose-induced cells which were returned to lactose broth with various amounts of galactose for an inhibitory effect on growth by galactose. Figure 7 illustrates the results. It was noted that the final population as seen by Klett units was approximately the same in each case, and the lag phase did not appear to be affected by the added galactose. Doubling times were: 54 minutes in 1 percent lactose, 63 minutes in 1 percent lactose plus 0.5 percent galactose, 69 minutes in 1 percent lactose plus 1 percent galactose, and 66 minutes in 1 percent lactose plus 2 percent galactose.

In examining growth of lactose negative mutants on various carbohydrates, pleitrophic mutants with an inability to use two or more carbohydrates were never encountered. Figure 8 is a typical growth response shown by one of the mutants, S. lactis C2 L13. A number of other mutants were studied and the growth response in each was the same.

A diauxic pattern of growth using S. lactis C2 and different amounts of glucose and galactose was never observed. Due to the growth response of S. lactis C2 as noted on Figure 9, it was thought that tne enzymes for galactose metabolism were either very quickly induced after glucose exhaustion or the organism was able to utilize the two sugars simultaneously. In S. lactis 7962 the picture was quite

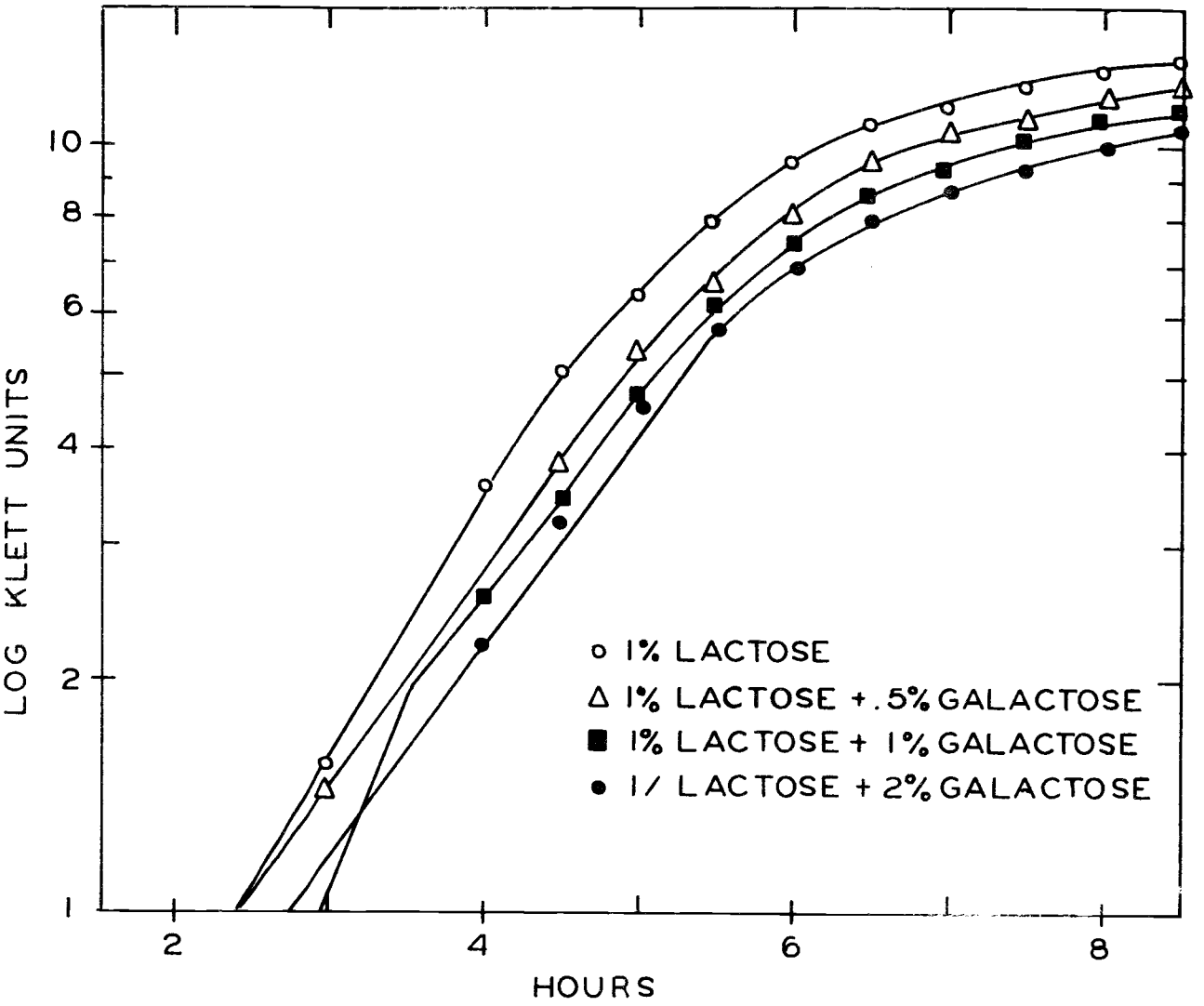


Figure 7. Growth curve of S. cremoris 459 induced in lactose and returned to 1 percent lactose broth with varying amounts of galactose.

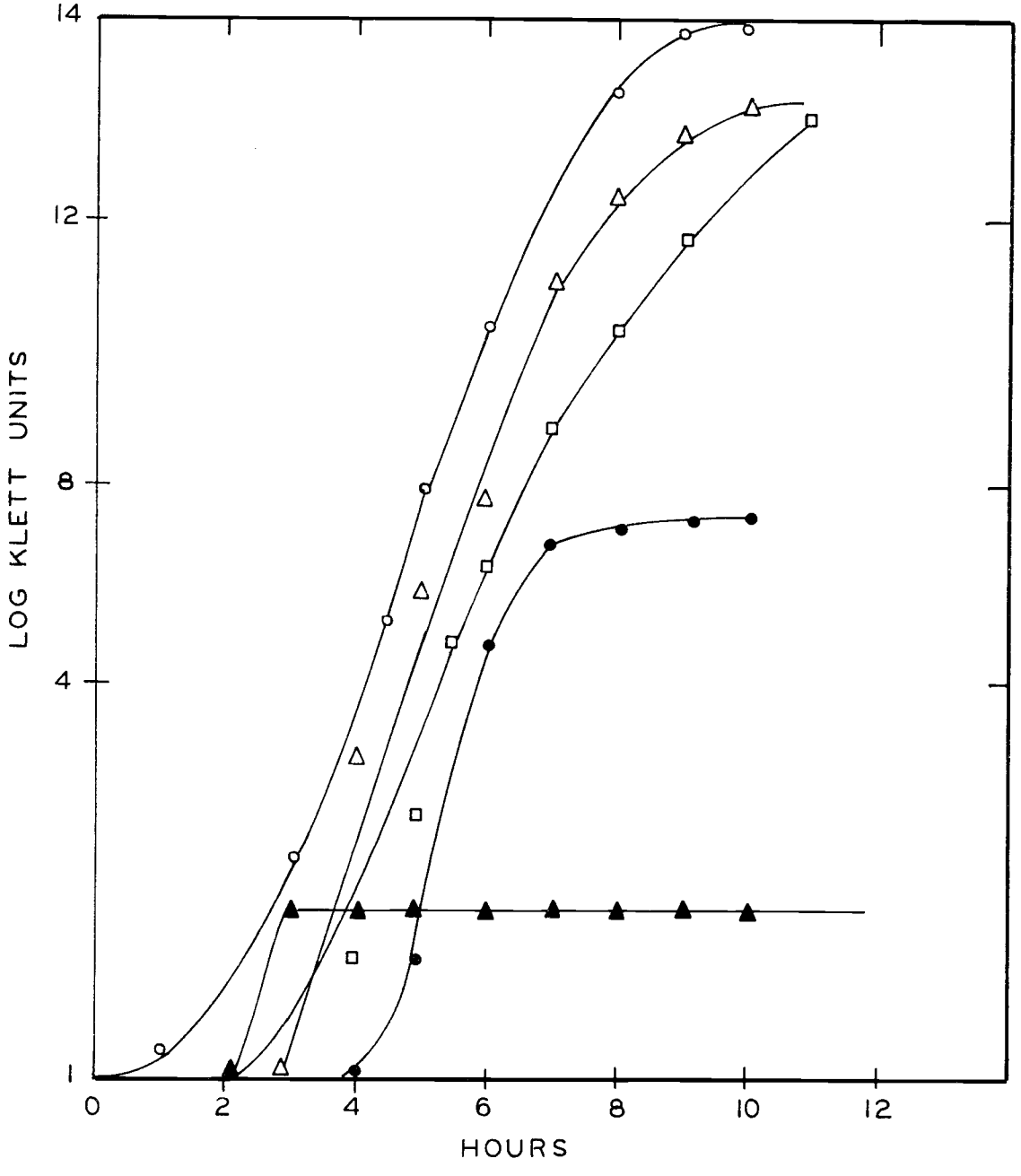


Figure 8. Growth of S. lactis C_2 L13 on five substrates, galactose (o-o), mannose (Δ - Δ), glucose (□ - □), maltose (●-●) or lactose (▲ - ▲).

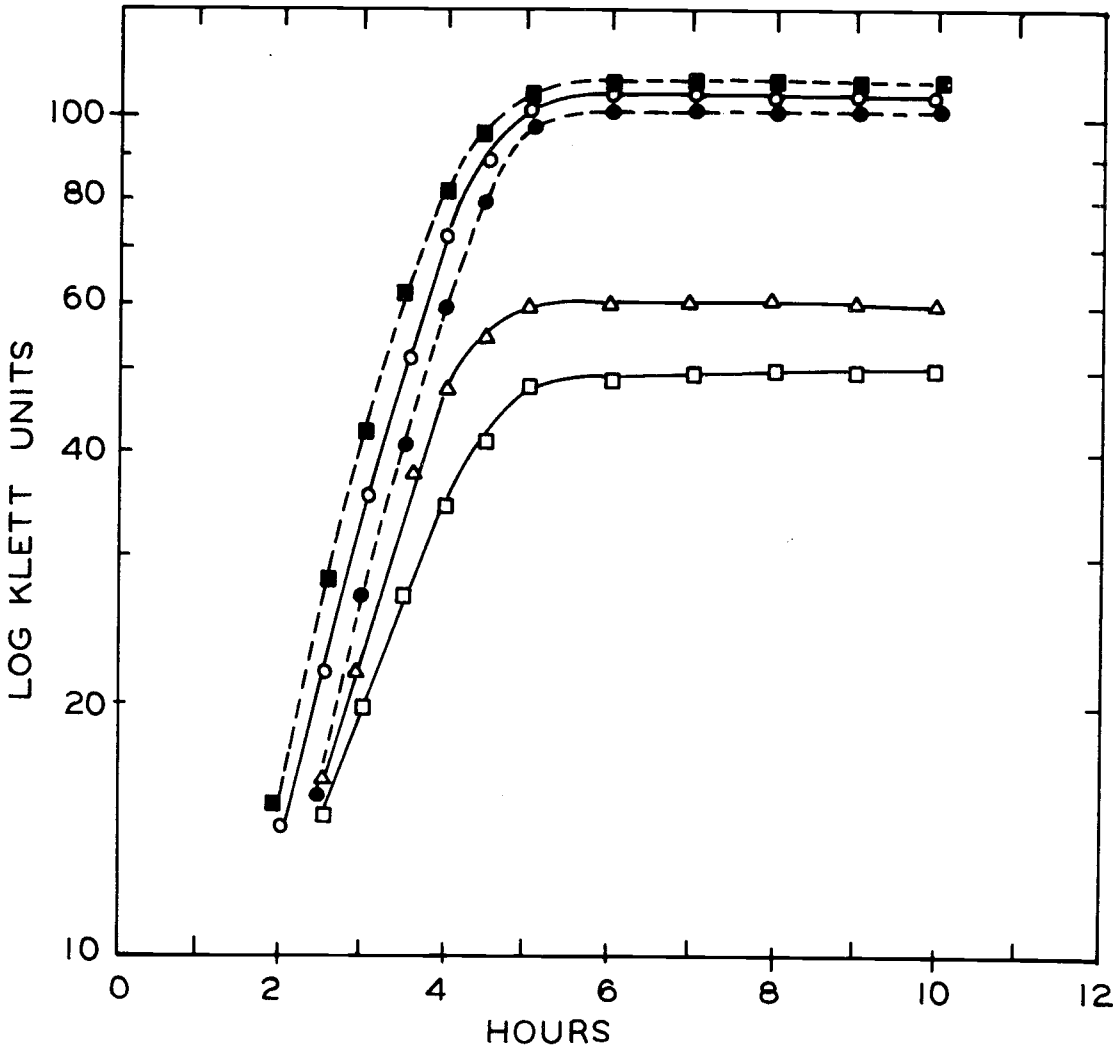


Figure 9. Growth curve of S. lactis C2 in lactic broth with varying amounts of glucose and galactose as follows: 0.02 glucose + 0.1 g galactose (●-●), 0.04 g glucose + 0.1 g galactose (o-o) 0.06 g glucose + 0.1 g galactose (■ - ■), 0.02 g glucose (□ - □) or 0.02 g galactose (△ - △).

different. As can be noted from Figure 10 there are two distinctly different slopes to the growth curves, and there is no real lag between the time glucose is exhausted from the medium and the time when galactose starts to be utilized.

Further efforts were made to determine if the lactic streptococci could utilize glucose and galactose simultaneously. Table 4 depicts an experiment in which S. diacetilactis 18-16 was used to test this hypothesis. This particular organism was used because it releases CO_2 from glucose (Busse, 1963). In order to determine if glucose and galactose were utilized simultaneously the reduction of the specific activity of the CO_2 had to be noted when the two sugars were added together as in the two bottom numbers in Table 4. It could be seen that the presence of glucose reduced the specific activity of CO_2 produced from galactose from 0.081 to 0.054, and the specific activity of CO_2 evolved from glucose was also reduced from 0.081 to 0.052 by the presence of galactose. Apparently galactose-induced cells can utilize both sugars at the same time. The ability of the cells to continue to do this has yet to be proved, for after some time, it is possible that the galactose-induced cells when provided with both sugars would degrade the galactose enzymes and preferentially use glucose.

