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Title: MECHANISMS OF LACTOSE UTILIZATION BY LACTIC ACID BACTERIA: ENZYMIC AND GENETIC STUDIES

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To learn more about the role β-galactosidase plays in rate of acid production in milk by lactic acid bacteria, several lactobacilli were examined for this enzyme. Cells from different species were grown in lactose broth to induce β-galactosidase activity. The specific activities of whole, solvent treated cells, and of cell-free extracts were compared using the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG). Among those tested, Lactobacillus helveticus possessed the greatest activity and properties of the enzyme in this species were studied. The optimum temperature for the enzyme in a cell-free extract prepared by sonication was 50 C and the optimum pH was about 6.6 when using sodium phosphate buffer (0.05 M) at 50 C; potassium phosphate buffer afforded 30% less activity. Galactose was an inducer of the enzyme but not as effective as lactose.

During the isolation of lactose negative mutants from *Streptococcus lactis* 7962, it was noted that a semi-synthetic medium, containing lactose as the sole carbon source, would not support growth if inoculated with cells previously grown on glucose. Lactose grown cells, however, grew in the medium after a 20 to 24 hour lag period. When glucose or galactose served as the sole carbon source, rapid growth by the glucose or lactose grown cells occurred. Addition of tryptophan to the basal medium permitted the glucose-grown cells to utilize the lactose. Thus tryptophan was stimulatory for growth of *S. lactis* 7962 on lactose, but was not required for rapid growth on glucose.

The mechanism of lactose utilization in two strains of lactic streptococci was examined. Sodium fluoride prevented lactose utilization by whole cells of *S. lactis* C_2F, but had no effect on *S. lactis* 7962. Sodium arsenate prevented lactose metabolism in *S. lactis* 7962 but had only a slight inhibitory response on *S. lactis* C_2F. Concentrated cell extracts from *S. lactis* C_2F hydrolyzed ONPG; this hydrolysis was inhibited by sodium fluoride, yet the addition of phosphoenolpyruvate (PEP) in the presence of sodium fluoride, restored maximal activity. Addition of acetyl-P, carbamyl-P, adenosine triphosphate (ATP), guanosine triphosphate (GTP), or uridine triphosphate (UTP) did not stimulate activity. The presence of cofactors did not stimulate nor did sodium fluoride inhibit the hydrolysis of ONPG in cell extracts of *S. lactis* 7962. The latter organism was shown to

hydrolyze lactose into glucose and galactose, whereas _S. lactis_ C_2F was unable to split the disaccharide. A nonmetabolizable analogue of lactose, thiomethyl-β-D-galactoside (TMG), was used to measure the transport process. Uptake of ^{14}C-TMG was inducible in both organisms. In _S. lactis_ C_2F ^{14}C-TMG rapidly accumulated as a derivative which was negatively charged and which chromatographed as TMG after treatment with phosphatase. The analogue uptake by _S. lactis_ 7962 was defective, yet the accumulated TMG still appeared primarily as a derivative. Galactose was also a better inducer than lactose in _S. lactis_ C_2F and in the several "β-galactosidase-less" lactic streptococci examined.

Lactose-negative mutants from _S. lactis_ C_2F all possessed the phenotype lac^-gal^-. Lactose negative mutants from _S. lactis_ 7962 were separated into several classes. Several mutants were unable to transport TMG, although they contained normal levels of β-galactosidase activity, suggesting they possessed the phenotype z^+y^-.

The instability of β-galactosidase in toluene treated cells or cell-free extracts of over 40 lactic streptococci was explained. These organisms did not hydrolyze lactose, but instead hydrolyzed lactose-P and consequently must possess a different enzyme. The significance of the TMG derivative in 7962, especially since extracts from this strain were shown to hydrolyze lactose, is unclear; it may explain the inability of TMG to induce lactose utilization in this strain.

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MECHANISM OF LACTOSE UTILIZATION BY LACTIC ACID BACTERIA: ENZYMIC AND GENETIC STUDIES

INTRODUCTION

Slow and insufficient acid production by lactic starter cultures often cause serious economic losses to the dairy industry. Natural inhibitors in milk, seasonal variation in the milk composition, residues of germicidal cleaning and sanitizing compounds, as well as the presence of bacteriophages may cause slow acid development. In some cases another important cause of insufficient acid production is noted; the organisms, for reasons unknown, become attenuated and are no longer capable of producing sufficient acid. When this occurs acid production by these slow strains may be enhanced by addition of protein hydrolysates to the milk, suggesting that a relationship exists between acid production and proteolysis.

Fast cultures of *Streptococcus lactis* coagulate milk within 18 hours at 21 C. If such a culture is streaked on an agar medium approximately 3% of individual colonies isolated will be unable to coagulate milk even within 48 hours after subculturing in milk. In a comparative study of these fast and slow strains it has been noted that the fast strains achieve four times the viable cell population and are four times more proteolytic than slow strains when grown in milk. This further suggests that a direct relation exists between available

nitrogen and total acid production by lactic streptococci in milk (Citti, Sandine and Elliker, 1965).

Lactic streptococci remain viable after storage at 3 C for ten days, however, the cells lose their proteinase enzyme system and become defective in acid production. This defect can be overcome by adding pancreas extract. From these observations, it has been assumed that decreased acid production results from the cells inability to obtain needed organic nitrogen from milk proteins and that the pancreas extract compensates for the altered proteinase system (Cowman and Speck, 1965). In contrast, experiments suggesting that extracellular proteinase activity was unnecessary for S. lactis to exhibit a high level of acid production in milk following storage at low temperatures were reported by Smittle and Koburger (1968). They contend that acid-producing activity is probably dependent on the physiological state of the organism; an organism containing high levels of endogeneous reserves would initiate rapid acid production, whereas a strain depleted in reserve material would initiate acid production more slowly.

In view of these apparent contradictions, it remains to be proven whether or not there exists a relationship between acid production and the extracellular proteinase activity of the lactic streptococci. It was considered necessary to first purify and characterize the

β-galactosidase from lactic streptococci, since growth of these bacteria in milk, their natural habitat, should depend on its function. A survey of over 40 strains of streptococci, however, revealed only one strain (S. lactis 7962) which produced an enzyme that could be assayed in toluene-treated cells or cell-free extracts. This enzyme was extensively examined by other workers in our laboratory, and found to be fairly unstable. Other studies, presented in the initial phase of this dissertation involved a survey of several lactobacilli for a more stable β-galactosidase.

The second phase of study considered essential was to investigate the factors involved in the fermentation of lactose by S. lactis 7962 and S. lactis C_2F. The latter strain was chosen to represent those 40 streptococci with an apparent "labile" β-galactosidase. The isolation of constitutive mutants as well as strains lacking galactoside permease and β-galactosidase would be strong evidence for operation of the same type of control over the lactose operon as occurs in Escherichia coli. It was hoped these investigations would contribute information as to the mechanism and regulation of lactose metabolism in lactic streptococci.

The results of the above study should provide an opportunity for a more detailed examination of the proteinase activity of lactic streptococci and its effect on the growth of these organisms. In this regard, the clarification of the apparent lability of β-galactosidase in lactic streptococci is of great importance.

HISTORICAL REVIEW

β-Galactosidase

β-galactosidase, originally designated lactase, catalyzes the hydrolysis of lactose to glucose and galactose. The growth of an organism with lactose as the sole carbon source has generally been thought to indicate the prior elaboration of β-galactosidase. This enzyme has consequently been identified in a wide variety of micro-organisms including Aeromonas formicans (Rohlfing and Crawford, 1966), Paracolobactrum aerogenoides (Anderson and Rickenberg, 1960), Shigella sonnei (Rickenberg, 1960; Clausen and Nakamura, 1963), Escherichia coli (Lederberg, 1950), Saccharomyces fragilis (Caputto, Lelour, and Trucco, 1947; Szabo and Rozsa, 1965), Neurospora crassa (Lester and Byers, 1965), Bacillus megaterium (Landman, 1957), B. subtilus (Auerma, 1964), and Aspergillus niger (Wallenfels and Malhottra, 1961).

The enzyme has also been identified in lactic acid bacteria. Monod and Cohen (1952) showed that β-galactosidase from Lactobacillus bulgaricus was activated by monovalent cations as well as divalent ions such as Mg++, Mn++, and Fe++. In addition, it did not react immunlogically with E. coli β-galactosidase. During enzymic studies of lactose metabolism of Streptococcus lactis UN, Vakil and Shahani (1962, 1969) found that the strain possessed β-galactosidase.

The enzyme from S. lactis 7962 also has been characterized and partially purified (Citti, Sandine, and Elliker, 1965; McFeters, Sandine and Elliker, 1967). However, this strain was the only organism among 40 lactic streptococci examined which possessed β-galactosidase activity in toluene-treated cells or cell-free extracts. Since the remaining strains could utilize lactose as a sole carbon source, it was assumed they produced an unstable enzyme in either toluene-treated suspensions or in cell-free extracts. A similar assumption was proposed for the apparent lability of the Staphylococcus aureus β-galactosidase in cell-free preparations (Creaser, 1955; McClatchy and Rosenblum, 1963).