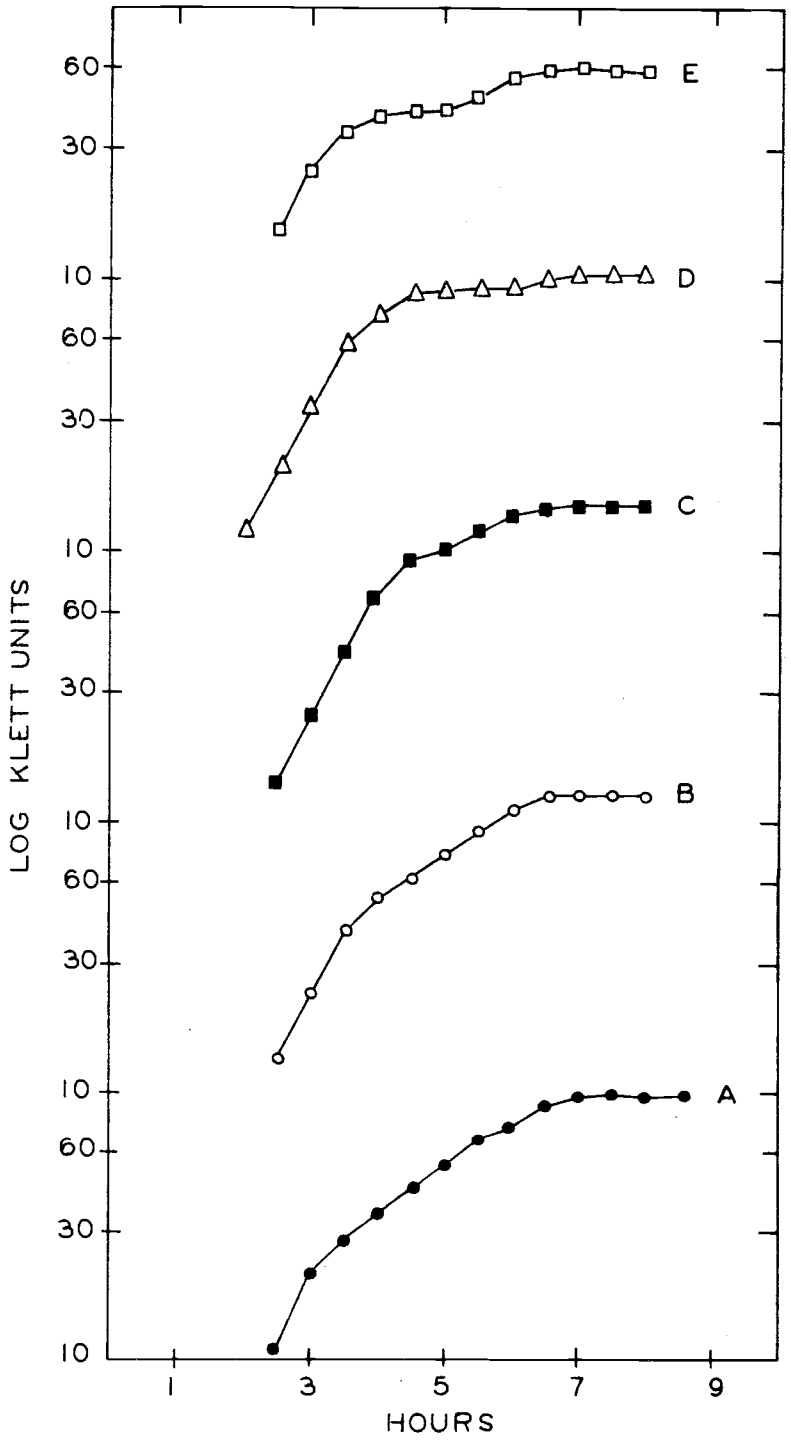


Figure 10. Growth curve of S. lactis 7962 in lactic broth with varying amounts of glucose and galactose as follows: 0.01 g glucose + 0.1 g galactose (●-●), 0.02 g glucose + 0.1 g galactose (o-o), 0.06 g glucose + 0.1 g galactose (■-■), 0.06 g glucose (Δ-Δ), 0.01 g glucose (□-□).

Table 4. The specific activity[a] of CO_2 evolved by galactose adapted cells of S. diacetilactis 18-16 over a 30 minute period at 30°C in different carbohydrates.

Sample	Medium	cpm[b]	Eff(%)	Corrected cpm	Average of 2 Trials (cpm)	ul of Total CO_2 produced[c]	Specific Activity of CO_2
1	33 m moles gal + .2μ C ^{14}C gal	24199	89	27190	26879	6.74	.081
2	33 m moles gal + .2μ C ^{14}C gal	22314	84	26564			
3	33 m moles gal + .2μ Ci ^{14}C glu	22011	85	25895	25373	6.29	.081
4	33 m moles gal + .2μ Ci ^{14}C glu	21121	85	24848			
5	33 m moles gal + .2μ Ci ^{14}C gal + 33 m moles glu	18612	87	21393	21205	7.81	.054
6	33 m moles gal + .2μ Ci ^{14}C gal + 33 m moles glu	18071	86	21013			
7	33 m moles glu + .2μ Ci ^{14}C glu + 33 m moles gal	17434	87	20039	20499	7.81	.052
8	33 m moles glu + .2μ Ci ^{14}C glu + 33 m moles gal	18232	87	20956			

[a]Specific activity is expressed as μ moles labeled CO_2/μ Moles total CO_2/mg cell dry weight/30 minutes.

[b]CPM were corrected to 100% efficiency by the channel ratio method.

[c]The total volume of gas was determined using a Gilson Differential Respirometer and was employed under identical conditions as when the labeled compound was used. The data are expressed as μ 1 CO_2/mg cell dry weight at a total time of 30 minutes and is the average of three determinations.

Differential Respirometry

Experiments were performed involving the collection and measurement of carbon dioxide evolved and oxygen absorbed when washed cells of S. diacetilactis 18-16 were returned to galactose, glucose, lactose, or a mixture of glucose and galactose. It was hoped that insight into the induction of different systems would be gained by observing the cells' respiratory capability when induced in the three different carbohydrates.

Figure 11 illustrates the CO_2 evolved from galactose-induced cells over a one-hour period. It appeared that galactose-induced cells could utilize all three sugars with equal effectiveness. Figure 12 shows the O_2 absorbed by galactose-induced cells in the presence of these same carbohydrates. A noteworthy observation was the high amount of O_2 absorbed when the cells were respiring galactose with >1500 µl/100 mg cell dry wt./hr. compared with the O_2 absorbed when the cells respired glucose (<850 µl/100 mg cell dry wt./hr.). When the cells respired lactose an intermediate amount of O_2 was absorbed (<1100 µl/100 mg cell dry wt./hr.).

Figure 13 depicts the CO_2 evolved from glucose-induced cells over a one hour period. These glucose-induced cells could not utilize all three sugars with the same efficiency. Endogenous cell control was 175 µl/100 mg cell dry wt./hr. and when the cells were placed in galactose, the result obtained was 210 µl/100 mg cell dry wt./hr.

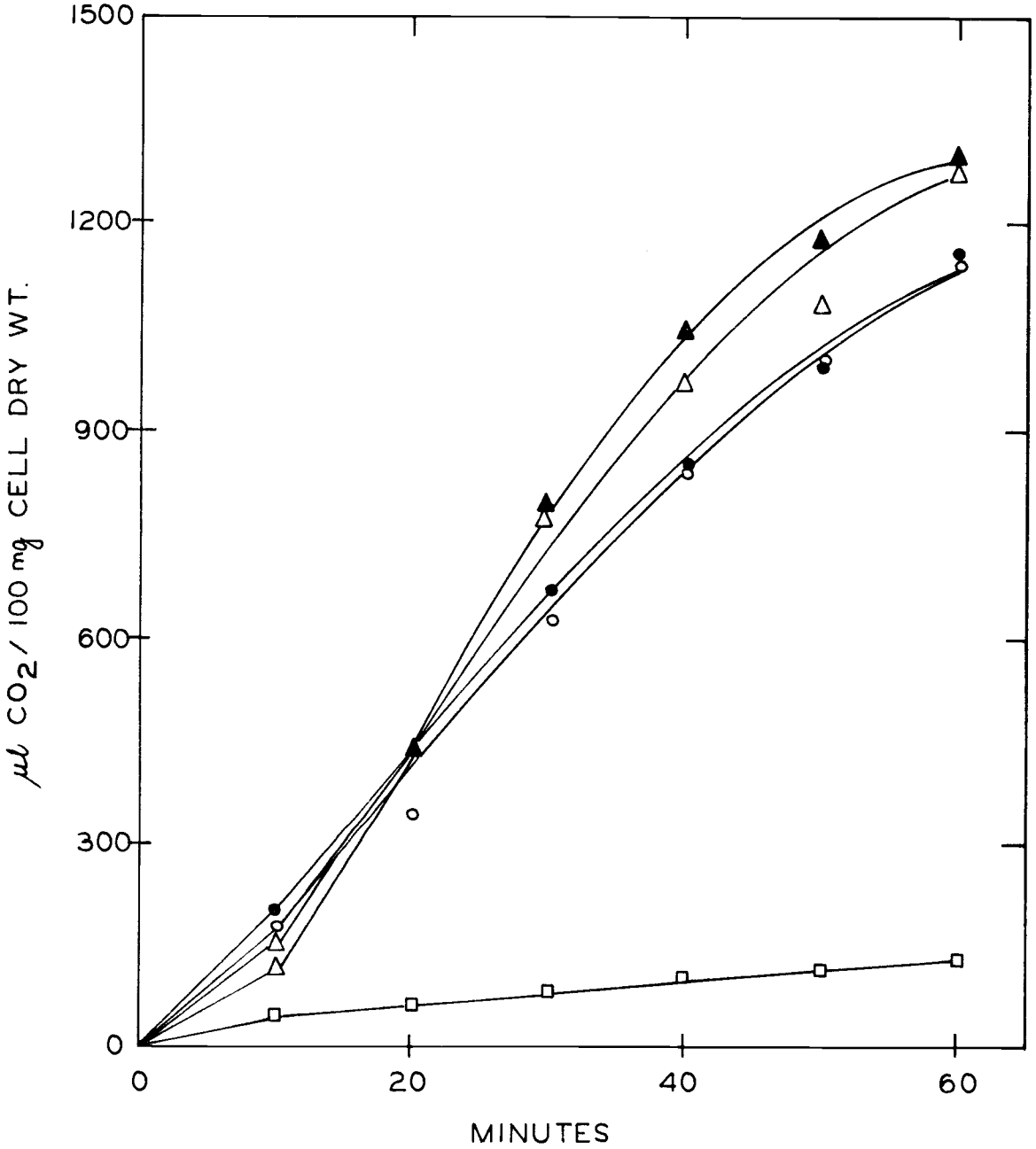


Figure 11. CO_2 evolution by galactose-grown resting cells of S. diacetilactis 18-16 in presence of galactose (●-●), glucose (o-o), lactose (▲ - ▲) glucose or galactose (△ - △); endogenous control: □ - □ .

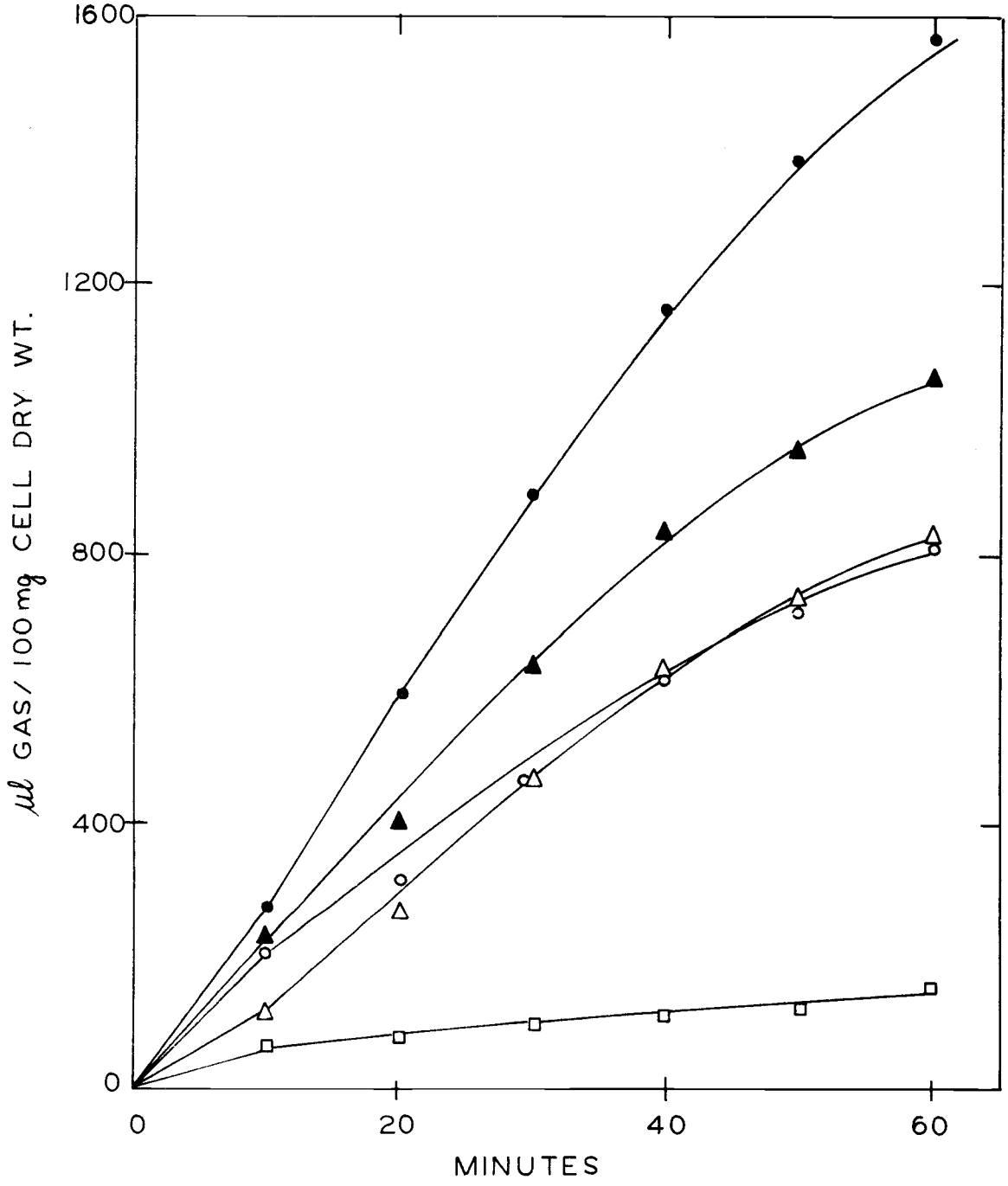


Figure 12. O_2 uptake by galactose-grown resting cells of S. diacetilactis 18-16 in presence of galactose (●-●), glucose (o-o), lactose (▲ - ▲) or glucose + galactose (△ - △); endogenous control: □ - □ .

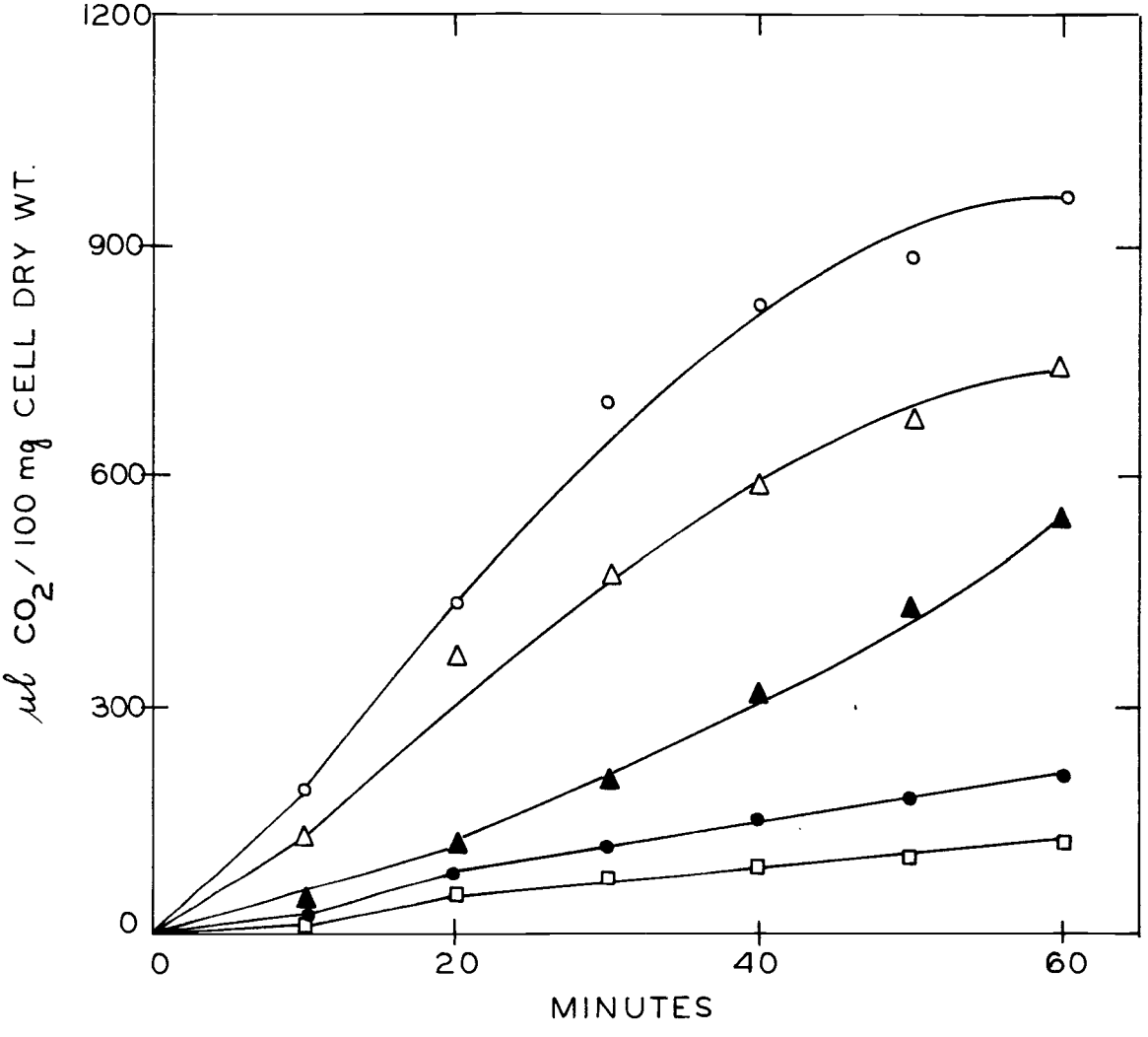


Figure 13. CO_2 evolution by glucose-grown resting cells of S. diacetilactis 18-16 in presence of galactose (●-●), glucose (o-o), lactose (▲ - ▲) or glucose + galactose (△ - △); endogenous control: □ - □ .

Apparently glucose-induced cells cannot utilize galactose. However, after being placed in lactose for 30 minutes, the glucose-induced cells began to metabolize lactose and 500 μl/100 mg cell dry wt./hr. of CO_2 was evolved from lactose compared to 950 μl/100 mg cell dry wt./hr. from glucose. When glucose and galactose were mixed each in a 33 mM final concentration, the amount of CO_2 evolved was only 740 μl/100 mg cell dry wt./hr. The galactose apparently interfered with the transport of glucose.

When uptake of O_2 was observed in glucose-induced cells (Figure 14) a striking thing was noted. When the cells were placed in galactose only, a small amount of O_2 was absorbed (< 400 μl/100 mg cell dry wt./hr.) as compared to the endogenous cell control of 150 μl/100 mg cell dry wt./hr. But when the cells respired lactose the amount of O_2 absorbed was >800 μl/100 mg cell dry wt./hr. Apparently the cells were utilizing the galactose from lactose since the galactose was being phosphorylated during transport.

Lactose-induced cells were able to use glucose and lactose most efficiently. In each case the CO_2 evolved (Figure 15) was approximately 1500 μl/100 mg cell dry wt./hr., whereas in the case of these cells using galactose, the CO_2 produced was only 800 μl/100 mg cell dry wt./hr. When a mixture of glucose and galactose were respired the CO_2 produced was only 700 μl/100 mg cell dry wt./hr. Figure 16 shows the O_2 absorbed by lactose-induced cells exposed to

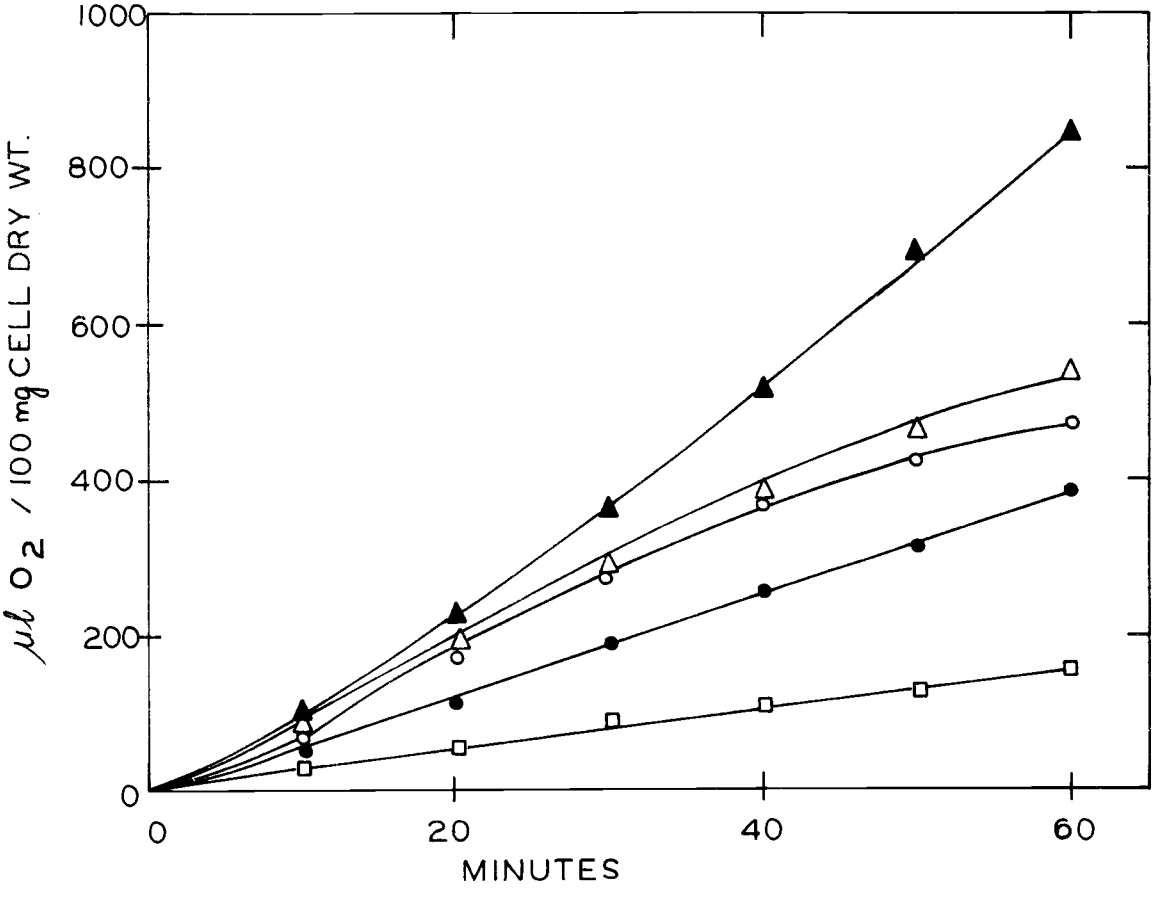


Figure 14. O_2 uptake by glucose-grown resting cells of S. diacetilactis 18-16 in presence of galactose (●-●), glucose (o-o), lactose (▲ - ▲) or glucose + galactose (△ - △); endogenous control: □ - □.

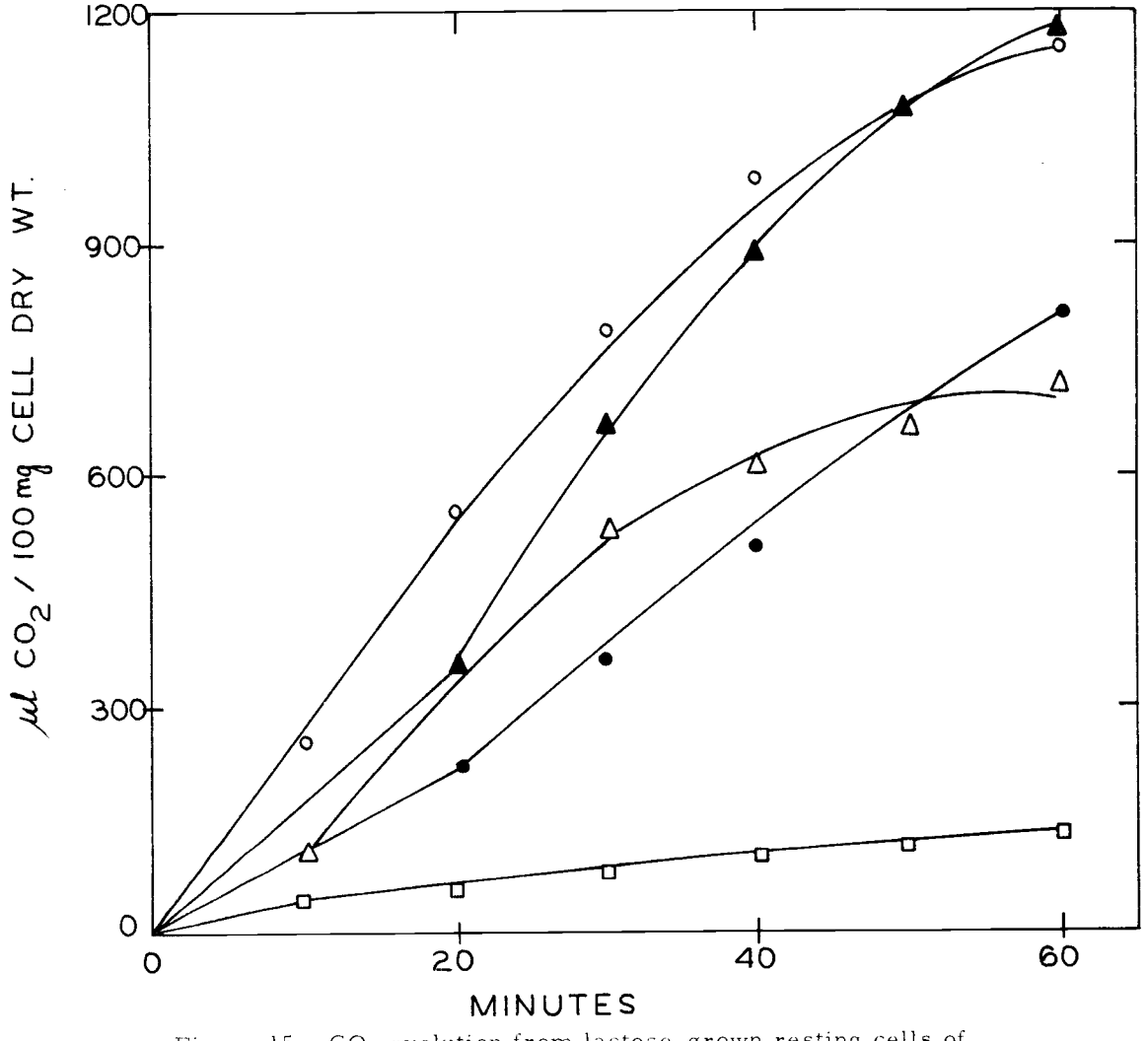


Figure 15. CO_2 evolution from lactose-grown resting cells of S. diacetilactis 18-16 in presence of galactose (o-o), glucose (o-o), lactose (▲ - ▲) or glucose + galactose (△ - △); endogenous control: □ - □ .

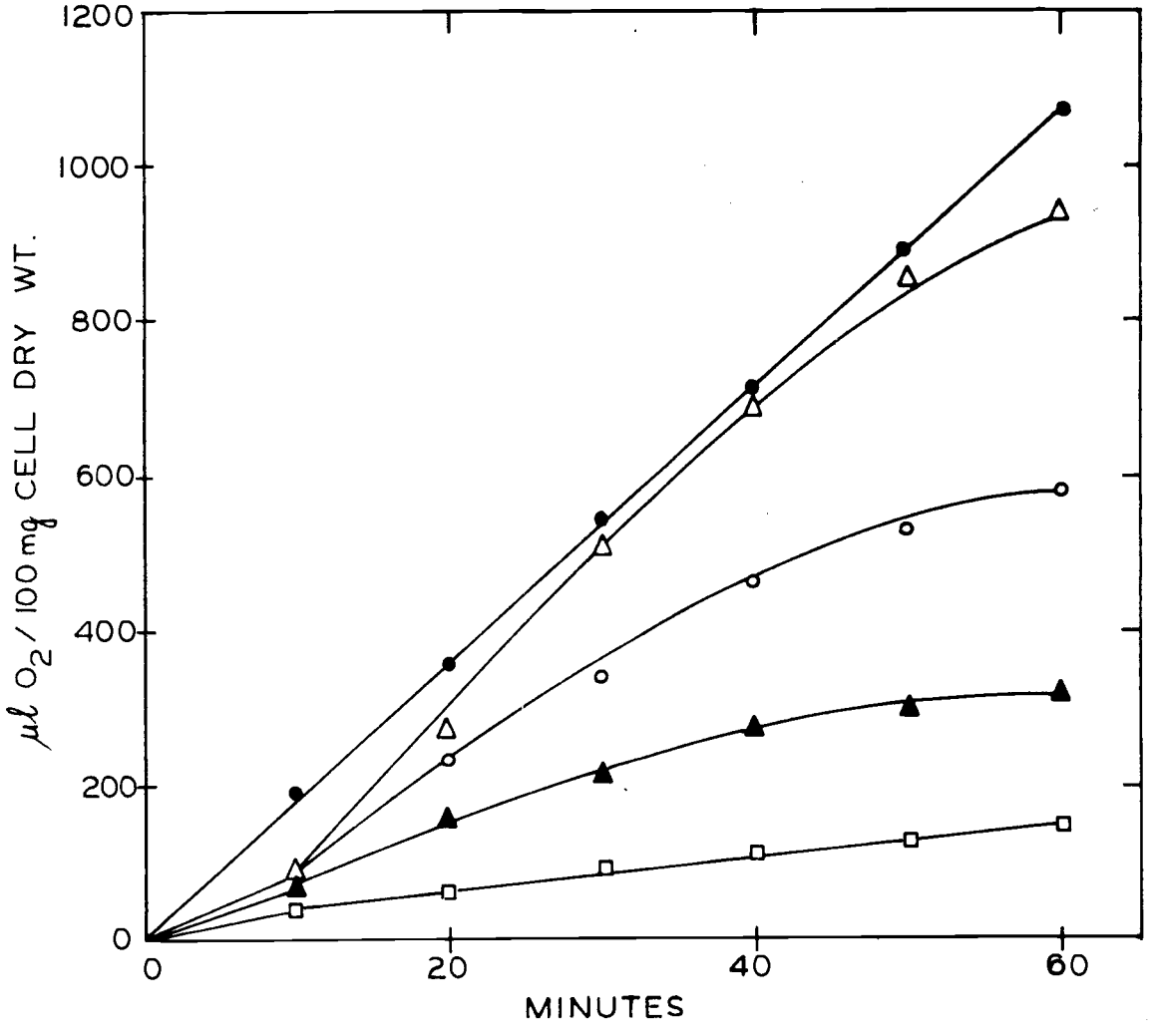


Figure 16. O_2 uptake of lactose-grown resting cells of S. diacetilactis 18-16 in presence of galactose (o-o), glucose (o-o), lactose (▲ - ▲) or glucose + galactose (△ - △); endogenous control: □ - □ .

the different substrates. When the cells were placed in galactose, the greatest amount of O_2 was absorbed (>1050 µl/100 mg cell dry wt./hr.). Apparently lactose-induced cells can utilize galactose in a mixture of glucose and galactose, for the O_2 absorbed approximated that from galactose alone (950 µl/100 mg cell dry wt./hr.). Lactose-induced cells took up very little O_2 (< 350 µl/100 mg cell dry wt./hr.).

Table 5 is included to illustrate the type of differential respirometry data obtained when galactose-induced cells were exposed to galactose, glucose, lactose, and a mixture of equal amounts of glucose and galactose. The table is included to show typical results obtained in these experiments. Averages of the three trials were obtained and accumulative totals tabulated. These data were graphed on Figures 11 and 12.

Since it was noted that galactose-induced cells absorbed a much greater amount of oxygen when exposed to galactose, an assay for galactose oxidase which requires elemental O_2 was performed and the results are given in Table 6. Data are recorded as units of enzyme activity on the various substrates tested. From the results of this experiment, it appeared that galactose oxidase was not present in S. diacetilactis and therefore not responsible for the higher absorption of O_2 in the presence of galactose.

Table 5. CO_2 evolution and O_2 uptake at ten-minute intervals by resting cells of S. diacetilactis 18-16 when induced in galactose broth and returned to four different substrates at 33 mM concentration[a].