The chemical and physical properties of β-galactosidase from various organisms has been reviewed (Cohen, 1957; Wallenfels and Malhotra, 1961). The enzyme from E. coli hydrolyzes lactose to glucose and galactose. Trisaccharides and digalactosides are also formed during lactose hydrolysis which indicated the enzyme catalyzes the transfer of the galactose moiety of lactose to an acceptor molecule such as water, glucose, lactose, galactose, or some other sugar. In addition, alterations in the glucose moiety of lactose can usually be made without greatly modifying the activity of E. coli β-galactosidase, but substitutions in the galactose moiety nearly abolishes enzyme activity.

Lactose Dehydrogenase

The conversion of lactose to lactobionic acid in *Pseudomonas graveolins* involves two different enzyme systems; 1) formation of lactobionic-δ-lactone by the direct oxidation of lactose via lactose dehydrogenase, and 2) formation of lactobionic acid by further hydrolysis of the lactobionic-δ-lactonase (Nishizuka, Kuno, and Hayaishi, 1962). These authors partially purified lactose dehydrogenase as well as lactonase and established that the enzyme system was associated with the cell envelope.

Stodola and Lockwood (1947) first reported that certain pseudomonads were directly able to oxidize lactose to the corresponding aldobionic acid without prior hydrolysis to monosaccharides. No evidence for the subsequent metabolism of the acid was presented. Bentley and Slechta (1950) observed that lactose would not support growth of *P. quercito-pyrogallica*; however the dissacharide was converted to lactobionic acid which accumulated in the medium with up to 75% yields. Iodoacetic acid (IAA), sodium fluoride (NaF), and p-chloromercuribenzoate had no effect on conversion, whereas sodium azide (NaN_3) and potassium cyanide (KCN) inhibited oxidation. Cort *et al.* (1965) suggested that lactobionic acid was an intermediate in lactose metabolism by *Penicillum chrysogenum*, although no direct proof was shown to support the hypothesis. Finally, the ability to

oxidize lactose to lactobionic acid was also reported in *Bacterium anitratum* and in the red alga *Iridophycus flaccidum* (Wallenfels and Malhotra, 1961).

In the above organisms, no contribution of the carbons in lactobionic acid for cell synthesis has been noted. *S. lactis* UN, on the other hand, has been reported to oxidize lactose to lactobionic acid which is then further hydrolyzed to galactose and gluconate (Valkil and Shahani (1962).

The Lactose Operon of *E. coli*

Knowledge regarding the metabolism of lactose by *E. coli* has revealed a complex system which must be understood to investigate the mechanism of lactose utilization in the lactic acid bacteria. Jacob and Monod (1961) and more recently Beckwith (1967) reviewed the experimental work which has led to the present concepts. This mechanism is briefly summarized here.

Lactose is hydrolyzed by β-galactosidase to glucose and galactose which can then undergo glycolytic fermentation. The organism also possesses a mechanism of accumulating lactose against a concentration gradient. This enzyme, termed galactoside permease, is associated with the cytoplasmic membrane and mediates the permeation of lactose by an energy dependent active transport system (Kepes and Cohen, 1962). The structure of the two proteins is

determined by two chromosomal genes, y for permease and z for β-galactosidase. When wild type cells are grown on any carbon source but lactose, the activity of these genese are repressed and their products are found only in small amounts. Growth on lactose, however, results in induction of gene expression. For a compound to be an inducer of the lac proteins in *E. coli*, it must possess an unsubstituted galactoside, as in thiomethyl-β-D galactoside (TMG) or isopropyl-β-D thiogalactoside (IPTG), which are excellent inducers but are not substrates for β-galactosidase (Herzenberg, 1959).

A third enzyme, thiogalactoside transacetylase, is also formed in the presence of inducer. This enzyme catalyzes the acetylation of a thiogalactoside by acetyl coenzyme A; however, the *in vivo* function is unknown. In this regard, Fox *et al*. (1966) isolated $i^+o^+z^+y^+a^-$ mutants of *E. coli* which lacked any noticeable impairment of lactose utilization.

The genetic map of the *E. coli* chromosome in the lac region has been extensively investigated. In addition to the structural genes for β-galactosidase (z), permease (y), and transacetylase (a) there is a gene for inducibility. Thus several types of mutations can occur:

$z^+ \longrightarrow z^-$, unable to produce β-galactosidase even in presence of inducer

$y^+ \longrightarrow y^-$, unable to synthesize permease even in presence of inducer

$i^+ \longrightarrow i^-$, gain in ability to maximally synthesize lac proteins even in the absence of inducer

All three mutations are independent of each other and reversible provided the mutation is not a deletion. The z and y genes behave as functional units in complementation tests but the i gene simultaneously affects both functions (Jacob and Monod, 1961). Many independent isolates of the various types have been obtained and the lac region has been mapped by recombination. The genes are closely linked and the wild type (+) alleles of z, y, and i are dominant.

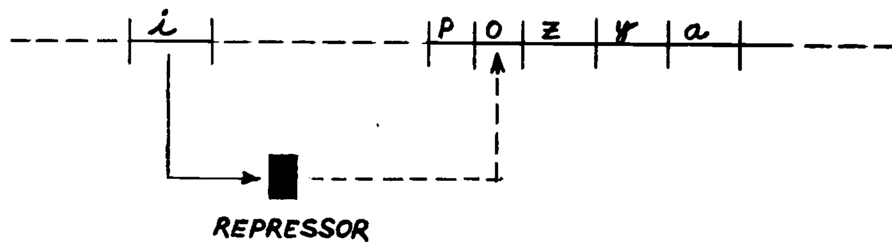
By means of genetic transfer employing the F episome, it is possible to produce heterogenotes (Jacob and Wollman, 1961). Partial diploids of the structure i^+z^-/i^-z^+ or i^+y^-/i^-y^+ form β-galactosidase or permease respectively only in the presence of inducer (Jacob and Monod, 1961). This reveals that the i^+ allele is dominant and active in the transposition with respect to z and y. The i gene thus appears to be a structural gene for a diffusible product which is responsible for repression of the lac operon. Genes of this type are termed "regulatory" genes. The product, termed the repressor, was isolated and recently shown to be a protein (Gilbert and Muller-Hill, 1966). The site of action of the repressor seems to be a genetic locus itself and is termed the operator or "o" locus. An

operon is a genetic unit of coordinate expression and in the lactose system includes the linked *o*, *z*, *y* and *a* genes. The operon model implies that the repressor has two sites, one which interacts with operator, and whose absence is indicated by the i^- allele, and another which interacts with the inducer. Mutations of the latter type would be in a continuously repressed state regardless of the presence of inducer and would be dominant over the i^+ allele. Such mutants, which result in non-inducibility of the lac operon in spite of intact operator and structural genes, have been isolated (Wilson *et al.*, 1964). These mutants termed i^s are thought to produce a repressor which has no affinity for inducer so that the operon is permanently shut off (Beckwith, 1967).

The nature of the operator was indicated by the finding that o^c mutants cause constitutive synthesis only for the lac genes which are linked to the operator. These mutants are dominant over the inducible wild type allele (o^+) and consequently were distinguishable from recessive i^- mutations. In o^c mutants the repressor no longer interacts with the operator; in fact, most o^c mutants are thought to be deletions (Beckwith, 1967).

Recently Beckwith (1967) introduced the term promotor (p) to indicate the site on the genome where synthesis of messenger RNA for the lac operon begins. This site was proposed to set the maximal potential for expression of the lac operon and mutants which altered

the maxinal level of the lac enzymes were isolated. The following diagram represents the most recent depiction of the lac operon from E. coli (Miller et al., 1968):



Although genetic recombination cannot yet be performed to characterize lac⁻ mutants of lactic streptococci, the isolation of constitutive mutants as well as strains lacking β-galactosidase and galactoside permease would be strong evidence for control over the lactose operon as occurs in E. coli.

Accumulation of Lactose by E. coli

To analyze lactose concentration mechanisms by E. coli requires that the compound be shown to accumulate within the cell. This may be done either by the use of an analogue which cannot be further metabolized or by the use of a bacterial strain which posesses the permease but lacks β-galactosidase and thus cannot further metabolize the accumulated disaccharide. Both methods have been applied to E. coli and the analogue TMG, as well as lactose, were accumulated as unaltered compounds. No derivatives were formed.

The entry process was sensitive to agents which uncouple oxidative phosphorylation, but was not affected by sodium fluoride (Kennedy and Scarborough, 1967). In addition, the entry of lactose into E. coli is the rate limiting step of lactose utilization (Kepes and Cohen, 1960). The latter point emphasizes the importance of the transport system.