		10		20		30		40		50		60	
Substrate	Trial	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2
Gal	1	1.92	2.65	2.84	3.26	2.25	3.07	1.90	2.91	1.38	2.08	1.59	2.50
	2	1.0	2.57	2.28	3.01	2.23	2.99	1.72	2.76	1.61	2.25	1.69	2.57
	3	2.16	3.03	2.43	3.21	2.06	3.00	1.74	2.61	1.38	2.13	1.68	2.49
Glu	1	1.7	2.03	1.81	1.09	2.86	1.38	2.43	1.48	1.69	1.07	1.48	1.02
	2	1.73	2.05	1.43	1.07	2.83	1.63	2.03	1.28	1.53	1.07	1.29	1.02
	3	1.91	2.19	1.79	1.17	2.79	1.86	2.03	1.36	1.52	.991	1.45	1.03
Lac	1	1.41	2.11	2.65	1.59	3.9	2.29	2.46	2.15	1.36	1.26	1.07	0.96
	2	1.92	2.47	2.61	1.69	3.62	2.41	2.21	1.89	1.30	1.22	1.02	0.95
	3	1.45	2.39	3.04	1.77	3.2	2.42	2.74	1.93	1.31	1.17	1.49	1.33
Glu + Gal[b]	1	1.02	1.02	3.28	1.63	3.38	2.09	1.97	1.53	1.11	.96	0.93	0.93
	2	1.28	1.28	3.22	1.48	3.39	2.16	1.79	1.41	1.28	1.11	0.88	0.88
	3	1.21	1.21	3.25	1.53	3.39	1.93	1.97	1.61	1.26	1.07	0.90	0.90
	AVERAGES OF THE THREE TRIALS												
Gal		2.04	2.75	2.52	3.16	2.18	3.02	1.79	2.76	1.46	2.15	1.65	2.52
Glu		1.78	2.09	1.68	1.11	2.83	1.62	2.16	1.37	1.58	1.02	1.41	1.02
Lac		1.59	2.32	2.77	1.68	3.57	2.37	2.47	1.99	1.32	1.22	1.19	1.08
Glu + Gal[b]		1.17	1.17	3.25	1.55	3.39	2.06	1.91	1.52	1.22	1.05	0.90	0.90
	ACCUMULATIVE TOTALS (FROM AVERAGES)												
Gal		2.04	2.75	4.56	5.91	6.74	8.93	8.53	11.69	9.99	13.84	11.64	16.36
Glu		1.78	2.09	3.46	3.20	6.29	4.82	8.45	6.19	10.03	7.21	11.44	8.23
Lac		1.59	2.32	4.36	4.40	7.93	6.37	10.40	8.36	11.72	9.58	12.91	10.66
Glu + Gal[b]		1.17	1.17	4.42	2.72	7.81	4.78	9.72	6.30	10.94	7.35	11.84	8.25
Cell Control		0.5	0.65	0.63	0.80	0.85	0.90	1.0	1.1	1.15	1.3	1.3	1.6

[a]Data are given as μ l/mg cell dry weight as determined by differential respirometry.

[b]16.5 mM concentration of each carbohydrate.

Table 6. Galactose oxidase activity[a] of S. diacetilactis 18-16 in various carbohydrates.[b]

Substrate	Units/mg Protein
Galactose	0.04
Glucose-1-phosphate	0.04
Glucose-6-phosphate	0.03
Galactose-6-phosphate	0.00
Glucose	0.00

[a]One unit of activity was defined as the amount of enzyme causing an absorbance change of 1.0 per ten minutes under assay conditions.

[b]Conditions of the assay were to add 1 ml of a chromogen-peroxidase mixture to 0.05 ml of substrate and 0.05 ml of crude enzyme in a cuvette. The mixture was incubated ten minutes at 30 C and absorbance read at 420 nm. Preparation of the chromogen-peroxidase mixture is described in the Materials and Methods section.

Galactose Metabolism

Galactose Utilization in S. lactis C2

Typical data showing the effect of phosphate donor on galactose utilization in S. lactis appear in Table 7. These experiments were designed to study the disappearance of free galactose in the presence of ATP or PEP. Results are expressed as μg/ml of galactose remaining. It should be noted that ATP is required for optimal removal of galactose; however, it was also apparent that some galactose was used when PEP was the phosphate donor. Since free galactose was disappearing with time, it became of importance to

determine its fate. A technique was devised using Glucostat and Galactostat reagents to determine the intermediates in its metabolism.

Table 7. The influence of PEP, ATP and KF on the utilization of galactose by resting, toluene-acetone-treated, galactose-grown (six hour) cells of Streptococcus lactis C2.

Time (min)	Conditions[a]			
	Galactose[b]	Galactose + KF	Galactose + KF + PEP	Galactose + KF + ATP
0	168	168	168	168
5	148	164	162	84
10	140	168	140	7
20	162	162	120	21

[a]Experimental conditions were as follows: galactose concentration, 168 μg/ml; KF 0.03 M and ATP or PEP, 0.01 M. 5 ml of a toluene-acetone treated suspension of S. lactis cells were added to each solution to a final O.D. of 0.45.

[b]Data are expressed in μg/ml of galactose

Conversion of Galactose to Glucose

Table 8 shows typical data for the utilization of galactose, suggesting that metabolism occurs via galactose-1-phosphate to glucose-1-phosphate and then to glucose-6-phosphate. The exact enzymatic mechanism for this conversion is not known; however, a key Leloir pathway enzyme was shown to be present, and it appeared that galactose was first phosphorylated by ATP to form galactose-1-phosphate. This appeared true because the level of free galactose declined very rapidly in the presence of ATP while the level of

galactose-1-phosphate increased and later declined as it was converted to glucose intermediates. To obtain these results, it was necessary to block the glycolytic pathway with KF as indicated.

Table 8. Compounds formed from galactose by toluene-acetone-treated, resting cells of Streptococcus lactis C2 grown (six hour) on galactose.

Time (min)	Compounds found (μg/ml)[a]					
	Glu	Gal	Gal-1-P	Glu-1-P	Glu-6-P	Gal-6-P
0	0	800	0	0	0	0
4	8	480	280	40	16	0
8	8	280	364	56	24	0
12	8	204	410	64	56	0
16	8	14	508	80	72	0
20	8	14	408	80	64	0
24	8	14	380	88	64	0
28	8	14	240	88	72	0
32	8	56	168	96	64	0

[a]Experimental conditions as follows: S. lactis cells were toluene-acetone treated and suspended in galactose solution (800 μg/ml) to an O. D. of 0.35 with 0.3 M KF and 0.01 M ATP. Incubation was at 37 C. Every four minutes, samples were removed, quickly frozen in acetone-dry ice and later thawed and assayed with Glucostat and Galactostat as indicated in the Materials and Methods section.

Additional evidence that galactose was converted to glucose was obtained by growing S. diacetilactis 18-16 in 0.2 percent lactose broth supplemented with 0.1 μCi of radioactive lactose

(glu-gal-$\mu^{14}C$) and testing for $^{14}CO_2$ evolution and synthesis of ^{14}C-labeled serine. Serine is synthesized by this organism from 3-phosphoglyceric acid which is derived from glucose (Davis et al., 1969). If labeled serine were found it would be direct proof that the galactose-^{14}C of lactose was converted to glucose. During such an experiment, CO_2 was evolved during six hours of growth and 1625 cpm were counted on the filter paper due to the $^{14}CO_2$. Proof that galactose was entering mainline metabolic pathways was less definite since a complex medium was required for growth and CO_2 evolution, and this medium apparently suppressed the synthesis of serine from glucose; when a protein hydrolysate of the above cells was tested for radioactivity, serine was not labeled.

For a long time during this research it was not known what was effecting the conversion of galactose to glucose. The enzymes of the Leloir pathway could be involved, but preliminary assays for these enzymes were unsuccessful. Since the enzymes could not be detected it was decided to check for the stimulation of growth in galactose by adding uracil to the growth medium in varying amounts. S. cremoris HP was chosen because it was known to grow slowly in galactose. This is true for most strains of S. cremoris. Figure 17 depicts growth curves of S. cremoris in 1 percent galactose with varying amounts of uracil added. Adding uracil did not stimulate growth, but in the two higher concentrations it appeared to inhibit growth.

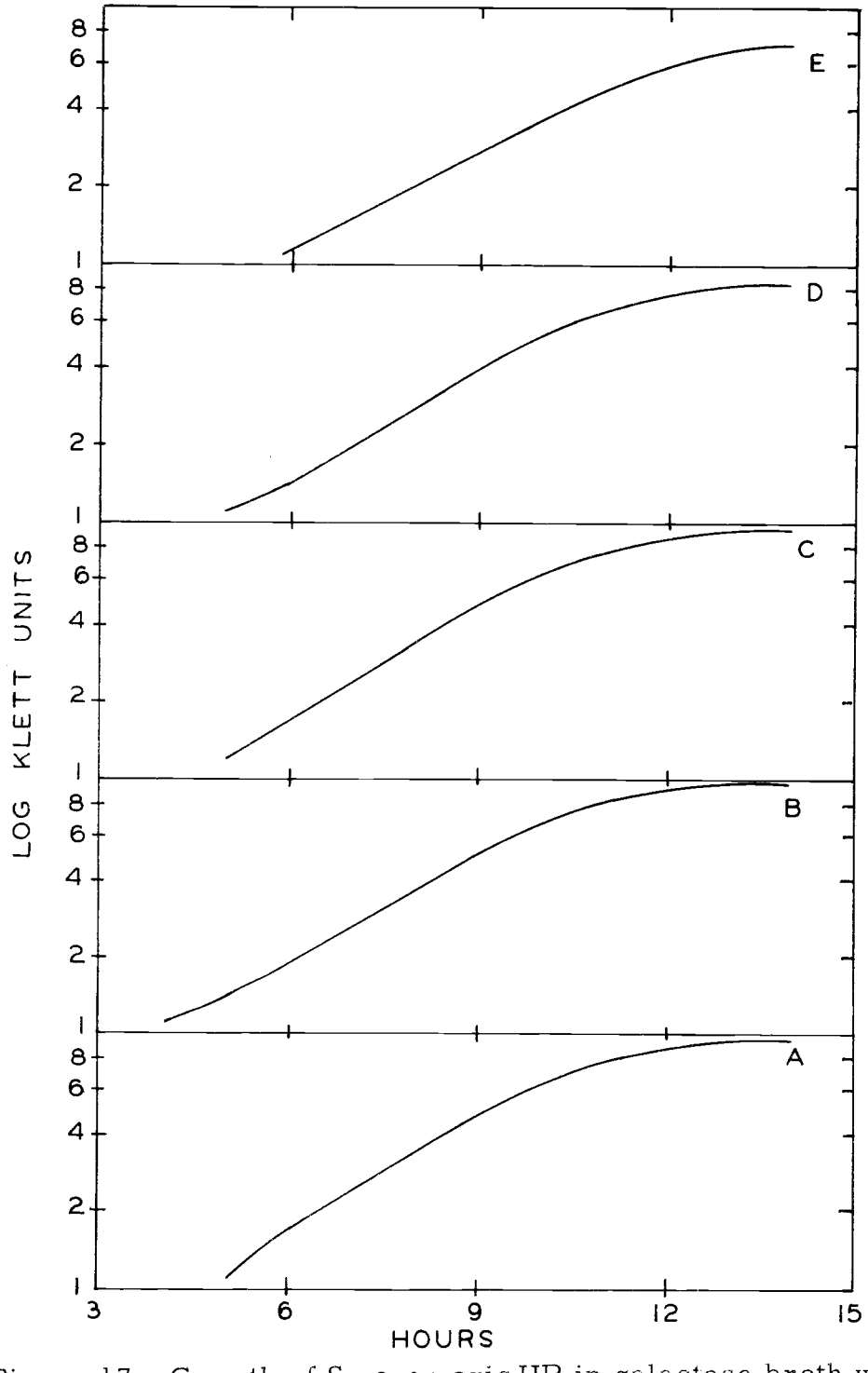
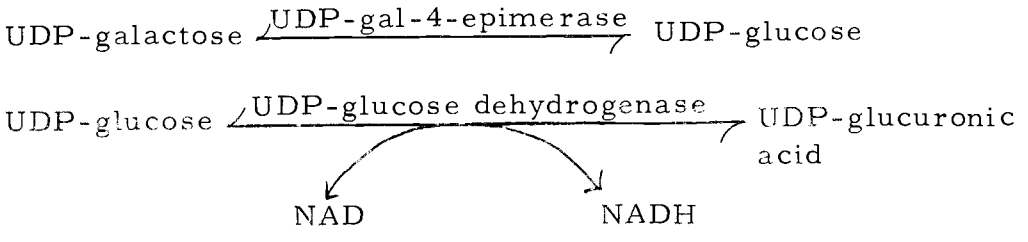


Figure 17. Growth of S. cremoris HP in galactose broth with varying amounts of uracil. One percent galactose (A) was supplemented with 0.02 g (B), 0.06 g (C), 0.2 g (D) or 0.6 g (E) of uracil.

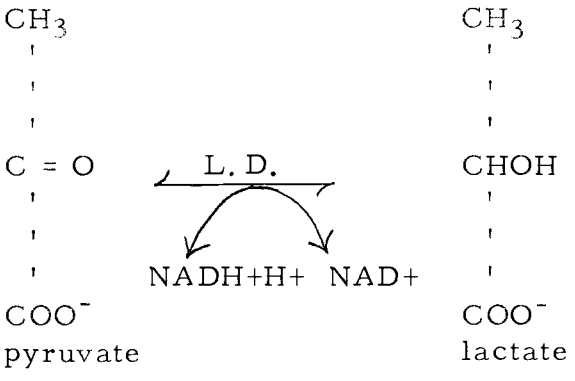
Doubling times were two hours for 1 percent galactose and 1 percent galactose plus 0.02 or 0.06 g of uracil. However, when 0.2 g of uracil was added to 1 percent galactose, the doubling time was increased to 130 minutes; when 0.6 g of uracil was added to 1 percent galactose, the doubling time increased to 144 minutes.

Uridine Diphosphogalactose-4-Epimerase (UDP gal-4-epimerase)

In the initial studies of enzymes it has been common practice to assay for the presence of the enzyme in crude cell preparations before beginning any purification techniques. In preliminary work involving galactose metabolism, this technique was tried in the assay of UDP gal-4-epimerase with no success. This enzymatic assay utilized UDP galactose as a substrate. If the epimerase were present, one would expect UDP glucose to be formed; therefore, the coupling of the epimerization to UDP glucose dehydrogenase would allow one to read the reaction spectrophotometrically because of the reduction of NAD by the dehydrogenation reaction. To clarify this, the following summary is included:



However, all attempts to follow reduction of NAD at 340 nm failed. In the crude cell preparation it was known that the enzyme, lactate dehydrogenase, was present. Lactate dehydrogenase catalyzes the following reaction:



This reaction has its equilibrium far to the right. However, it was known from the report by Mattsson (1965) that pyruvate can be complexed by the addition of 0.2 M semicarbazide, and the reaction's equilibrium shifted to the left. It seemed reasonable to assume that NAD reduction was not observed in our assay procedure because lactate dehydrogenase oxidized NADH back to NAD as fast as it was produced.