Fox and Kennedy (1965) isolated a protein component from E. coli which was controlled by the i gene. This protein, the M protein, was membrane bound and an essential component of the lac operon. Later experiments (Fox, Carter, and Kennedy, 1967) indicated the y gene was responsible for its synthesis. Upon the isolation of the protein responsible for lactose transport in E. coli, Fox and Kennedy proposed a model for β-galactoside transport in E. coli based on the original theories of Kepes and Cohen (1962), and Koch (1964). This model has a molecule of lactose in the medium combining with the "M protein" on the exterior surface of the membrane as a lactose-M complex. On the inner surface the complex would dissociate. If the enzyme β-galactosidase is present in the cell, the continuous removal of lactose by this reaction would lead to substantial influx of lactose from the medium into the cell. This process, known as facilitated diffusion, does not require energy, and the transport of lactose would be directly linked to its subsequent metabolism by the cell.

In E. coli, however, lactose is accumulated by the cell against a concentration gradient (Rickenberg et al., 1956). Such a process

requires the continuous expenditure of energy by the cell and is termed "active transport." Scarborough, Rumley and Kennedy (1968) suggested a mechanism to account for such a process in *E. coli*. The lactose-M complex would dissociate at the inner surface of the membrane yielding intracellular lactose and M_i. M_i can no longer function as a carrier because of its greatly reduced affinity for lactose. However, by the continuous expenditure of energy, M_i is converted back to the functional carrier M at the external side of the membrane where it now functions to bring in another molecule of lactose.

"β-Galactosidase" of *Staphylococcus aureus*

The enzyme system responsible for lactose metabolism in *S. aureus* was originally investigated by Creaser (1955). ONPG hydrolysis by whole cells was preferentially induced by galactose; lactose was only a partial inducer. The "β-galactosidase" had a higher affinity for lactose than the synthetic β-galactoside; in fact, ONPG hydrolysis was prevented by the presence of lactose, galactose or glucose. Since exhaustive attempts to obtain the enzyme in cell-free extracts were unsuccessful, the authors concluded the organism contained a very labile enzyme.

McClatchy and Rosenblum (1963) confirmed this conclusion but in addition further attempted to characterize the lactose system in

S. aureus by the isolation of lactose negative mutants. Mutants defective in "β-galactosidase" as well as galactoside permease were isolated and shown to belong to separate but closely linked groups by transduction analysis. In addition, a constitutive strain was isolated which simultaneously produced large amounts of both enzymes in the absence of inducer, suggesting the existence of a regulatory gene which controlled the expression of the two structural genes. The gene responsible for constitutivity was not closely linked to the structural genes, as is the operator locus of *E. coli*. Although these results suggested to the authors that *S. aureus* utilized lactose as occurs in *E. coli*, another mutant type was isolated which affected lactose utilization. These mutants were pleiotropic and did not ferment lactose, sucrose, mannitol, fructose, or galactose. The significance of this phenotype, however, was not realized at that time.

The existence of pleiotropic mutants affecting carbohydrate utilization were known prior to this report. In fact, the occurrence of such mutants had been reported from time to time since one of the earliest reports by Doudoroff *et al.* (1949). He reported the isolation of a mutant from *E. coli* which simultaneously lost the ability to utilize lactose, glucose, and maltose. In 1951, Lederberg *et al.* also reported the isolation of pleiotropic mutants. Monod and Cohen (1952) pointed out that further studies were needed on those organisms which simultaneously lost the ability to ferment a group of

carbohydrates. In 1962 Morse and Egan first reported pleiotropic mutations in _S. aureus_, although a detailed report was not published. Korman (1962) also isolated pleiotropic mutants from _S. aureus_. These mutants, obtained by UV irradiation or as spontaneous variants from stock cultures were unable to ferment mannitol, fructose, galactose, sucrose, lactose, and maltose. Reversion of a single mutant on one carbohydrate, however, resulted in the ability to ferment all the carbohydrates. Transductants of the pleiotropic mutant isolated on galactose also restored the ability to utilize all the carbohydrates. These results suggested the pleiotropic phenotype was the cause of a single gene effect.

In 1963, Mukai and Margolin reported the isolation of mutants from Salmonella which failed to ferment glucose, arabinose, galactose, maltose, mannose, sorbitol, and mannitol. This pleiotropic locus mapped near the cluster of tryptophan genes and this region on the chromosome identified with carbohydrate utilization was designated as the car locus.

The first detailed report characterizing the pleiotropic mutation was in _S. aureus_ (Egan and Morse, 1965a). The car$^-$ mutant was unable to ferment eight different carbohydrates, and in agreement with the work of Korman (1962), the genetic defect was shown to be a single gene effect. Since the organism grew on glucose it was assumed the glycolytic enzymes were present and although sucrose

and maltose were not fermented, the hydrolytic enzymes, sucrase and maltase, were present in cell-free extracts. Hence the inability of the car$^-$ mutant to ferment eight different sugars did not appear to be related to their intracellular metabolism, but rather the transport of the carbohydrate into the cell. Indeed, when the latter was examined, it was observed that the car$^-$ mutant was impermeable to the carbohydrates. This transport defect was not associated with the cell wall nor were the uptake of nutrients other than carbohydrates affected (Egan and Morse, 1965b). In accordance with the theory of sugar transport as suggested by Kepes and Cohen (1962) and Koch (1964), the authors suggested the car$^-$ mutant lacked the common carrier responsible for the transport of carbohydrates into the cell, while the inducible nature of lactose utilization reflected permease function, specific for each carbohydrate, whose role was to increase the affinity between the carbohydrate and the carrier protein.

Efforts to support the hypothesis that carbohydrate transport was dependent upon specific permeases as well as a common carrier, Egan and Morse (1966) studied the uptake of carbohydrates by the wild type (car$^+$) strain of S. aureus. Although data was presented supporting the above hypothesis, the most significant finding was the nature of the intracellular state of the accumulated sugars. Using mutants defective in β-galactosidase, one would expect lactose to accumulate within the cell as the free disaccharide since it cannot be further

metabolized. In S. aureus, however, lactose accumulated as a derivative which no longer chromatographed with the parent compound. The formation of this derivative required the integrity of the cell. In addition, the concept of "active transport" for lactose utilization in S. aureus could no longer be used, since the sugar accumulated as a derivative and not as the free disaccharide. Sucrose, α-MG, and maltose were also shown to accumulate partially or wholly as derivatives.

The characterization of the lactose derivative was performed by Hengstenberg, Egan, and Morse (1967, 1968) by isolating large quantities of the substance from a lac^- mutant lacking "β-galactosidase." The derivative was negatively charged and when treated with alkaline phosphatase chromatographed as lactose-1-^{14}C. This result, coupled with the observation that in double labeling experiments the derivative contained labeled material from both lactose-1-^{14}C and ^{32}P, suggested the derivative was lactose-PO_4. E. coli β-galactosidase could not hydrolyze the derivative, however, extracts from wild type S. aureus readily split the phosphorylated derivative to glucose-1-^{14}C and a ^{32}P labeled hexose. The apparent lability of "β-galactosidase" in cell-free extracts as reported by various workers (Creaser, 1955; McClatchy and Rosenblum, 1963; and Egan and Morse, 1965a) was therefore explained; the organism contained an enzyme which hydrolyzed lactose-PO_4 and not the free disaccharide.

Since the car$^-$ mutants were unable to transport or phosphorylate certain carbohydrates (Hengstenberg, Egan, and Morse, 1967 and 1968), it was suggested that phosphorylation was involved in the transport process similar to the phosphoenolpyruvate (PEP) dependent-phosphotransferase system as reported earlier in E. coli (Kundig, Ghosh, and Roseman, 1964).

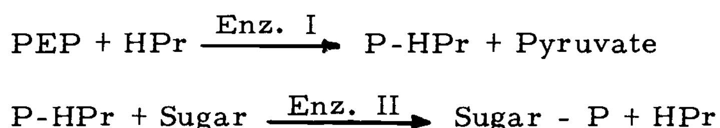
As already noted, whole cells of S. aureus hydrolyze ONPG; however, loss of activity is observed upon treatment of the cell with reagents which destroy the permeability barrier (Creaser, 1955; Egan and Morse, 1965a). In 1967, Kennedy and Scarborough reported a new mechanism for the hydrolysis of ONPG in S. aureus. They discovered that the addition of PEP to toluene treated cells restored the ability to hydrolyze ONPG; other high energy compounds could not replace the apparent PEP requirement. In addition, NaF, an inhibitor of the enolase reaction, inhibited ONPG hydrolysis in whole cells but had no effect on toluene-treated cells in the presence of PEP, which was consistent with the idea that a supply of PEP was necessary for the hydrolytic reaction. The authors suggested that S. aureus possessed an enzyme hydrolyzing ONPG-P and not ONPG. The phosphorylation step was suggested to occur via the PEP-dependent system of Kundig, Ghosh, and Roseman (1964).

The o-nitrophenyl-B-D-galactoside-6-phosphate (ONPG-P) was synthesized by Hengstenberg and Morse (1968) and shown to be a

substrate for the staphyloccal "β-galactosidase," but not a substrate for the β-galactosidase of E. coli. Since the enzyme from S. aureus hydrolyzes the phosphorylated derivatives and not the unsubstituted compounds, an appropriate name for the staphylococcal "β-galactosidase" would be β-D-phosphogalactoside galactohydrolase (Laue and MacDonald, 1968).

The PEP-Phosphotransferase System

Kundig, Ghosh, and Roseman (1964) and Kundig et al. (1965 and 1966) described a PEP-dependent phosphotransferase system from E. coli which catalyzed the transfer of phosphate from PEP to certain sugars. This system was separated into three protein fractions, enzymes I and II and a histidine containing protein (HPr) which accepted phosphate from PEP. In the presence of Mg^{++} enzymes I and II catalyzed the following reactions:



$$PEP + HPr \xrightarrow{\text{Enz. I}} P\text{-}HPr + Pyruvate$$

$$P\text{-}HPr + Sugar \xrightarrow{\text{Enz. II}} Sugar - P + HPr$$

Preliminary experiments suggested more than one type of enzyme II existed, while no differences in enzyme I or HPr were observed. Enzyme II was associated with the membrane fraction, whereas enzyme I and HPr were in the soluble extract. Cold shocked cells of E. coli became defective in the ability to transport sugars, and

this defect was overcome by the addition of HPr, which was lost from the cells during the shock treatment. This suggested the transferase system was involved in the transport of sugars into the cell. Sodium fluoride also inhibited the sugar transport which was consistent with the idea that the PEP system was linked to sugar transport if the inhibitor acted on enolase. The same system was also detected in *Aerobacter cloacae*, *A. aerogenes*, and *Lactobacillus arabinosus*.

The above findings triggered a series of papers characterizing the PEP-system by the isolation of specific mutants. The metabolism of mannitol in *A. aerogenes* was shown to be dependent on the phosphotransferase system and that in the absence of an inducible enzyme II specific for mannitol, the organism failed to grow (Tanaka, Lerner, and Lin, 1967). In a subsequent communication Tanaka and Lin (1967) reported on the phenotypic consequences of mutations affecting enzyme I and HPr. Both types resulted in the inability of *A. aerogenes* to utilize a group of carbohydrates, i.e., the pleiotropic mutation. An explanation for these mutants may now exist. For example, if the carbohydrates were phosphorylated during transport, then a mutation affecting this process (lack of enzyme I or HPr) would be pleiotropic with respect to sugar utilization. The multiple sugar defects observed in *A. aerogenes* were also the result of single gene effects.

In 1964, Fraenkel, Falcoz-Kelly, and Horecker reported the

isolation of a mutant from *E. coli* which grew poorly on glucose, and not at all on fructose or mannose. This mutant was subsequently shown to lack enzyme I of the phosphotransferase system (Tanaka, Fraenkel, and Lin, 1967).

Simoni *et al.* (1967) reported on a single mutation in *Salmonella typhimurium* which failed to grow on nine carbohydrates with the loss of enzyme I. This mutant was defective in the transport of sugars into the cell, even though the enzymes necessary for their subsequent metabolism were present. The mutant was cryptic. The authors thus linked the phosphotransferase system with sugar transport.

As judged by their occurrence, pleiotropic mutants are not rare events. The question arises however, as to why several different pleiotropic mutants can be isolated from the same organism. The suggestion by Tanaka and Lin (1967) that there exists more than one kind of enzyme I-HPr system in the same organism and that each system governs the metabolism of a different family of carbohydrates could account for this observation. For example, Wang and Morse (1968) described a pleiotropic mutant from *E. coli* unable to ferment ten different carbohydrates including lactose. Yet *E. coli* is not dependent on the phosphotransferase system for the fermentation of lactose (Kennedy and Scarborough, 1967). Pleiotropic mutants not affecting lactose metabolism in *E. coli* were also described by Tanaka and Lin (1967). The meaning of the latter two mutations

with respect to lactose utilization is as yet unclear.

In 1968, Simoni, Smith, and Roseman linked the car$^-$ mutant from S. aureus to the PEP-dependent phosphotransferase system. This mutant was deficient in enzyme I. In addition, a new protein (fraction III) required in the phosphorylation of TMG was isolated. It was not clear whether the Km factor from A. aerogenes (Hanson and Anderson, 1968) and fraction III functioned in the same or different manner. The Km factor increased the affinity of fructose for enzyme II in A. aerogenes.

Lactose Metabolism in S. aureus

Galactose was originally observed to be the preferential inducer of lactose utilization in S. aureus (Creaser, 1955). McClatchy and Rosenblum (1963) confirmed this observation and in addition, found that IPTG, a potent inducer in E. coli, was ineffective as an inducer in S. aureus. Morse et al. (1968) observed that galactose-6-P induced approximately eightfold higher "β-galactosidase" activity than galactose which suggested this compound may be the true inducer. During further experimentation, galactose-6-P was shown to induce high levels of "β-galactosidase" in a gal$^-$ lac$^-$ strain; galactose was not an inducer. This suggested the gal$^-$ lac$^-$ mutant was unable to phosphorylate the sugars and hence the inducer galactose-6-P was not produced. Likewise, a "β-galactosidase" negative strain, unable

to hydrolyze lactose-P to galactose-6-P, was not induced by lactose; galactose, however, induced lactose uptake. These findings were confirmed by Simoni, Smith, and Roseman (1968) using a car$^-$ mutant unable to phosphorylate galactose. Galactose-6-P induced enzyme II and fraction III for lactose utilization in this mutant, whereas galactose could not.

Since lactose is utilized via the PEP-dependent phosphotransferase system in S. aureus, several types of mutations can be described which affect its metabolism. Deficiency in enzyme I or HPr results in the car$^-$ phenotype, unable to utilize a series of carbohydrates including lactose (Egan and Morse, 1965a; Simoni, Smith and Roseman, 1968). A defect in the membrane associated enzyme II gives rise to a gal$^-$ lac$^-$ phenotype. Such mutants, however, synthesize and hydrolyze ONPG-P in extracts supplemented with membrane fractions from induced wild type cells (Morse et al., 1968; Hengstenberg et al., 1968). A second class of gal$^-$ lac$^-$ mutants can be isolated which differ from the above in that activity in extracts is restored by addition of a soluble component (fraction III) present in induced wild type cells (Hengstenberg, 1968; Simoni, Smith and Roseman, 1968). Strains unable to hydrolyze lactose-P are designated z$^-$ and possess the phenotype gal$^+$ lac$^-$ (McClatchy and Rosenblum, 1963; Morse et al., 1968). In addition, regulatory mutants which are constitutive for the uptake and hydrolysis of

β-galactosides were isolated. These different mutants suggest the lactose operon of S. aureus could be represented as $r^+ z^+ II^+ III^+$. Transduction analysis has shown that z, II, and III are closely linked whereas the r gene could not be determined whether it represented mutations comparable to i^- or o^c mutations in E. coli (McClatchy and Rosenblum, 1963; Morse et al., 1968).

Lactose Metabolism in S. lactis

Enzymes of lactose metabolism in S. lactis were initially studied by Novikova (1957a); hydrolysis of lactose was determined using Saccharomyces globosus, which ferments glucose and galactose but not lactose. Using this technique, three of four cultures were found to hydrolyze lactose to glucose and galactose, whereas a fourth was unable to cleave the disaccharide. The same author (1957b) also tested S. lactis for hydrolysis of lactose-1-P. Neither whole cells nor extracts were able to hydrolyze the derivative and it was assumed S. lactis did not utilize lactose by way of direct phosphorylation.

No definite information is yet available as to how lactose is catabolized by S. lactis. It is generally assumed that in S. lactis, lactose is hydrolyzed by β-galactosidase and the resulting monosaccharides enter the glycolytic cycle where they are assimilated. Shahani and Vakil (1962) studied the effect of several antibiotics on the carbohydrate metabolic patterns of S. lactis UN when grown on

lactose and its constituent monosaccharides, glucose and galactose. The results suggested that this organism metabolized the sugars partly through classical glycolysis and partly through the hexosemophosphate shunt pathway. This hypothesis was strengthened by their observation that S. lactis UN converted lactose to lactobionic acid which was cleaved to form gluconate and galactose (Vakil and Shahani, 1962).

The earlier observations of Novikova (1957), suggested that β-galactosidase was a variable enzyme in different strains of S. lactis, since in one organism acid production was directly related to its lactose decomposition, but he could not detect any β-galactosidase. Similar observations were made by Citti, Sandine and Elliker (1965) and McFeters, Sandine, and Elliker (1967). They reported that in S. lactis the synthesis of β-galactosidase was induced by lactose and that it was a very unstable enzyme since of over 40 strains examined only one strain (7962) produced enzyme with sufficient stability to permit purification.