Figure 18 depicts an assay for UDP-gal-4-epimerase by the standard method but with 0.2 M semicarbazide incorporated in the glycine buffer. From this figure it can be seen that activity is only demonstrable with the semicarbazide added. To prove that lactate dehydrogenase was preventing the reaction from being observed,

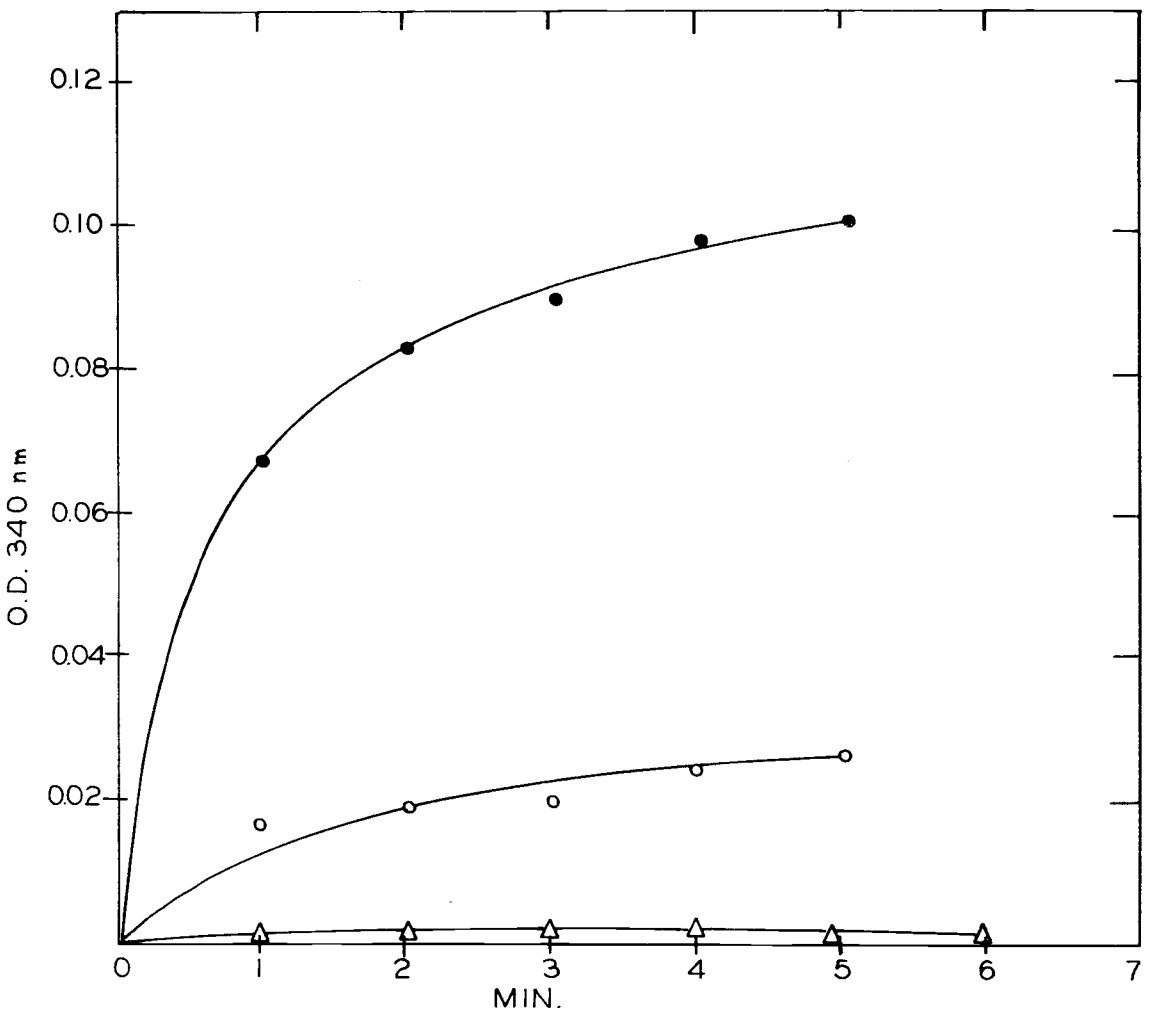


Figure 18. Activity of UDP gal-4-epimerase in crude cell-free extracts of S. lactis C2 with (●-●) and without (△-△) semicarbazide in the buffer. A control commercial enzyme preparation also was assayed (o-o) with added semicarbizide.

pyruvate was added in excess along with the semicarbazide. Under these conditions, it was noted that activity of UDP-galactose-4-epimerase was reduced to almost nothing in the presence of 0.4 M pyruvate. When 0.004 M pyruvate was added there was essentially no reduction in activity (results not shown). It was of concern that the results obtained with the addition of 0.4 M pyruvate might be caused by a pH change, since pyruvate in this concentration would lower the pH. To eliminate this possibility another system was derived in which pyruvate added as a buffered solution was used. Figure 19 depicts the results of such an assay. Reduction of pH was therefore ruled out as the cause of reduction in activity.

Figure 20 depicts the activity of UDP gal-4-epimerase in cells grown in three different carbohydrates. The activities of the enzyme were very similar in lactose and galactose, both of which induced the enzyme. Some activity was noted in glucose-grown cells, but it could have been due to the presence of a small amount of inducer in the complex medium. Table 9 gives the specific activity of UDP gal-4-epimerase in four different substrates. The highest specific activity (0.66) was noted in galactose-grown cells and the lowest specific activity (0.14) in cells grown in a mixture of glucose and galactose.

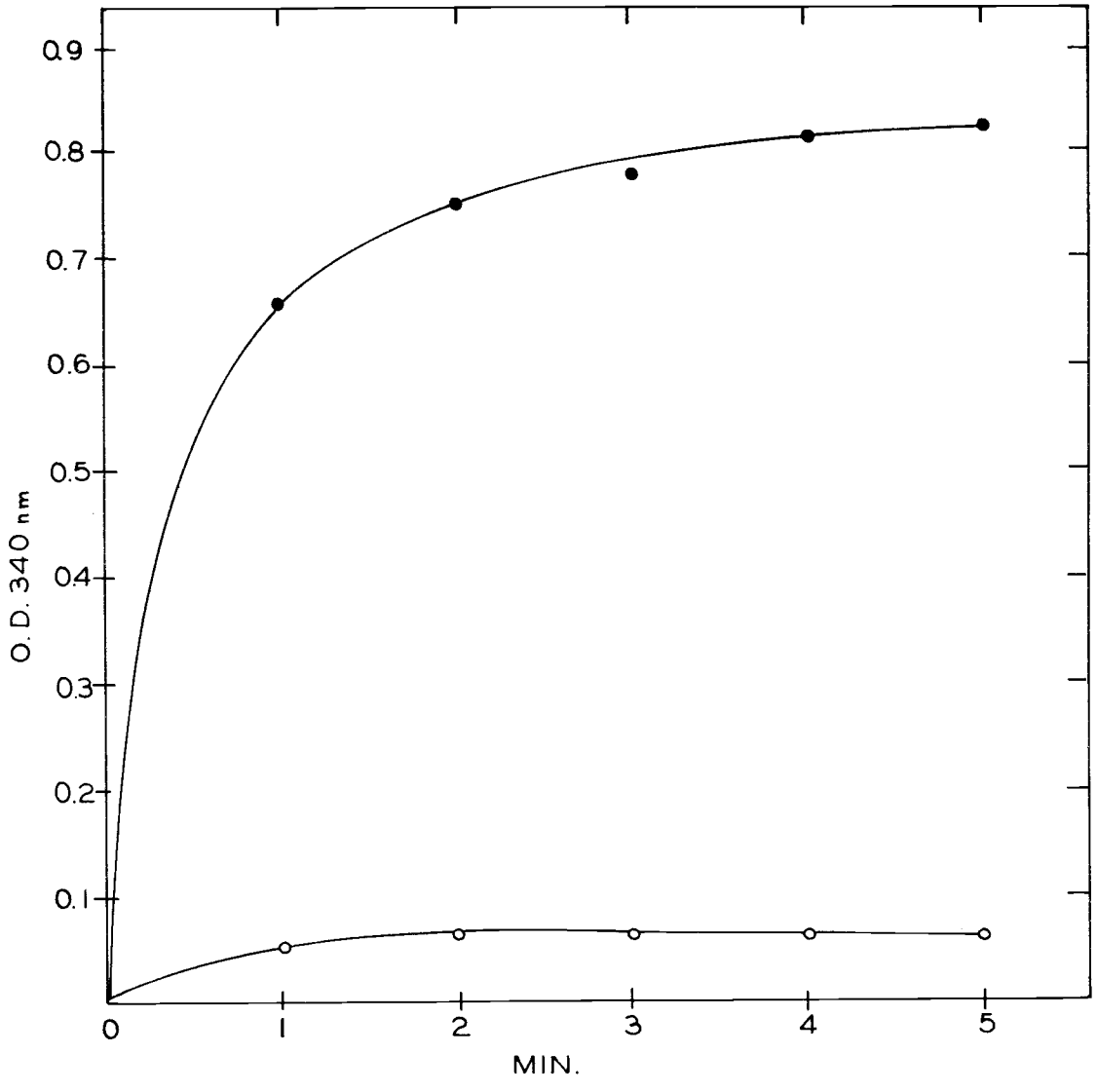


Figure 19. Activity of UDP gal-4-epimerase in crude cell-free extracts of S. lactis C10 in glycine-semicarbazide buffer with (o-o) and without (●-●) 0.01 M sodium pyruvate added.

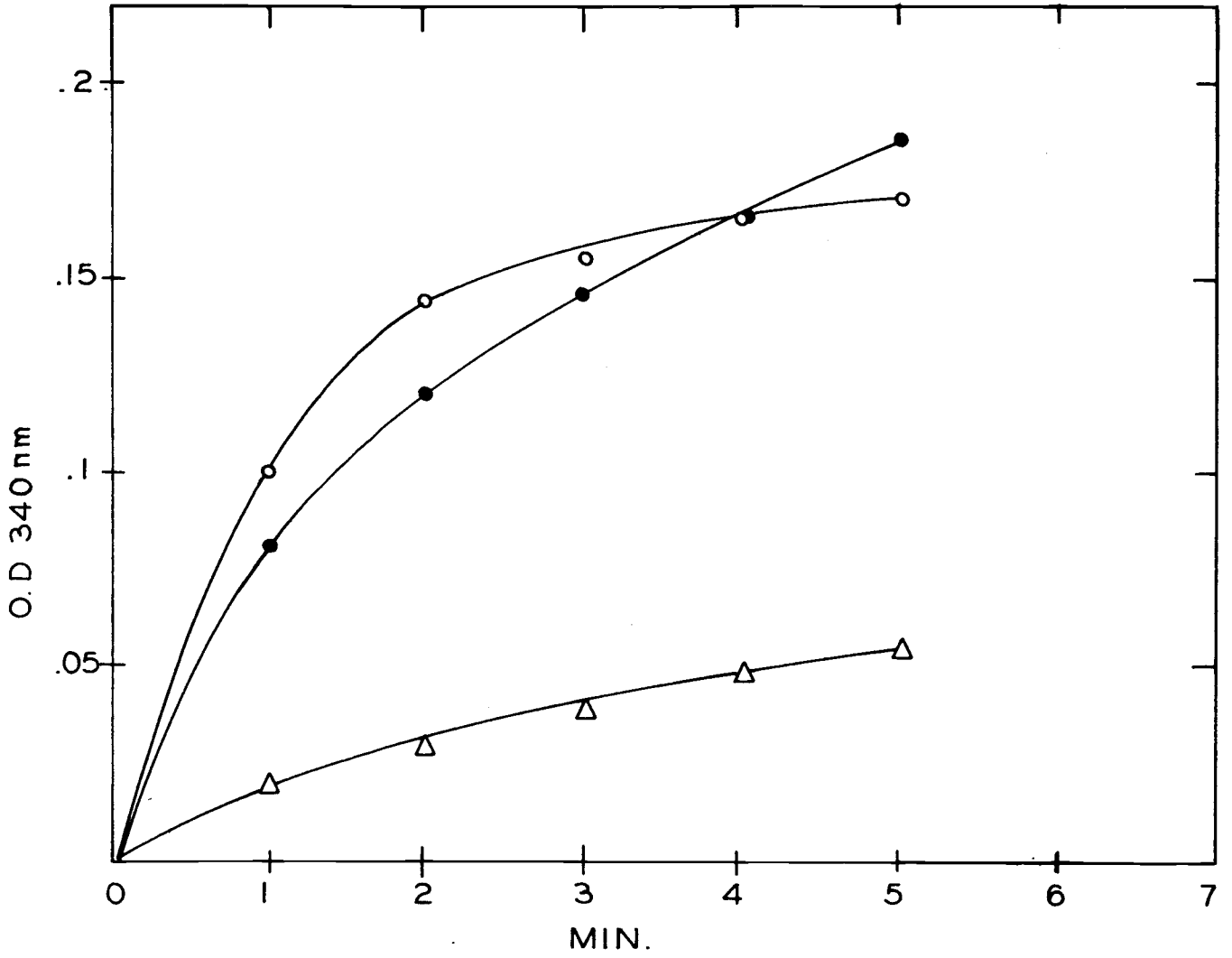


Figure 20. Activity of UDP gal-4-epimerase in crude cell-free extracts of S. lactis C2 when cells were grown on galactose (o-o), lactose (●-●) or glucose (Δ - Δ).

Table 9. Activity[a] of UDP-galactose-4-epimerase in S. lactis C2 grown in different carbohydrates to late log phase.

Substrate Grown On	Specific Activity (units[b]/mg protein)
1% galactose	0.66
1% glucose	0.24
1% lactose	0.51
0.5% galactose + 0.5% glucose	0.14

[a]Assay was performed as described in Materials and Methods.
[b]A unit is defined in Materials and Methods and Table 10.

In studying the induction of this enzyme in the lactic streptococci, cells were grown in glucose, washed, and returned to 1 percent galactose broth. It was found that the enzyme was very quickly induced in S. lactis (Figure 21). This figure also shows that near maximal activity was reached in S. lactis in 20 minutes. This same type of induction pattern could not be obtained in S. cremoris even after repeated attempts. A fairly high level of the enzyme was found to be present in the S. cremoris cells at time of introduction to the inducing medium, and the specific activity did not appear to increase over a one-hour time interval.

UDP gal-4-epimerase was found to be present in a number of lactic streptococci (Table 10). The highest specific activity of the enzyme was present in S. lactis C10 and lowest in S. diacetilactis $3D_1$. The enzyme was also found present in cells grown in milk.

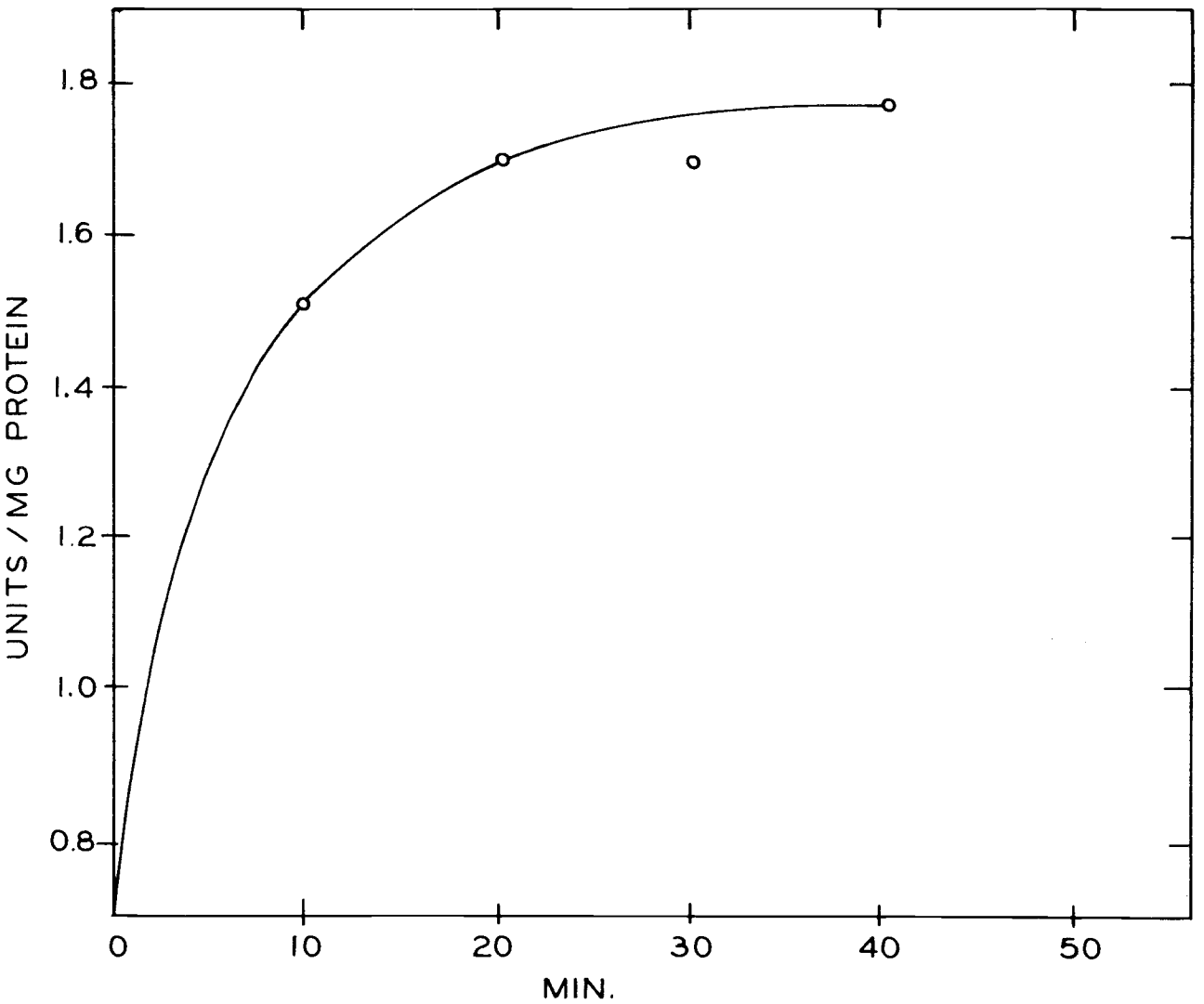


Figure 21. Induction of UDP gal-4-epimerase in glucose-grown cells of S. lactis C10 when returned to a medium with 1% galactose.

Cells were grown for ten hours hours in 11 percent nonfat milk, centrifuged, washed several times to remove debris, and suspended in 30 ml of buffer. S. lactis C10 showed a specific activity of 0.87 units/mg protein, and S. cremoris 75, a slow strain, showed 0.69 units/mg protein. These cells were induced in lactose before being introduced into the milk; however, when S. lactis C10 was induced in glucose and then grown for ten hours in milk, the specific activity of UDP gal-4-epimerase was reduced to 0.68 units/mg protein.

Table 10. Activity[a] of UDP galactose-4-epimerase from various lactic streptococci grown to late log phase in galactose broth.

Organism	Specific Activity (units[b]/mg Protein)
Streptococcus lactis	
C2	0.66
7962	0.57
UN	0.82
C10	1.76
Streptococcus cremoris	
459	0.44
Hp	0.95
163	0.56
Streptococcus diacetilactis	
1816	0.65
3D1	0.36
DRC1	0.44

[a] Method of assay is described in Materials and Methods.

[b] A unit was defined as the amount of enzyme needed to convert 1 μMole of UDP galactose to UDP glucose per minute at pH 8.8 and 25 C.

Galactose-1-phosphate Uridyl Transferase in Lactic Streptococci

During assays for a second enzyme of the Leloir pathway, Galactose-1-phosphate uridyl transferase, it was determined that a small amount was present in crude extracts of S. lactis C2. Evidence for its presence was obtained when more residual UDP-glucose (detected by adding UDP-glucose dehydrogenase and NAD^+ and reading reaction at 340 nm) was found when crude extract of S. lactis C2 was added to UDP-glucose alone as compared to when the extract was added to UDP-glucose and galactose-1-phosphate. The amount of NAD^+ reduced when crude extract was added to UDP-glucose alone was 7.74×10^{-5} M/l as calculated from the equations: Δ O.D. = $\varepsilon \times l \times C$, in which Δ O.D. = change in optical density, ε = molar extinction coefficient for NADH, l = length of light path in cuvette (1 cm), and C = concentration in Moles/l of reduced NAD. The amount of NAD^+ reduced when crude extract was added to UDP glucose and galactose-1-phosphate was 7.22×10^{-5} Moles/l. It was clear therefore that some transferase was present to remove some of the UDP glucose resulting in less NAD^+ reduction in this latter case.

Galactose Transport in S. lactis C2

In an attempt to better characterize the transport of galactose by lactic streptococci, experiments were performed using whole cells

of S. lactis C2 and membrane filter techniques to determine which sugars competed, which energy sources stimulated, and which metabolic inhibitors interfered with the uptake of galactose-1-^{14}C. Table 11 illustrates the CPM obtained per mg cell dry weight per minute of uptake when various sugars were studied for completion. Galactose, as expected, competed the most. All other sugars listed competed to some extent, with TMG and glucose being the next most effective.

Table 11. Galactose transport in S. lactis C2 and the effect of competing carbohydrates.

Competing Sugar	cpm/mg Cell Dry Wt./Min.
Galactose	1506
Fucose	45510
Mannose	29609
Glucose	17910
Lactose	26891
ONPG	57922
TMG	7761
None	79708

The uptake of galactose was performed according to the Methods section with each competing sugar at a final concentration of 2 mM. Final reaction mixture was in 2 ml volumes containing 0.2 μCi/ml of galactose-1-^{14}C, 2 mM of a competing sugar, and approximately 0.1 mg/ml of intact S. lactis C2 cells in 0.1 M sodium phosphate buffer, pH 7.0.

Table 12 depicts the results of experiments performed to elucidate which metabolic inhibitors interfered with galactose transport. DNP in a final concentration of 10^{-3} M was found to be an inhibitor, but at a concentration of 10^{-4} M it did not appear to inhibit transport. Sulfhydryl inhibitors did not appear to inhibit galactose uptake, nor did arsenate or rotenone. The most effective inhibitor was KCN at 10^{-2} M. Azide, fluoride, and iodoacetate were also inhibitors of galactose uptake.

In a search for energy sources which influenced galactose uptake (Table 13), it was found that ATP and arginine stimulated uptake of galactose the most. Ascorbate and phenazine methosulfate, a potent stimulator of galactose uptake in E. coli (Kerwar et al., 1972), did not stimulate uptake in S. lactis. D-lactate also did not stimulate the uptake of galactose. Sodium acetate appeared to stimulate slightly but sodium citrate and acetyl phosphate appeared to have no effect. PEP inhibited uptake as did fructose 1,6-diphosphate and galactose-6-phosphate.

Figure 22 depicts a time course study of the uptake of galactose in S. lactis C2 with varying concentrations of fucose (6-deoxygalactose) added as a competing sugar. As can be seen, increasing the concentration of fucose enhanced the competitive effect.

Table 12. Galactose transport in S. lactis C2 and the effect of metabolic inhibitors.[a]

Inhibitor and Concentration	cpm/mg Cell Dry Wt. /Min. of Uptake	% Inhibition[c]
DNP[b] 10^{-3}	37,404	41
DNP 10^{-4}	65,140	None
Arsenate 10^{-2}	63,362	None
PCMB 10^{-3}	61,198	3
PCMB 10^{-4}	66,924	None
Rotenone 10^{-2}	62,814	None
Rotenone 10^{-3}	67,854	None
NEM 10^{-3}	60,840	4
KCN 10^{-2}	12,158	81
NaN_3 10^{-2}	31,980	49
NaN_3 10^{-3}	41,546	34
KF 3×10^{-2}	40,856	35
Iodoacetate 8×10^{-3}	52,044	18
Control (no inhibitor)	63,094	---

[a]Assay was performed as described in Table 11 and Methods.

[b]Abbreviations used:
DNP - dinitrophenol
PCMB - p-chloromercurobenzoate
NEM - n-ethylmalamide
KCN - potassium cyanide
NaN_3 - sodium azide
KF - potassium fluoride

[c]$\%\ \text{inhibition} = \left(\frac{63,094 - \text{observed counts}}{63,094}\right) 100$

Table 13. Galactose transport in S. lactis C2 and the effect of energy sources.

Energy Source	cpm/mg Cell Dry Wt. /Min. of Uptake	% Stimulation
Ascorbate + phenazine methosulfate	9869	None
D-lactate	44393	None
PEP	36298	None
ATP	91297	54
Sodium acetate	69192	17
Arginine	117840	99
Sodium citrate	59274	None
Fructose 1, 6-diphosphate	27276	None
Galactose-6-phosphate	14404	None
Acetyl phosphate	55763	None
Control (nothing added)	59244	---

[a]Assay was performed as described in Table 11 and Methods, and the final concentration of each energy source was 20 mM.

[b]% Stimulation = $\left(\frac{\text{observed count} - 59244}{59244}\right) 100$

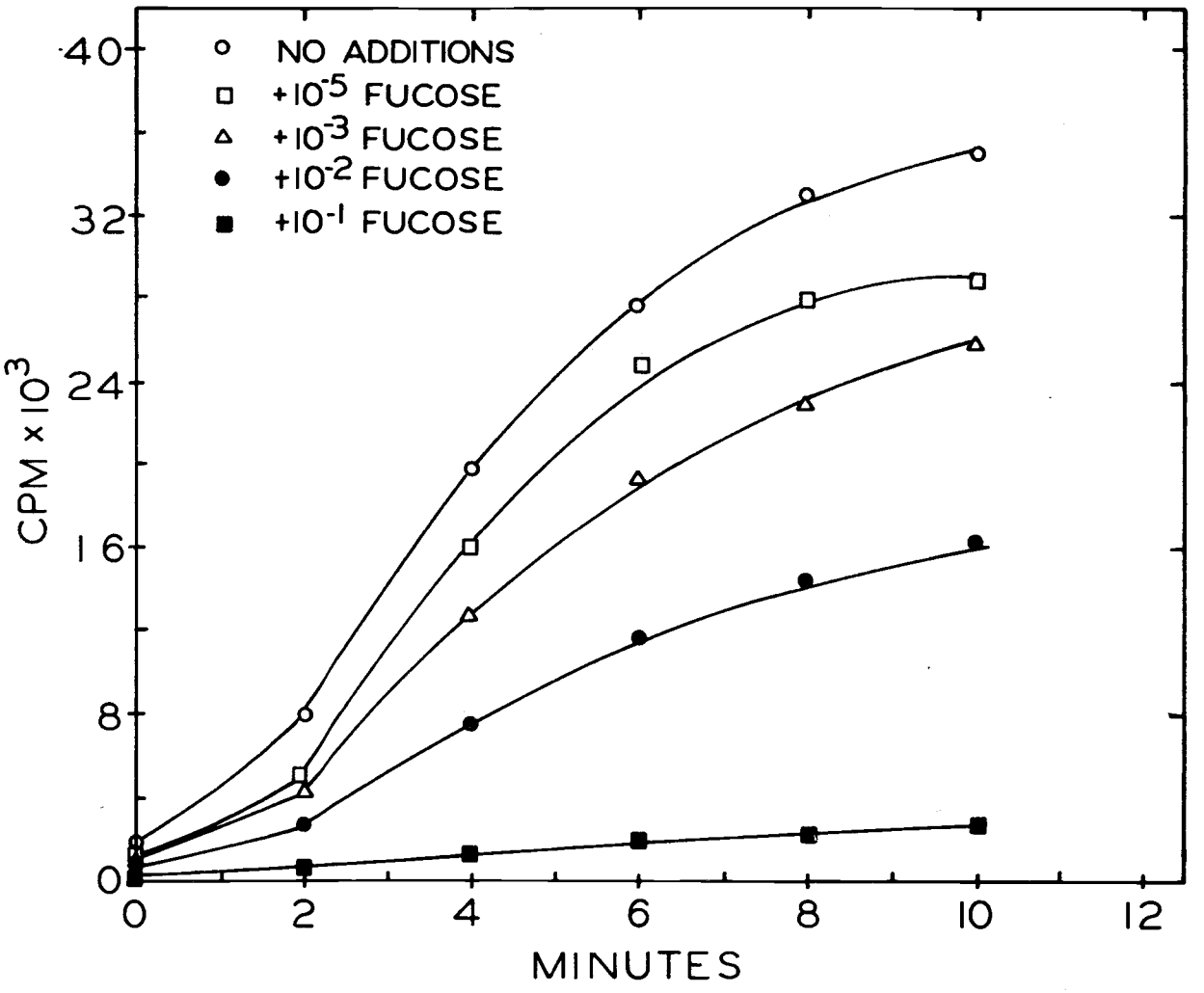


Figure 22. Inhibition of galactose uptake by 6-deoxygalactose in resting cells of S. lactis C2.

Phosphorylation Studies

In this research a major objective was to determine which if any of the common growth carbohydrates for the lactic streptococci were transported by the PTS of Roseman. The most common approach used has been to examine the phosphorylation of the sugars by different phosphate donors. Cells of S. lactis C2 were first made permeable to the donors by treatment with toluene-acetone; substrates utilized were commonly available radioactively labeled sugars and the two phosphate donors examined were ATP and PEP. It was necessary to study both these donors simultaneously because a question had arisen concerning the conversion of PEP to ATP in this system. Table 14 records the results. The data are presented as percent phosphorylation and were obtained by dividing the total counts coming off the ion-exchange column into the counts coming off in the formate buffer wash, the phosphate fraction. All data were obtained after 30 minutes of reaction time. In the case of galactose, ATP was the effective phosphate donor, but glucose- and mannose-grown cells were unable to phosphorylate galactose. Galactose-grown cells showed a small amount of phosphorylation of galactose by PEP, however. Galactose, lactose, and maltose-grown cells were all able to preferentially utilize ATP as the phosphate donor for galactose. In the phosphorylation of glucose both phosphate donors were utilized equally well. In lactose

Table 14. Role of ATP and PEP in phosphorylation of various sugars by S. lactis C2 grown on five different carbon sources.[a]

	Substrate Phosphorylated									
	Galactose		Glucose		Lactose		Maltose		Mannose	
Inducer	ATP	PEP	ATP	PEP	ATP	PEP	ATP	PEP	ATP	PEP
Galactose	95	32	95	94	45	96	34	56	71	95
Glucose	21	14	96	89	23	95	13	16	36	31
Lactose	93	11	97	97	97	95	9	13	83	89
Maltose	80	5	95	96	24	84	39	31	46	84
Mannose	13	12	96	97	35	96	18	13	60	88

[a]Data are expressed as percent phosphorylation which was calculated by dividing the total counts eluted from the column into the counts in the phosphate fraction. These data were obtained after the substrates and toluene-acetone treated cells were mixed and held for 30 minutes.

phosphorylation, PEP was the preferred phosphate donor, except in the case of lactose-grown cells in which ATP was equally effective as the donor of phosphate. Maltose was not effectively phosphorylated in any case. In all cases with mannose phosphorylation it appeared that PEP was the most effective phosphate donor, but in the case of lactose-grown cells again ATP was nearly as effective a donor of the phosphate.

Figure 23 details a time course study of phosphorylation of galactose and lactose by galactose-grown cells of S. lactis C2. The experiment was carried out over a 30-minute period and data are plotted as Cpm/mg cell dry weight x 10^2 vs. minutes. It can be seen that the effective phosphate donor for galactose was ATP; no phosphorylation of galactose was noted with PEP. The effective donor of phosphate to lactose was PEP; however, some phosphorylation also was observed with ATP.

Table 15 illustrates typical data on the percent phosphorylation of four growth sugars of S. cremoris HP and S. cremoris Hp Lac$^-$ mutant. These data corresponded fairly well with the data in Table 14, but maltose was not included since it is not a carbon source for S. cremoris. It can be seen that the Lac$^-$ mutant was a phosphorylation mutant lacking either Enzyme II and/or Factor III of the PTS.