More recently Vakil and Shahani (1969) described the enzymatic utilization of lactose in S. lactis UN. This organism possessed β-galactosidase and lactose dehydrogenase activity. The organism did not exhibit lactose phosphorylase activity, nor was lactose-1-P an intermediate in the metabolism of lactose. These results suggested S. lactis UN utilized lactose by two pathways: (1) by

hydrolytic cleavage of lactose to glucose and galactose via β-galactosidase, and (2) by oxidation to lactobionic acid via lactose dehydrogenase, prior to cleavage to gluconate and galactose.

Lactose dehydrogenase activity, as measured by the reduction of 2, 6 dichlorophenolindophenol, has also been observed among lactic streptococci in our laboratory (Wagner, 1965; Walter, 1969). This procedure, however, was found not to be specific for lactose, since other carbohydrates also reduced the dye.

The observations reported here indicate that lactic streptococci may possess different mechanisms for lactose utilization, functioning separately or together in a given strain. However, the "labile" β-galactosidase of many lactic streptococci remained unanswered. Working on this problem, Walter (1969) suggested the involvement of the PEP-phosphotransferase system in lactose utilization by _S. lactis_ C_2F, a strain containing "labile" β-galactosidase.

MATERIALS AND METHODS

Microorganisms

Strains were obtained from the stock culture collection maintained by the Department of Microbiology, Oregon State University. *Lactobacilli* species included *L. bulgaricus* (GA), *L. acidophilus* (FARR), *L. arabinosus* (17-5), *L. lactis* (39-A), *L. casei* (OSU), and *L. helveticus*. Cultural characteristics of these organisms were previously described (Briggs, 1953; Wheater, 1955a; Wheater, 1955b). Strains of lactic streptococci used were *S. lactis* 7962, C_2F, C10, C_2S, 11454, 7963, E, b, and *S. cremoris* 144F; their physiological characteristics were determined by Sandine, Elliker, and Hays (1962). *S. thermophilus* Mc, C3, and S, as well as *E. coli* B, were also used during this study.

Media and Conditions of Growth

The cultures were maintained in sterile nonfat milk. The organisms were incubated near their optimum growth temperature until milk coagulation occurred. At this time, 1.0 ml of the culture was transferred to 10.0 ml of sterile milk in a screw cap tube. All cultures were stored at 0 C for two weeks after which the above procedure was repeated. To maintain active cultures of *S. lactis*

C_2F it was necessary to store the culture at -20 C, since repeated transfer of this strain would eventually give rise to a slow culture.

For induction experiments the organisms were propagated in lactose broth consisting of lactose, 10 g; Difco tryptone, 20 g; Difco yeast extract, 5 g; gelatin, 2.5 g; sodium chloride, 4 g, sodium acetate, 1.5 g; ascorbic acid, 0.5 g, and distilled water to 1.0 liter. The medium was prepared without lactose, sterilized, and then a sterile solution of lactose was added to give a final sugar concentration of 1%. In later experiments, galactose was used to induce lactose utilization in the parent and mutant strains of S. lactis C_2F. The lactobacilli were grown in milk until coagulation, after which a 1% inoculum was made into maltose broth and incubated at the appropriate temperature for 12 hours. At this time a 1% inoculum was delivered into lactose broth to induce for β-galactosidase activity. L. lactis, L. acidophilus, and L. helveticus were incubated at 37 C while L. casei, L. arabinosus, E. coli, and S. lactis 7962 were incubated at 32 C. All cultures were grown for ten hours.

For induction of β-galactosidase activity in L. bulgaricus, the organism was grown at 37 C in milk for 12 hours. A 1% inoculum was then delivered into 100 ml lactose broth which was incubated under 12 to 14% CO_2 tension for six to eight hours at 40 C. Cells were then harvested and tested for enzyme activity.

Measurement of β-Galactosidase

Harvesting Cells

The cells grown in the lactose broth were harvested using a RC-2 Sorvall refrigerated centrifuge (1 C) at 3,000 x g. The cells were washed twice with the appropriate suspending medium and resuspended in the buffer.

Preparation of Whole Cell Suspensions

Washed cells were resuspended in buffer to obtain approximately 65 μg dry weight per ml.

Preparation of Toluene-treated Cell Suspensions

Washed cells were resuspended as with whole cells. Four-ml volumes of these suspensions were treated with 0.2 ml toluene-acetone (1:9) (v/v) and incubated at room temperature with vigorous agitation. The treated suspensions were immediately assayed for β-galactosidase.

Preparation of Cell-free Extract

Cells were suspended in 0.05 M sodium phosphate buffer (pH 7.0) to obtain approximately 150-160 μg dry weight per ml, and then

broken in a Raytheon sonic oscillator for 20 minutes. The cell debris was removed by centrifugation at 12,000 x g for ten minutes at 1 C. The supernatant fluid was assayed for enzyme activity.

β-Galactosidase Assay

The chromogenic substrate, ONPG (Lederberg, 1950), was used to measure enzyme activity. Solutions of 0.005 M ONPG were prepared in the appropriate buffer system. A 0.5 ml volume of whole cells, toluene-treated cells, or cell-free extracts was incubated with 2.5 ml ONPG for 15 minutes at the appropriate temperature. The reaction was stopped by adding 2.5 ml of 0.5 M sodium carbonate and where necessary cells were removed from the reaction mixture by centrifugation. The absorbancy of the supernatant liquid was measured at 420 mμ and the μmoles of o-nitrophenol (ONP) liberated from ONPG determined from a standard curve prepared by measuring the change in absorbancy produced by various concentrations of ONP. The dry weight of the cell suspension was obtained from a standard curve relating cell suspension optical density at 420 mμ to cell dry weight (Society of American Bacteriologists, 1957). Protein was measured by the method of Lowry and Rosebrough (1951). The specific activity of β-galactosidase was expressed as μmoles ONP liberated from ONPG per mg of cell dry weight (or protein) per minute. One unit of enzyme is equivalent to one μmole

of ONP liberated from ONPG per minute.

Uptake of Lactose

Essentially, the experimental procedure consisted of inducing a culture of bacteria, preparing a concentrated cell suspension, exposing the suspension to lactose and, after given time intervals, assaying for residual extracellular lactose. A similar procedure was used by Goodman and Pickett (1966) to study lactose uptake by various Enterobacteriaceae. The method has the obvious disadvantage that lactose is non-gratuitous and therefore intracellular metabolism of the sugar will occur. However, the technique has several advantages over the conventional method of assaying for galactoside permease (uptake of gratuitous radioactive β-galactosides such as TMG) namely (1) the natural substrate lactose is employed rather than an analogue, and (2) the method is simple and requires no elaborate equipment or reagents. In later experiments, the uptake of ^{14}C-TMG was measured.

Suspending Media

Cells were washed, suspended, and assayed in one of the following solutions: 0.05 M sodium phosphate buffer (pH 6.6 for the lactobacilli and pH 7.0 for the streptococci): 0.05 M sodium arsenate, pH 7.0; 0.15 M sodium chloride, pH 7.0; 0.15 M KCl, pH 7.0; H_2O,

or 0.05 M sodium phosphate buffer at pH 7.0 plus one of the following inhibitors: 0.01 M sodium azide, 0.03 M sodium fluoride, or 0.001 M iodoacetic acid.

Measurement of Lactose Uptake

The organism to be studied was inoculated into 100 ml lactose broth. After ten to 12 hours of incubation at 37 C (lactobacilli) or 32 C (streptococci) the cells were centrifuged, washed with the appropriate buffer system containing 100 μg of chloramphenicol per ml and resuspended in the buffer. The concentration was adjusted to a cell dry weight of 1.60 mg ml for the lactobacilli and 2.5 mg ml for the streptococci. For the test 2.0 ml cells plus 1.0 ml lactose (3 mg ml) were mixed and then incubated at 37 C. At appropriate time intervals, 0.4 ml samples were diluted 1/10 in ice cold water and centrifuged. The supernatants were assayed in duplicate for lactose by the anthrone test. In some cases glucose grown cells were also measured for lactose uptake.

Anthrone Test for Hexose

A modification of the anthrone test described by Dische (1955) was used which allows the assay of small samples. The unknown sample in a total volume of 1.2 ml was chilled and 2.4 ml cold anthrone reagent (0.2% anthrone in 95% H_2SO_4 prepared fresh daily)

were added by layering over the surface of the unknown solution. The reaction mixture was vigorously mixed and then heated in a boiling water bath for 10 min after which the tubes were iced. The OD was read at 625 mμ on a Bausch and Lomb Spectronic 20. The μg lactose per tube was obtained by referring to a standard curve prepared by carrying out the above procedure with known concentrations of lactose.

Measurement of TMG Uptake

^{14}C-TMG uptake was measured at 37 C in 4 to 6 ml cell suspensions containing 1.0 to 2.0 mg cell dry weight per ml. At zero time the radioactive substrate was added and 0.5 ml samples were collected at intervals by filtration through nitrocellulose membrane filters (0.45 μ pore size) in order to stop the reaction quickly. The filters were washed with 4.0 ml of ice cold 0.05 M sodium phosphate buffer (pH 7.0) and then dried at 60 C for three hours. The radioactivity accumulated by the cells was determined by liquid scintillation spectrometry employing 4.0 g of 2.5-diphenyloxazole (PPO) and 0.05 g p-Bis 2-(5-phenyloxazole) (POPOP) per liter of toluene as the fluor solution.