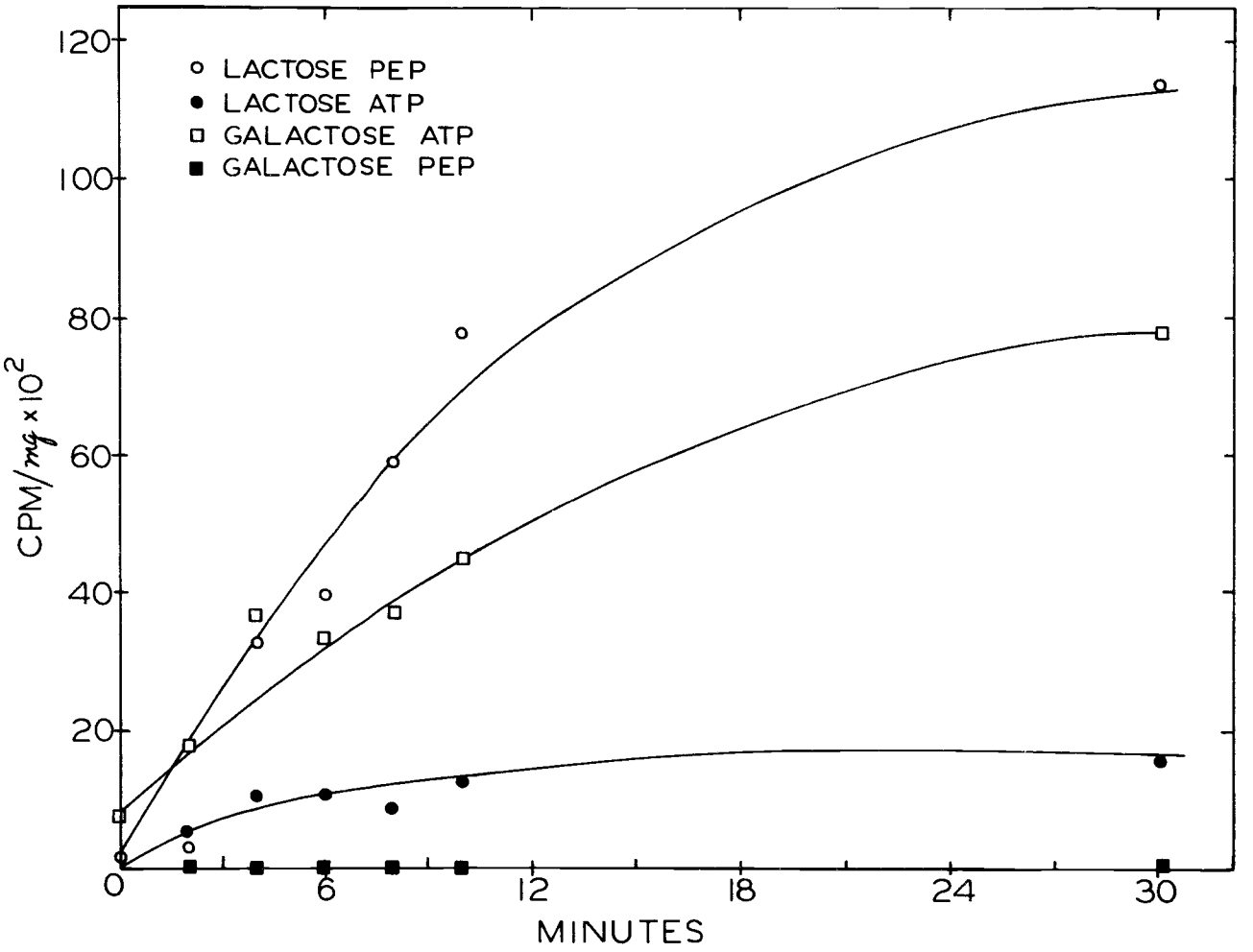


Figure 23. Phosphorylation of lactose and galactose by toluene-acetone treated cells of S. lactis C2 with .01 M ATP or PEP in presence of 0.03 M KF.

Table 15. Phosphorylation of four growth substrates by S. cremoris HP and a S. cremoris HP Lac⁻ mutant.[a]

	Organism			
	Wild Type		Lac⁻ Mutant	
	ATP	PEP	ATP	PEP
Galactose	52	23	77	16
Glucose	94	94	64	94
Lactose	48	94	8	11
Mannose	89	87	35	88

[a]Data are expressed as percent phosphorylation; the wild type was lactose grown whereas mutant was grown in galactose.

A number of lactose negative mutants were examined for their ability to phosphorylate several substrates using ATP and PEP as phosphate donors. In growth studies pleitrophic mutants have never been observed. In characterizing some of the lactose negative mutants obtained, it was found that they all were phosphorylation negative mutants. Table 16 presents data on percent phosphorylation of five substrates using both ATP and PEP as phosphate donors and three Lac⁻ mutants. It was interesting that phosphorylation of TMG did not occur. Apparently toluene-acetone treated cells lose affinity for TMG. Even in a time course study (data not shown) of S. lactis C2, phosphorylation of TMG was not noted.

Table 16. Percent phosphorylation of various carbohydrates by S. lactis C2 mutants.[a]

	C2L13		C2FG2		C2FW	
	ATP	PEP	ATP	PEP	ATP	PEP
Galactose	95	6	89	34	96	21
Glucose	96	73	81	95	80	88
Lactose	11	19	8	9	14	7
Mannose	56	86	62	87	31	83
TMG	6	9	8	11	3	8

[a]These data were obtained as described in Materials and Methods and Table 14.

Since there seemed to be no phosphorylation of TMG by toluene-acetone-treated cells, the procedure was modified to that of McKay (1969). Three mg of S. lactis C2 cells (dry weight) were suspended in 1 ml of 0.1 M sodium PO_4 buffer at pH 7.0. One-tenth μCi of ^{14}C TMG was added and final concentrations of TMG was brought to 6×10^{-4}M with unlabeled TMG, and the mixture incubated at 37 C for one hour. Very few counts < 5000/ml remained in supernatant, but when the cells were extracted with 1 ml of 80 percent ethyl alcohol by boiling for five minutes and the supernatant placed on ion-exchange column, 230,160 cpm/ml were obtained in the formate-buffer wash (phosphate fraction). When 1 ml of the phosphate fraction was acid hydrolyzed at 100°C for ten minutes in 1 N HCl, approximately one-third of the counts remained in phosphate fraction and two-thirds in the water wash of an ion-exchange column; this indicated that the

phosphate had been removed and the sugar was now unphosphorylated.

As noted in Table 16 lactose phosphorylation by all three mutants was reduced to almost nothing. No pleitrophic mutants were observed; however, in C2L13, glucose phosphorylation appeared to be reduced with PEP as the phosphate donor. Several experiments were performed using this mutant and the percent phosphorylation of glucose was always lower than in the wild type cells when PEP was the phosphate donor.

For the successful completion of one of the objectives of this research, it seemed necessary to isolate a lactose negative mutant which still possessed the ability to transport and phosphorylate lactose but was unable to cleave the lactose-PO_4. This mutant would be a β-P-gal mutant. In the use of three mutagenic agents, UV irradiation, EMS, and acriflavin, no such mutant was detected. A large number of lactose negative mutants were examined for the inability to cleave ONPG-6-P. This was the first step in selection of the proper mutant. All Lac$^-$ mutants examined lacked any ability to cleave this substrate; therefore, they lacked β-P-gal. The second step in selection of the proper mutant was to check the organism for lactose transport. All these mutants examined were unable to transport lactose as determined by the membrane filter technique with radioactive lactose. Table 17 illustrates the results. As can be seen, no pleitrophic mutants were observed, none could cleave ONPG-6-P, and the

Table 17. Characteristics of some selected lac⁻ mutants of S. lactis C2.

Mutant	Fermentation in glucose	galactose	lactose	ONPG-6-P Hydrolysis	CPM/mg cell dry wt. /15 Mins. of lactose uptake[a]
C_2F (Wild Type)	+	+	+	+	69265
C_2F SH	+	+	-	-	1413
C_2F tm	+	+	-	-	639
C_2F rl	+	+	-	-	490
C_2F 2	+	+	-	-	500
C_2F 3	+	+	-	-	669
C_2F 4	+	+	-	-	614
C_2F 5	+	+	-	-	921
C_2F 6	+	+	-	-	750
C_2F 7	+	+	-	-	542
C_2F 8	+	+	-	-	405
C_2F 10	+	+	-	-	433
C_2F 12	+	+	-	-	358
C_2F 13	+	+	-	-	543
C_2F 14	+	+	-	-	402
C_2F 15	+	+	-	-	556
C_2F 16	+	+	-	-	562
C_2F 18	+	+	-	-	381
C_2F 19	+	+	-	-	557
C_2F 20	+	+	-	-	444
C_2F 21	+	+	-	-	324
C_2F 23	+	+	-	-	342

[a] The uptake of lactose was determined by a membrane filtration technique. Cells were placed in 1 ml of .05 M sodium phosphate buffer and allowed to equilibrate for five minutes. At 0 time 0.1 μ Ci of lactose-1-^{14}C was mixed with cells and uptake at 25 C was allowed to proceed for 15 minutes after which time 1 ml of the mixture was filtered, washed with 10 ml cold phosphate buffer and radioactively determined by liquid scintillation.

transport of lactose by whole cells was negative in each case.

In further experiments involving phosphorylation, it was found that S. lactis 7962 apparently phosphorylated lactose-1-^{14}C. The experiments were preliminary in nature and detailed investigations of the ability of this strain to phosphorylate lactose were not undertaken.

Metabolism of Galactose-6-Phosphate

As expected, galactose-6-phosphate could not be utilized as a carbon source for growth. Crude cell extracts were used to check for ability to convert galactose-6-phosphate into another galactose form. Free galactose or galactose-1-phosphate were not detected in these experiments. Also experiments which tested the ability of crude extracts to convert galactose-6-phosphate into some glucose form were performed. Never was free glucose, glucose-1-phosphate or glucose-6-phosphate detected. The determination of inorganic phosphate when galactose-6-phosphate was incubated with a crude cell extract also showed that the phosphate was not cleaved to form galactose and inorganic phosphate.

When experiments were performed utilizing galactose-6-phosphate as a substrate and observing the reduction of $NADP^+$ with a crude enzyme preparation, it was noted that $NADP^+$ was reduced to NADPH; NAD^+ was not reduced. This was first thought to be the method by which galactose-6-phosphate was metabolized until it was

discovered that the galactose-6-phosphate used in the experiments was contaminated by at least 0.2 percent glucose-6-phosphate. This fact was later confirmed for us by Sigma Chemical Co., St. Louis, Missouri, the supplier of the chemical.

In experiments in which crude extract was used to oxidize glucose-6-phosphate, it was noted that the increase in O. D. at 340 nm with glucose-6-phosphate alone was 0. 06/minute; however, if 0. 1 ml of a 0. 01 M galactose-6-phosphate solution were added to the reaction, a sudden rate decrease in O. D. change to 0. 035/minute was noted. From this it appeared that the galactose-6-phosphate was not being oxidized or if it were the rate of oxidation was much lower than the rate for glucose-6-phosphate. Further experiments were performed to test for enzyme inhibition by competition for substrates in a crude extract of S. lactis C2 using glucose-6-phosphate, galactose-6-phosphate, and an equal mixture of each. Optical density after the first minute of reaction with glucose-6-phosphate was 0. 12; with galactose-6-phosphate, it was 0. 015 and with an equal mixture of glucose-6-phosphate and galactose-6-phosphate it was 0. 095. In calculating, the concentration of NADPH it was found with glucose-6-P alone to be 1.9×10^{-5} moles/l; with galactose-6-P alone, 2.4×10^{-6} moles/l and with an equal mixture of both, 1.5×10^{-5} moles/l.

DISCUSSION

Involvement of the PTS in Carbohydrate Metabolism in the Lactic Streptococci

Before this research was undertaken, preliminary evidence had been obtained that lactose was transported by the PTS of Roseman. Further evidence implicating this transport system in lactose utilization by the lactic streptococci was needed and provided by the present study. In a survey conducted on representatives of the four major groups of streptococci, it was determined that β-P-gal was the most frequently occurring lactose cleavage enzyme; however, in all members of the viridans streptococci, it was noted that β-gal was the enzyme involved. The significance of this fact is not understood. Speculation could be given that the utilization and assimilation of galactose might be involved. Galactose is a required component for cell wall structure of the streptococci. It is possible that in the viridans streptococci, glucose does not repress the phosphorylation of galactose, and a PTS is not necessary to supply a phosphorylated form of galactose. This is merely speculation, but could be tested through experimentation.

The most convincing evidence for this system of transport being functional in lactic streptococci came from studies in which a system was devised to show the phosphate donor for given sugars. From

such studies it was seen that PEP was the effective phosphate donor for lactose. Some concern was expressed that phosphorylation was not a criterion of proof for implication of the PTS in transport of various sugars; however, Kornberg and Reeves (1972) recently reported a direct correlation between PEP dependent hexose transport and PTS activity in E. coli.

Several other criteria may have also been used to implicate the PTS in lactose transport. The isolation of galactose-6-phosphate from an extract of a lactic streptococcus would have provided very convincing evidence. Repeated attempts to accomplish this proved unfruitful apparently due to the fact that the compound rapidly entered metabolic pathways or was otherwise removed. It was recognized early that a mutant defective in β-P-gal but normal for transport of lactose was needed from which to isolate lactose-phosphate in sufficient quantity to detect it by chromatographic techniques. The lactose-phosphate could be cleaved by a purified β-P-gal, and galactose phosphate should be one of the end products of this cleavage.

The isolation of such a mutant proved to be very difficult. Of all the mutants selected for inability to utilize lactose not one was of the desirable phenotype. The mutants all seemed to fall in one class or type and all appeared to be mutants lacking the ability to phosphorylate lactose and also the ability to transport the disaccharide. This could be due to a lack of either Enzyme II^{Lac} and/or Factor

III^{Lac}. The explanation for the inability to obtain a β-P-gal mutant is difficult and at most speculative, but McKay et al. (1972) recently postulated that the enzymes for the fermentation of lactose reside on extrachromosomal material or episomes. My results tend to support this due to the fact that when acriflavin was used as a mutagen, a large number of lactose negative mutants were produced and these mutants were all in the same class. Never was a pleitrophic mutant, one lacking Enzyme I of the PTS, found. Also mutants unable to utilize glucose or galactose were never obtained by the same method (acriflavin) utilized to obtain lactose mutants. Apparently, Enzyme I of the PTS is not carried on an episome and therefore is not as easily mutated as the enzymes for β-P-gal and other components of the PTS. In this same regard there could be some merit in the statement by Simoni and Roseman (1973). These authors stated that they believed Factor III^{Lac} to be important in the control of transcription and/or translation of the lactose operon in S. aureus. If this is true, then Factor III^{Lac} could easily be removed simply because it might be a hot spot for mutation and after removal β-P-gal would not be formed.

A good control lactic streptococcus in which to study the phosphorylation of lactose would have been S. lactis 7962. This strain was studied early as the only representative of these bacteria which possessed a typical β-gal for cleaving lactose (Citti et al., 1965;

McKay et al., 1969, 1970). This strain was questioned as being a lactic streptococcus by McKay et al. (1970) because it was the only representative of this group which possessed a typical β-gal; also, with Lancefield serotyping it did not give a positive Group N reaction. However, the present research indicated that cells of this strain were agglutinated by Group N antiserum when whole-cell agglutination studies were performed. It proved to be similar in most respects to other lactic streptococci with some notable exceptions. It possessed the ability to cleave ONPG, and it fermented certain carbohydrates not fermented by the lactic streptococci. These differences do not seem to be sufficient, however, to reclassify the organism. In a personal communication with Dr. Otto Kandler, Munich, Germany, assurance was given that the organism gave typical lactic streptococcal reactions when tested in his laboratory; also, he indicated that it possessed the typical lactic streptococcal peptidoglycan in the cell wall.

Since S. lactis 7962 possessed a typical β-gal, lactose should not be phosphorylated during transport. However, in experiments involving phosphorylation, it was found that lactose-1-^{14}C appeared to be phosphorylated by this strain. McKay (1969) also noted that TMG accumulated in this organism as an apparent phosphate derivative. He was unable to explain this phenomenon.

Experiments performed on phosphorylation in S. lactis 7962 in the present study were only preliminary in nature. It would be interesting to study the simultaneous phosphorylation of lactose, radioactively labeled in the galactose moiety only, by ATP and PEP. Since it is known that ATP is the phosphate donor for galactose and not PEP, this experiment might provide evidence that lactose is first cleaved into its two monosaccharides and these in turn are phosphorylated. From other results, it would be reasonable to assume that lactose is cleaved and the glucose is phosphorylated by ATP and PEP; however, galactose is phosphorylated only by ATP. Convincing evidence for the cleavage of lactose and subsequent phosphorylation of its monosaccharides could be obtained by use of lactose labeled only in the galactose moiety and show phosphorylation of the labeled galactose by ATP alone.