Effect of β-Galactosidase on Acid Production

S. lactis C_2F (fast acid producer) and *S. lactis* C_2S (slow acid

producer) were grown at 21 C in 11% reconstituted nonfat milk for eight and 12 hours, respectively. The cultures were diluted 1:1 with sterile 0.05 M sodium phosphate buffer (pH 7.0) and 6.0 ml of diluted culture was added to 94 ml 11% milk supplemented with β-galactosidase, heat inactivated enzyme, or 0.05 M sodium phosphate buffer. These samples were incubated at 32 C and the percent titratable acidity determined after 2.5 and 3.5 hours. The change in percent acidity during this time interval was calculated for each culture and the percent increase over the control was then determined for the enzyme supplemented cultures.

The β-galactosidase was a crude extract prepared from *L. helveticus* and the final concentration in the milk culture was 0.025 units/ml. The heat inactivated enzyme was prepared by placing a tube containing the extract in a boiling water bath for ten minutes.

Isolation of Lactose Mutants of *S. lactis*

Mutagenesis

Ultraviolet Light. Cells in 100 ml lactic broth were grown at 32 C for four to six hours. Cells were collected, washed, and resuspended in 0.85% saline. Forty ml of the cellular suspension were then added to a sterile six inch petri dish and irradiated with a UV lamp positioned 12 inches above the plate. After a 30 to 45 second

exposure (99.999% kill), 1.0 ml samples were added to 10.0 ml lactic broth. Incubation was continued at 21 C for six hours, samples were removed, diluted, and spread over the surface of indicator plates.

Acriflavin. Cultures were grown at 32 C for ten hours after which a 5% inoculum was added to 10 ml lactose broth. Acriflavin was added to give a final concentration of 10 and 20 μg per ml. Cultures were incubated at 40 C for 24 hours and then 0.1 ml samples removed and spread over the surface of lactose agar plates (Hirota, 1960) (Table 1).

N-Methyl-N-nitro-N-nitrosoguanidine (NTG) *S. lactis* 7962 was grown for 4.5 hours and *S. lactis* C_2F for 3.5 hours at 32 C in glucose broth. Twenty ml of culture were then removed and filtered through a 0.45 μ Millipore filter, the cells were washed with 10.0 ml TM buffer (0.50 M Tris, 0.05 M maleic acid, 1.0 g $(NH_4)_2 SO_4$, 0.1 g $Mg SO_4 \cdot 7H_2O$, and 0.25 mg $FeSO_4 \cdot 7H_2O$ per liter of distilled water, pH 7.0), and the filter placed in 18.0 ml of the buffer to remove the cells. The filter was removed and NTG (Adelberg, Mandel, and Chen, 1965) was added to a final concentration of 200 μg per ml (*S. lactis* 7962) or 100 μg per ml (*S. lactis* C_2F). The flasks were incubated at 32 C for two hours (*S. lactis* 7962) or 30 minutes (*S. lactis* C_2F) and then samples removed, diluted, and plated on indicator agar. Figure 1 shows the survival curves of the two strains exposed to NTG.

Table 1. Effect of acriflavin on *S. lactis* C_2F.

μg acriflavin per ml	No. of colonies per plate	No. of lactose negative colonies
10	54	38
	49	41
20	15	8
	16	8
10	387	6
20	111	1
0	509	0
0	668	0

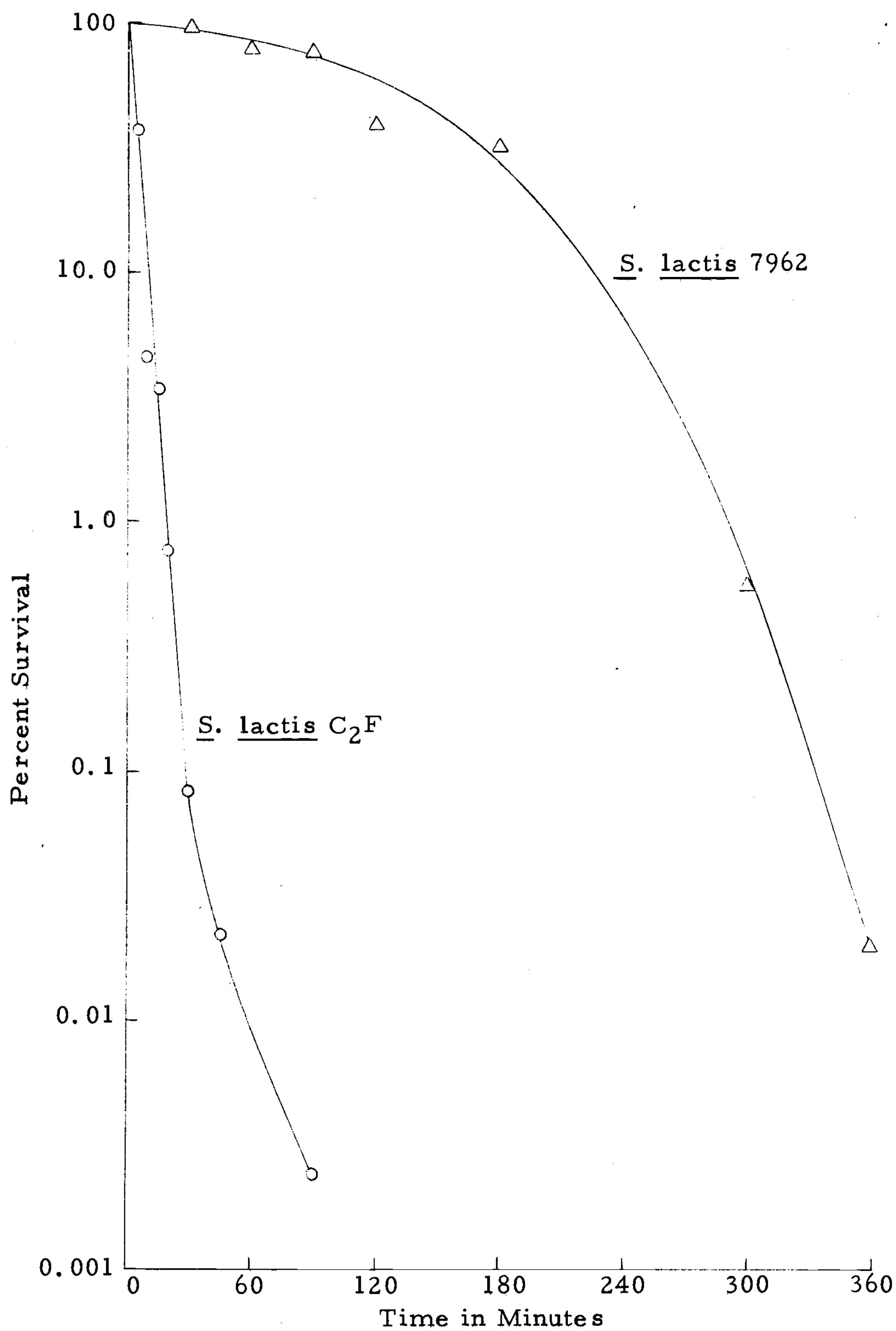


Figure 1. Treatment of S. lactis 7962 and S. lactis C_2F with NTG.

Selection of Lac^- or Gal^- Mutants

Lactic agar containing 1% lactose or galactose as the sole carbon source supplemented with 0.004% brom cresol purple served as the indicator medium. On this medium colonies producing acid were yellow in contrast to the white non-acid forming colonies. Plates were incubated at 32 C for approximately 30 hours.

Test for Constitutive β-Galactosidase Production

Cells were spread over the surface of lactic agar containing 0.5% glucose, 0.5% lactose plus 0.5% glucose, or 0.2% lactose plus 1% glucose. The plates were incubated at 32 C for 36 hours and then treated with 4.0 ml 0.01 M ONPG for five to ten minutes (Loomis and Magasanik, 1965). A faint yellow zone appeared around each colony producing a β-galactosidase constitutively or which was resistant to catabolite repression.

Test of Purity

To avoid the isolation of contaminants, only those mutants which were catalase negative and sensitive to their specific parent phages were selected. The *S. lactis* C_2F phage was obtained from the stock culture collection, whereas the phage for *S. lactis* 7962 was isolated from a whey sample.

Enrichment for β-Galactosidase Constitutive Mutants

Kessler and Rickenberg (1964) described a technique for the selection of _E. coli_ mutants constitutive for β-galactosidase synthesis. This method was based on the observation that α-methylglucoside (α-MG) would inhibit adaptation to lactose. Preinduced cells or those which formed β-galactosidase constitutively were unaffected by α-MG in the presence of lactose. Thus by growing the cells in glucose broth, harvesting, and resuspending the cells in broth containing lactose plus α-MG a lag of 18 to 24 hours was observed before growth resumed. From this new growth β-galactosidase constitutive mutants were isolated. This approach was used in attempting to isolate constitutive strains from _S. lactis_ 7962.