Further studies involving phosphorylation of sugars by the lactic streptococci implicated the PTS of Roseman in the utilization of glucose and mannose in addition to lactose. The experiments involving phosphorylation were always performed simultaneously with the two phosphate donors, ATP and PEP, being utilized as the phosphate donor. The results showed that the effective phosphate donor for galactose was different than for lactose, glucose, or mannose. Galactose phosphorylation required only ATP, whereas lactose phosphorylation required PEP as evidenced by increasing counts in

the formate buffer wash with PEP (Figure 23). It must be noted that these data showed some phosphorylation of lactose with ATP also but at a much lower level. It is not known for certain what this means; however, speculation at least can be made that there are two systems of transport functionally active in these organisms. It is known that the transport of galactose in bacteria is complicated by various transport systems and is an area where research attention is needed. It seems reasonable to assume that a lactic streptococcus could possess more than one functionally active transport system for sugars. After all, it is known that the lactic streptococci possess at least two systems for the transport of lactose. One system involves the PEP-dependent PTS in which the sugar is phosphorylated as it enters the cell. The other system could be carrier-mediated with the sugar entering as the free sugar, later being phosphorylated by a kinase with ATP the effective phosphate donor. Another possibility might exist in which lactose is transported as the free disaccharide, cleaved into its component monosaccharides, and phosphorylated by an ATP dependent kinase, as apparently is the case in S. lactis 7962, the lactic streptococcus possessing a typical β-gal.

In further experiments involving a study of the effect of carbon source on phosphorylation, glucose was shown to be effectively phosphorylated by ATP and PEP. This was another reason for believing that two systems of transport existed in the lactic streptococci. Also

mannose was shown to be phosphorylated by both phosphate donors in most cases, but PEP appeared to be the most effective phosphate donor in this case. Glucose-grown cells repressed the phosphorylation of mannose and galactose. In this regard, Wilkins (1970) showed that glucose-grown cells of S. faecalis did not accumulate phosphorylated galactose and it is a well-known fact that glucose represses many enzyme systems through the familiar mechanism of catabolite repression.

In every case where phosphorylation was apparent, galactose used ATP as the donor of the phosphate, whereas lactose was effectively phosphorylated by PEP. However, in lactose-grown cells ATP also was as effective in phosphorylating lactose. Perhaps galactokinase was effecting the phosphorylation of lactose. It should be noted that glucose-, maltose-, and mannose-grown cells were greatly deficient in their ability to phosphorylate lactose with ATP. Glucose and mannose appeared to repress galactokinase.

To prove that two systems of transport existed for glucose in the lactic streptococci would require the isolation of a mutant which could still grow on glucose but could not phosphorylate glucose with PEP or conversely with ATP. Several lactose negative mutants were examined for inability to phosphorylate other substrates. None were found which had this characteristic but a detailed search was not undertaken. The S. lactis C2L13 mutant isolated by McKay seemed

to be defective in the phosphorylation of glucose with PEP. Several experiments were performed using this mutant and the glucose phosphorylation with PEP was always much lower than with the wild type or other mutants. It seems reasonable to assume that a mutant defective in the glucose PTS Enzyme II^{glu} could be isolated, and experiments designed to do this should be performed.

In studying phosphorylation of several lactose negative mutants, the defect in the lactose operon was partially elucidated. Several S. lactis mutants and one S. cremoris mutant were shown to be deficient in phosphorylating lactose. The mutants all lacked activity for β-P-gal or β-gal. The deficiency in phosphorylation could be due to a lack or impairment of Enzyme II^{Lac} and/or Factor III^{Lac} of the PTS. All the lactose negative mutants were normal for the utilization of glucose, galactose, and mannose except the mutant C2L13 which apparently was defective in the phosphorylation of glucose by PEP.

The inability of toluene-acetone treated cells to phosphorylate TMG was surprising. However, when whole cells were allowed to take up TMG over an hour time period, TMG accumulated as a phosphate derivative. It was hoped that TMG could be utilized as a substrate with which to study the position of phosphorylation on the galactose. Since TMG is not a metabolizable substrate for the lactic streptococci, the β linkage should not be cleaved and no further conversion of the phosphorylated molecule should occur. To show that TMG was

phosphorylated on the sixth carbon of the galactose moiety, the hydrolysis of the phosphate in 1N HCl at 100 C for ten minutes would be required. The strong ester linkage on the sixth carbon would not be hydrolyzed by this treatment; however, the weaker bond on the first carbon is entirely hydrolyzed by this treatment. Results of the hydrolysis of the TMG phosphate were not conclusive because some of the phosphate was hydrolyzed by mild acid hydrolysis. These results are very difficult to explain because the one carbon in the galactose moiety of TMG is tied up through the β linkage and should not be available for phosphorylation. The results indicating that toluene-acetone treated cells do not phosphorylate TMG might be explained by the fact that these cells lose the affinity for TMG due to denaturation of destruction of some component necessary for recognition of this compound.

In all these phosphorylation studies involving ATP and PEP as phosphate donors, it must be realized that PEP could be converted into ATP by the action of the enzyme, pyruvate kinase. Evidence that this does not occur comes from the fact that galactose was phosphorylated by ATP, not PEP, in lactose-grown cells. If PEP were being converted to ATP by the action of pyruvate kinase in our system, then PEP would also appear to be an effective phosphate donor for galactose.

Metabolism and Transport of Free Galactose

The rapid decline of free galactose in the presence of ATP and the concommitant increase of galactose-1-PO_4 with time which subsequently decreased indicated that phosphorylated intermediates were formed and metabolized when cells were provided with the sugar. Since PEP did not cause the same response at ATP, it appeared that galactose was transported by a system other than the PTS. Inhibition of galactose uptake in whole cells of S. lactis C2 by fucose (6-deoxy-galactose) also supported this idea. The six carbon of galactose is believed to be the position of phosphorylation of the hexose when the PTS operates; however, since fucose lacks an oxygen on the six carbon, it is not phosphorylated. If the PTS were functional, fucose would not be expected to inhibit the uptake of galactose unless there was some competition for the initial binding of the enzyme for the substrate due to the structural similarity of the two molecules.

The enzyme responsible for phosphorylation of galactose is apparently the classical galactokinase, described as the first enzyme of the Leloir pathway which initiates reactions for the conversion of galactose to glucose. By blocking the glycolytic pathway with fluoride and supplying toluene-acetone treated cells with galactose and ATP, it was possible to show conversion of galactose to galactose-1-PO_4 and subsequently to glucose-1-PO_4 and glucose-6-PO_4. The data

obtained by analyzing for substrates and intermediates is direct evidence for galactose being converted into glucose. Leloir pathway enzymes were functioning in these conversions. First attempts to assay for UDP gal-4 epimerase were not successful; however, this was remedied when oxidation of NADH was prevented by trapping pyruvate with semicarbazide.

It seemed reasonable to expect lactate dehydrogenase activity being involved in oxidizing NADH back to NAD^+ and preventing an increase in absorbance at 340 nm. By knowing that the PTS of Roseman is functional in the transport of lactose, glucose, and mannose, it was reasoned that a large amount of pyruvate would be available as a substrate for the enzyme. Semicarbazide added in the buffer at 0.2 M final concentration should complex the pyruvate and make it unavailable for the action of lactate dehydrogenase. When assays were performed with 0.2 M semicarbazide incorporated in the buffer, good activity was noted; however, if an excess of pyruvate was added along with the semicarbazide no activity was noted. A very small amount of pyruvate (0.004 M) added showed essentially no reduction of activity when added along with semicarbazide.

The ability to show the induction of the epimerase in glucose-grown cells and the difference in specific activities of the enzyme in different sugars are added proof that the enzyme is present and that the activity measured at 340 nm is real and not just an artifact due to

addition of the semicarbazide in the system. Further evidence is taken from the fact that in a control reaction without substrate (UDP-galactose) no apparent increase in O. D. was noted.

From growth curves of S. lactis C2 it was determined that this enzyme is very quickly induced; however, the rapid induction of this enzyme in S. cremoris was not shown. The time interval for studying induction in S. cremoris, which was one hour, may not have been long enough because growth curves of S. cremoris showed a longer lag in galactose than S. lactis.

Further evidence that Leloir pathway enzymes were functioning in the conversion of galactose to glucose was taken from the coupled enzyme assay for galactose-1-phosphate uridyl transferase. The results of the assay showed activity of the transferase because in the assay procedure more reduction of NAD^+ was evidenced in a reaction quantitating residual UDP glucose when UDP glucose was added alone with a crude extract. Contrastingly, when UDP glucose and galactose-1-PO_4 were added together with crude extract, less reduction of NAD^+ was noted indicating that less UDP glucose remained. Since the UDP moiety was transferred to galactose to form UDP galactose and glucose-1-phosphate, the transferase was apparently functioning.

The transport of galactose appeared to be effected through a carrier-mediated permease. The transport was inhibited by several inhibitors of enzymes in the glycolytic pathway. Among these

inhibitors were potassium cyanide, sodium azide, potassium fluoride, and iodoacetate. The uncoupler, dinitrophenol, inhibited transport at 10^{-3} M final concentration. These inhibitors must function by some way interfering with energy formation in the cell, for it is very obvious that energy was required for the transport of galactose in S. lactis. This observation had also been noted as a requirement for the transport of galactosides by S. lactis by Kashket and Wilson (1972) and for the transport of galactose by S. faecalis (Wilkins, 1970). The reason energy was believed to be required for the transport of galactose was taken from the results of stimulation of galactose uptake by arginine and ATP. Arginine is metabolized by S. lactis and ATP is a direct product of the reactions. ATP stimulated transport externally, in agreement with the report of Scarborough et al. (1968). It was of interest to note that PEP added externally inhibited the uptake of galactose. These results are in contrast with the findings for S. faecalis reported by Wilkins (1970) in which PEP increased the accumulation of free galactose, but ATP added extracellularly inhibited the reaction. The results of this research, therefore, indicate that galactose is transported by a system other than the PTS; this system requires energy and bears no resemblance to the galactose uptake system reported by Kerwar et al. (1972) for E. coli.

Growth Studies and Differential Respirometry

In studies involving growth of the lactic streptococci in five carbohydrates, the cell dry weight yield was greatest in glucose and poorest in galactose. In interpreting these results it seemed that these differences were mainly due to different growth rates on the various carbohydrates. There have been reports by deVries and Stouthamer (1968) that molar growth yield in Bifidobacterium was approximately twice more in lactose than in glucose or galactose. Our research did not show this in lactic streptococci, but this could have been due to the way the experiments were designed. Gilliland et al. (1972) inferred that when lactic streptococcal cells were growing in milk and supplied with glucose and galactose, the cells will not use the galactose or will use it at a much slower rate. These authors reported that when fermenting milk was treated with exogeneously-added β-gal, the amount of free galactose appearing in the whey increased with time. The amount of glucose increased and later decreased. The authors did not state that galactose supplied as lactose was not utilized, but only showed that lactose-induced cells preferred glucose over galactose. This was substantiated in the present study by differential respirometry which showed that lactose-induced cells produced a greater amount of CO_2 from glucose (>1100 μl/100 mg cell dry wt./hr.) than from galactose (< 825 μl/100

mg cell dry wt./hr.).

Several explanations may be given for the fact that diauxic growth patterns could not be detected in S. lactis C2. The galactose enzymes could have been induced very rapidly; a fact substantiated in S. lactis but not in S. cremoris. However, S. cremoris appeared to grow very slowly on galactose. Other experiments involving studies of diauxic growth need to be performed using S. cremoris. The organism could be utilizing the two sugars simultaneously. This was substantiated by experimentation in S. diacetilactis 18-16 induced on galactose. Specific activity of CO_2 evolved was measured when glucose and galactose were added simultaneously to respiring cells and the specific activity was found to be reduced. It was seen that the presence of glucose reduced the specific activity of the CO_2 produced from galactose, and the presence of galactose reduced the specific activity of CO_2 produced from glucose. These data indicated that galactose-induced cells could simultaneously utilize glucose and galactose. McBrien and Moses (1968) have shown this same thing with E. coli. However, these authors pointed out that the cells are required to be in the proper state of induction. It would be interesting to perform these experiments on cells induced in various sugars. This would possibly be one way to determine the inducers of the enzymes for the galactose pathway.

In further studies using differential respirometry, it was shown that galactose-induced cells absorbed more O_2 than cells in other states of induction. Glucose-induced cells of S. diacetilactis 18-16 were able to oxidize glucose and lactose but not galactose; when glucose-induced cells were placed in galactose, little CO_2 was evolved nor was an appreciable amount of O_2 absorbed. The reason for this is not known, but experiments to test for the presence of galactose oxidase, an enzyme which requires O_2, were negative. Demko et al. (1972) reported a two- to three-fold increase in the amount of oxygen utilized by cells of S. lactis in similar respiration experiments.

An interesting result was noted when glucose-induced cells were placed in lactose. After approximately 20 minutes, it appeared that the lactose use began with uptake of more O_2 (>800 l/100 mg cell dry wt./hr.) than in glucose (< 500 l/100 mg cell dry wt./hr.). This fact would lead one to speculate that the cells are utilizing the galactose which is being phosphorylated during transport, so galactokinase need not be present for galactose use. This could be one important function of a PTS in lactic streptococci. When cells are respiring lactose, the glucose present would tend to repress galactokinase and galactose would not be utilized, but when a PTS exists the galactose is phosphorylated and further metabolism of the galactose molecule is then possible even when glucose is present.

Metabolism of Galactose-6-Phosphate

The route of metabolism of galactose-6-phosphate was not elucidated. The speculation of Simoni and Roseman (1973) that the compound could be converted to galactose-1-phosphate through the action of a mutase seems unlikely in lactic streptococci. No galactose-1-phosphate was ever detected when crude extracts were incubated with galactose-6-phosphate. Also these same authors suggested that the molecule was oxidized to 6-phosphogalactonic acid and metabolized through the Entner-Doudoroff pathway. This appeared to occur in the lactic streptococci, but contamination of the galactose-6-phosphate with glucose-6-phosphate prevented a definitive conclusion.

The recent work of Bissett and Anderson (1973) concerning the metabolism of galactose-6-phosphate in S. aureus has been the only report which detailed the metabolism of this compound in any organism. Since the S. aureus and S. lactis PTS are very similar, as shown by McKay et al. (1970), it is likely that galactose-6-phosphate metabolism proceeds through the same pathway as shown by Bissett and Anderson (1973) for S. aureus. The metabolism of galactose-6-phosphate in the lactic streptococci needs further research attention.

SUMMARY

Further evidence that the lactic streptococci utilize lactose through the PTS was provided; PEP was shown to be the phosphate donor but the position of phosphorylation on the molecule was not shown. A survey of the streptococcal groups for β-P-gal and β-gal was performed using the chromogenic substrates ONPG and ONPG-6-phosphate. All organisms possessed greater specific activity for β-P-gal except members of the viridans group which contained only β-gal.

In a study of sugar transport by the lactic streptococci, it was found that glucose and mannose, in addition to lactose, were transported by the PTS. Galactose was transported by a different system; this system was partially characterized and shown to be stimulated by ATP and arginine but not PEP. Common carbohydrates such as lactose and glucose competed with galactose in this uptake system. Potassium cyanide was shown to be a very potent inhibitor of the transport system. Other common inhibitors of metabolism which functioned in this system were azide, fluoride, and iodoacetate. Dinitrophenol (10^{-3}M) also inhibited the system. From the results, it was speculated that two systems of transport functioned in the accumulation of glucose and mannose.

The metabolism of free galactose in the lactic streptococci was mediated by the Leloir pathway; galactose entered the cell as the free sugar, was phosphorylated by ATP, and subsequently was converted into glucose derivatives. The epimerase and transferase of the Leloir pathway were determined to be present.

In differential respiration studies, catabolite repression of galactose enzymes was shown; however, it also was shown that cells of S. diacetilactis 18-16 which were induced in galactose could utilize galactose and glucose simultaneously. Galactose-grown cells required more oxygen when metabolizing galactose than cells in other states of induction or cells respiring other sugars. Galactose oxidase was not present in these cells so the reason for this increased amount of oxygen consumption in the respiration of galactose was not explained.

The metabolism of galactose-6-phosphate in the lactic streptococci was not elucidated. Free galactose and inorganic phosphate were not formed upon the incubation of galactose-6-phosphate with crude extracts or toluene-acetone treated cells. It seemed that galactose-6-phosphate was dehydrogenated to 6-phospho galactonic acid and possibly further metabolized by way of the Entner-Doudoroff pathway; however, contamination of the substrate with glucose-6-phosphate cast doubt on this possibility.

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