To test such a system, _S. lactis_ 7962 which form β-galactosidase inducibly was grown at 32 C in lactic broth containing 1% lactose (adapted cells) or 1% glucose unadapted cells) for ten hours. Cells were harvested, washed, and resuspended in 0.05 M sodium phosphate buffer (pH 7.0) to obtain an absorbancy of approximately 0.1 when a semi-synthetic medium containing 10 mM α-MG and 10 mM of lactose was inoculated. These tubes were incubated at 32 C and the increase in turbidity during the next 48 hours was recorded using a wavelength of 420 mμ. The semi-synthetic medium contained acid hydrolyzed casamino acids, 10 g; carbohydrate, 10.0 mM; sodium acetate,

0.67 g; niacin, 500 μg; calcium pantothenate, 545 μg; pyridoxamine, 500 μg; biotin, 50 μg; Na_2HPO_4, 0.2 g; NaH_2PO_4, 0.2 g; $MgSO_4$, 80 mg; NaCl, 4.0 mg; $FeSO_4 \cdot 7H_2O$, 4.0 mg; $MnSO_4 \cdot 4H_2O$, 4 mg; and distilled water to one liter. The pH was adjusted to 7.0 prior to sterilization.

Test for Pleiotropic Carbohydrate Mutations

Fermentation tests were initially conducted in lactic broth (Elliker, Anderson, and Hannessen, 1956) containing 0 004% brom cresol purple. Later experiments utilized the more convenient technique of streaking the organism on lactic agar plates supplemented with the acid indicator, in the presence of one percent carbohydrate.

Serological Identification

Lancefield's Streptococcus Group N capillary precipitin test was performed using extracts from S. lactis C_2F, S. lactis C_2FW (a lac^- mutant), and S. lactis 7962. Extracts of the latter strain were also tested against Bacto Streptococcus Antisera from serological groups A, B, C, D, E, F, G, H, K, M, O, and MG. The cells were grown in Bacto-Todd Hewitt broth at 32 C for 24 hours and the antigen prepared by the autoclave or hot HCl method (Difco Laboratories, Inc., 1968). Positive controls were group specific antisera and antigen, whereas negative controls consisted of antisera

or antigen layered with 0.85% saline.

Hydrolysis of Lactose by Cell-Free Extracts

S. lactis 7962 or *S. lactis* C_2F was grown in one liter of lactose broth for ten hours. Cells were harvested, washed, and suspended in 50 ml of 0.05 M Na_2HPO_4 buffer (pH 7.0) and ruptured in a Raytheon 10 KC sonic oscillator for 30 minutes. The cell debris was removed by centrifugation at 12,000 × g for ten minutes at 1 C. The supernatant fluid was used as the cell-free extract. Four ml of extract plus 4.0 ml of lactose (3.0 mg/ml) were mixed and incubated at 37 C for 1 hr. At the end of this period samples were placed in an ice bath. The extracts were spotted on thin layer chromatographic (TLC) plates prepared by dissolving 30 g of Silica Gel G in 60 ml M/15 phosphate buffer at pH 8.0. Plates were developed in n-butanol-dioxane-water (4:5:1) (v/v/v) for 3 hr (Heftman, 1967). Carbohydrates were detected by the periodate-benzidine method (Clark, 1964).

Studies Using Labeled Lactose and TMG

Accumulation of Labeled Lactose and TMG

S. lactis C_2F was grown at 32 C for four hr. in the presence of 1% galactose to induce for lactose utilization. Cells were harvested and washed twice with 0.05 M, pH 7.0 sodium phosphate

buffer, containing 100 μg of chloramphenicol (CAP) per ml and resuspended in the same buffer. For measurement of accumulation of lactose the assay consisted of 1.62 mg cell dry weight per ml and 5.2×10^{-4} M lactose-1-^{14}C in a total volume of 1 ml. In studies on the accumulation of TMG, the system consisted of 2.0 mg cell dry weight per ml and approximately 6×10^{-4}M ^{14}C-TMG in a total volume of 1.0 ml. Both samples were incubated at 37 C in a water bath for one hour after which they were centrifuged and the supernatant fluid removed. The cells were then treated with boiling 80% ethanol for five minutes, the debris was sedimented by centrifugation and the extracts were removed for further analysis. Measurement of the accumulation of ^{14}C-TMG by _S. lactis_ 7962 was performed as above except cells were induced by growth in 1% lactose. In later experiments, the entire 1.0 ml sample was filtered through a Millipore membrane filter and washed with 25 ml of ice cold sodium phosphate buffer (pH 7.0). The filter containing the filtrate, was then treated as above to extract the labeled TMG from the cells.

Chromatography and Detection of Radioactive Compounds

To examine the distribution of ^{14}C -label from accumulated lactose, a portion of the ethanol extract was spotted on one-inch strips of Whatman No. 1 paper approximately 29 cm in length. The chromatograms were developed by ascending chromatography for

5 hr in 1 M sodium acetate buffer (pH 3.8)-95% ethanol (2:5). TMG accumulation was studied in the same manner except chromatograms were developed in ethyl acetate-pyridine-water (12:5:3)(v/v/v) for 2.5 hr. After development chromatograms were scanned using a Packard Radiochromatogram Scanner (Model 7201).

Electrophoresis of the ^{14}C-TMG Derivative

Portions of the ethanol extracts containing ^{14}C-TMG and the TMG derivative were spotted on cellulose acetate strips. Electrophoretic characteristics were determined employing 0.05 M sodium phosphate buffer (pH 7.3) at a voltage of 300 with an amperage of 15 ma.

Concentration of the ^{14}C-TMG Derivative and Treatment with Phosphatase

The derivative was separated from contaminating ^{14}C-TMG by chromatography as previously described for the TMG system. A portion of the strip containing only the derivative was then eluted with water for 16 hr. The collected fraction (2.7 ml) was lyophilized to dryness and then resuspended in 0.4 ml water.

Highly purified alkaline phosphatase (Mann Research Laboratories) was dissolved in 0.2 M glycine buffer, pH 8.8, containing 0.014 M $MgCl_2$ at a concentration of 1 mg per ml. Acid phosphatase

(Calbiochem) was prepared in 0.15 M sodium acetate buffer, pH 5.0, at a concentration of 0.2 mg protein per ml. For the test, 0.1 ml substrate (^{14}C-TMG derivative) and 0.1 ml of the appropriate enzyme were mixed and incubated at 30 C for 1 hr. The entire 0.2 ml from each system was then spotted on separate chromatogram strips and developed in the TMG solvent system. The distribution of the label was determined using the strip scanner.

Effect of Cofactors on the Hydrolysis of ONPG in Cell-Free Extracts

Cell-free extracts from galactose grown cells of *S. lactis* C_2F were prepared by breaking the cells in an Eaton press (Eaton, 1962). The frozen mixture was removed from the press, thawed, and centrifuged at 20,000 × g for 20 min. The supernatant was diluted to contain 10 to 15 mg protein per ml as measured by the method of Lowry *et al.*, 1951. Cell-free extracts from *S. lactis* 7962 were prepared from lactose grown cells by sonic oscillation. The debris was sedimented by centrifugation and the supernatant fluid diluted to contain 100-200 μg of protein per ml.

The assay system contained 11.4 mg soluble protein for strain C_2F (128 μg of soluble protein in the case of strain 7962), 10 μmoles ONPG, 20 μmoles cofactor, and when used 20 μmoles NaF in a total volume of 2.0 ml. After incubation at 37 C for 10 min, the reaction

was terminated by the addition of 2.0 ml of 0.5 M Na_2CO_3 and optical densities recorded at 420 mμ.

RESULTS

Studies on Lactobacilli

Optimal Enzyme Assay Conditions

In preliminary experiments, L. helveticus was shown to possess the greatest amount of β-galactosidase activity of several lactobacilli examined; consequently lactose grown cells of this organism were used to determine the optimal enzyme assay conditions. Since treatment of whole cells with toluene, to destroy permeability barriers prior to assay, resulted in an increase in specific activity over untreated whole cells, the maximum time for toluene treatment of L. helveticus was determined (Figure 2). The optimum time was found to be five minutes and increased toluene treatment time up to 20 minutes did not markedly change the activity of the enzyme. The enzyme from this organism was also stable in cell-free extracts prepared by sonic treatment or by breakage on the Eaton press. Figure 3 shows the effect of sonic treatment time on enzyme and protein release from a washed cell suspension. Protein was liberated to a maximum level within ten minutes and then increased only slightly during the next 25 minutes; the enzyme activity in solution, however, reached a maximum level in five minutes and then decreased slowly.

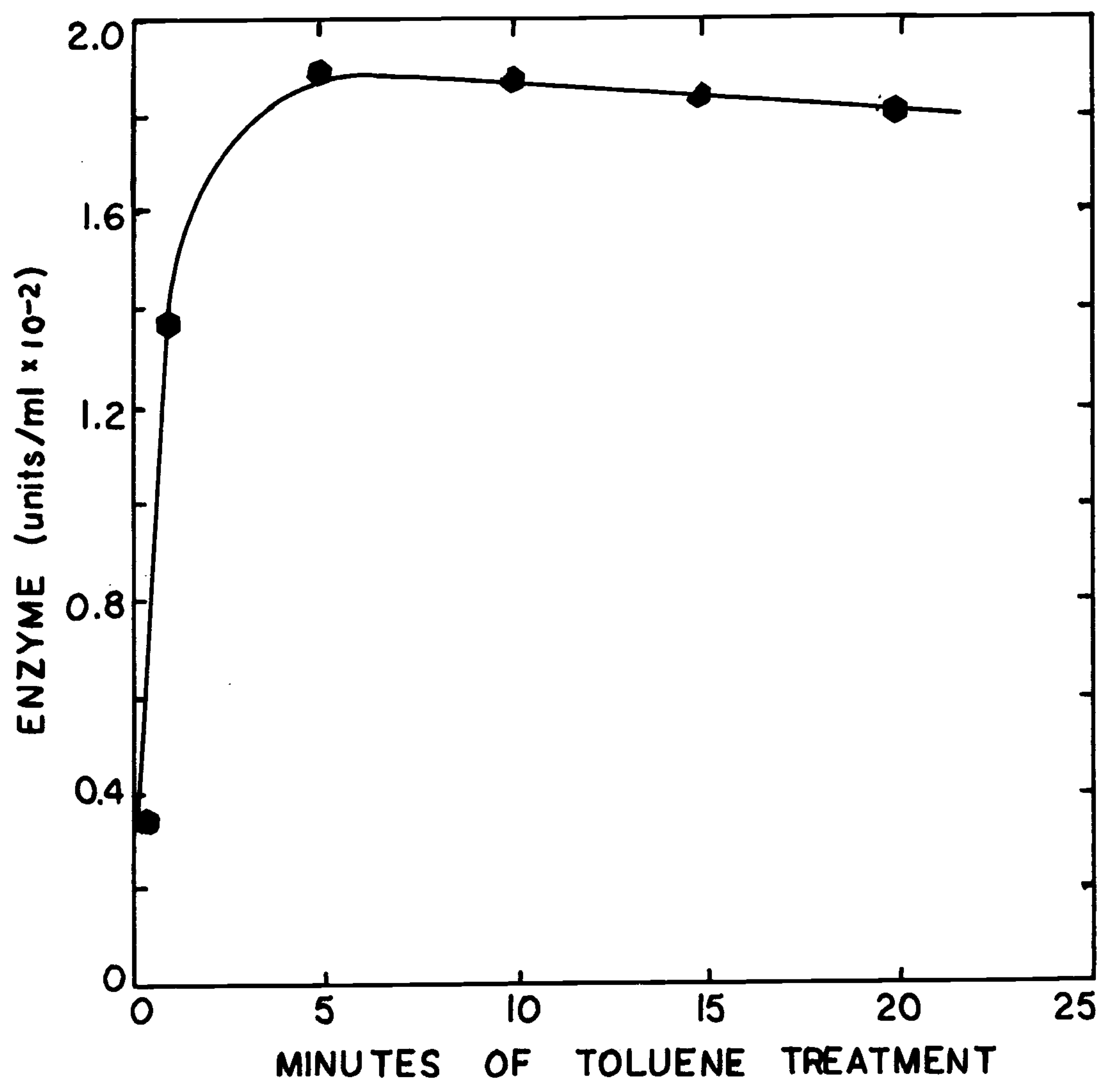


Figure 2. Effect of toluene treatment time on β-galactosidase activity in L. helveticus. Four ml washed cells (approximately 60 μg dry weight per ml) were brought to 25 C and treated with 0.2 ml toluene-acetone (1:9). Tubes were vigorously shaken and at different time intervals samples were removed for enzyme assay.

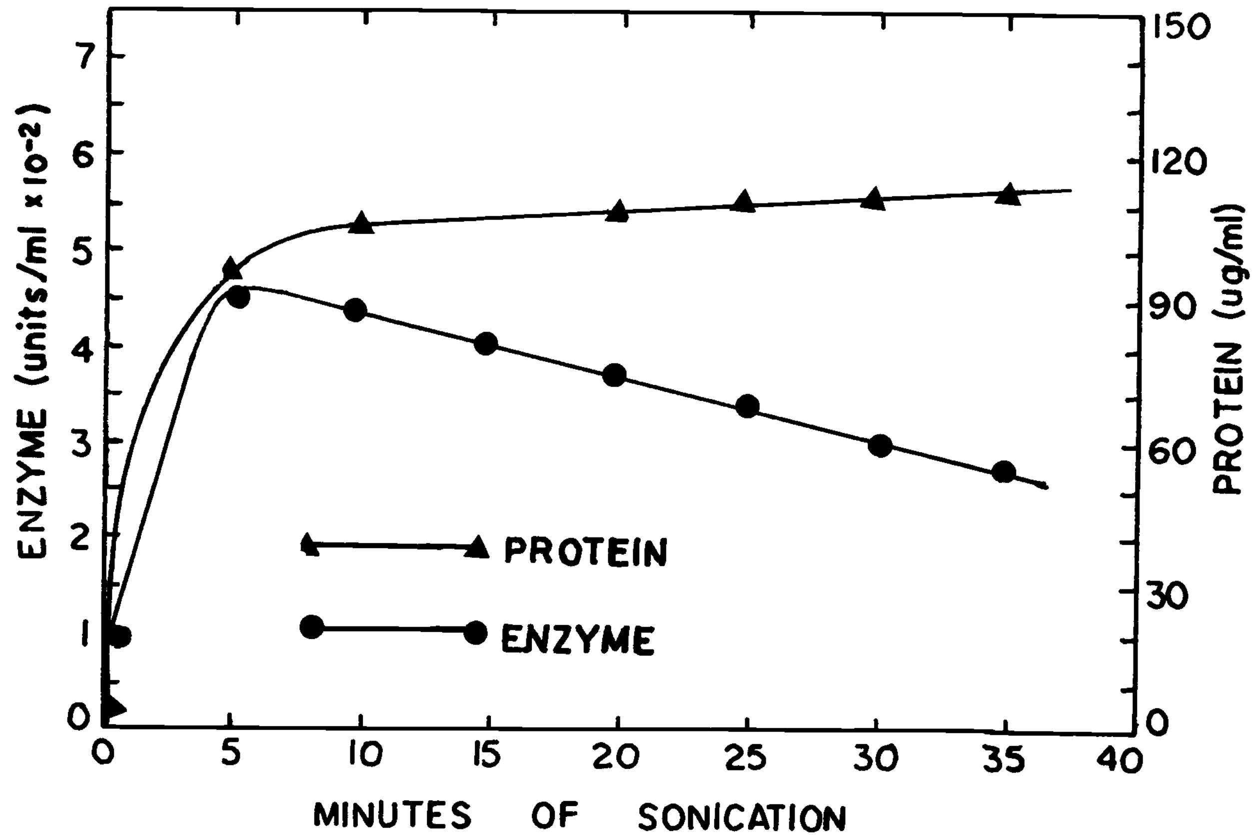


Figure 3. Effect of sonication time on release of β-galactosidase from L. helveticus. Fifty ml cell suspension (approximately 150 μg dry weight per ml) was treated with sonic oscillation and samples were periodically removed for protein and enzyme assay.

The effect of assay incubation temperature on enzyme activity is shown in Figure 4. The activity in cell-free extracts increased with temperature up to 50 C, and then dropped rapidly. The effect on cell-free extracts of the assay solution pH is shown in Figure 5. The optimal pH was about 6.6 when using sodium phosphate buffer (0.05 M) at 50 C; potassium phosphate buffer afforded 30% less activity.

β-Galactosidase of Several Lactobacilli

Since *L. helveticus* possessed the greatest enzyme activity at 50 C and pH 6.6, a comparison was made between this organism and other lactobacilli. The enzyme activities were determined at 50 C and 37 C (Figure 6). *L. helveticus*, *L. lactis*, and *L. acidophilus* possessed greater enzyme activity at 50 C than at 37 C, whereas *L. arabinosus* and *L. casei* had greater activity at 37 C. *S. lactis* 7962 and *E. coli*, the controls, also had greater activity at 37 C. The lactobacilli were then compared to *E. coli* using toluene treated and untreated whole cells at assay conditions of pH 6.6 and 50 C (Figure 7). The specific activity of enzyme was markedly increased by toluene treatment in *L. helveticus*, *L. lactis*, *L. acidophilus*, and in the control *E. coli*. In fact, under these assay conditions the above organisms possessed greater enzyme activity than *E. coli*. The enzyme from *L. arabinosus* was not greatly stimulated by toluene

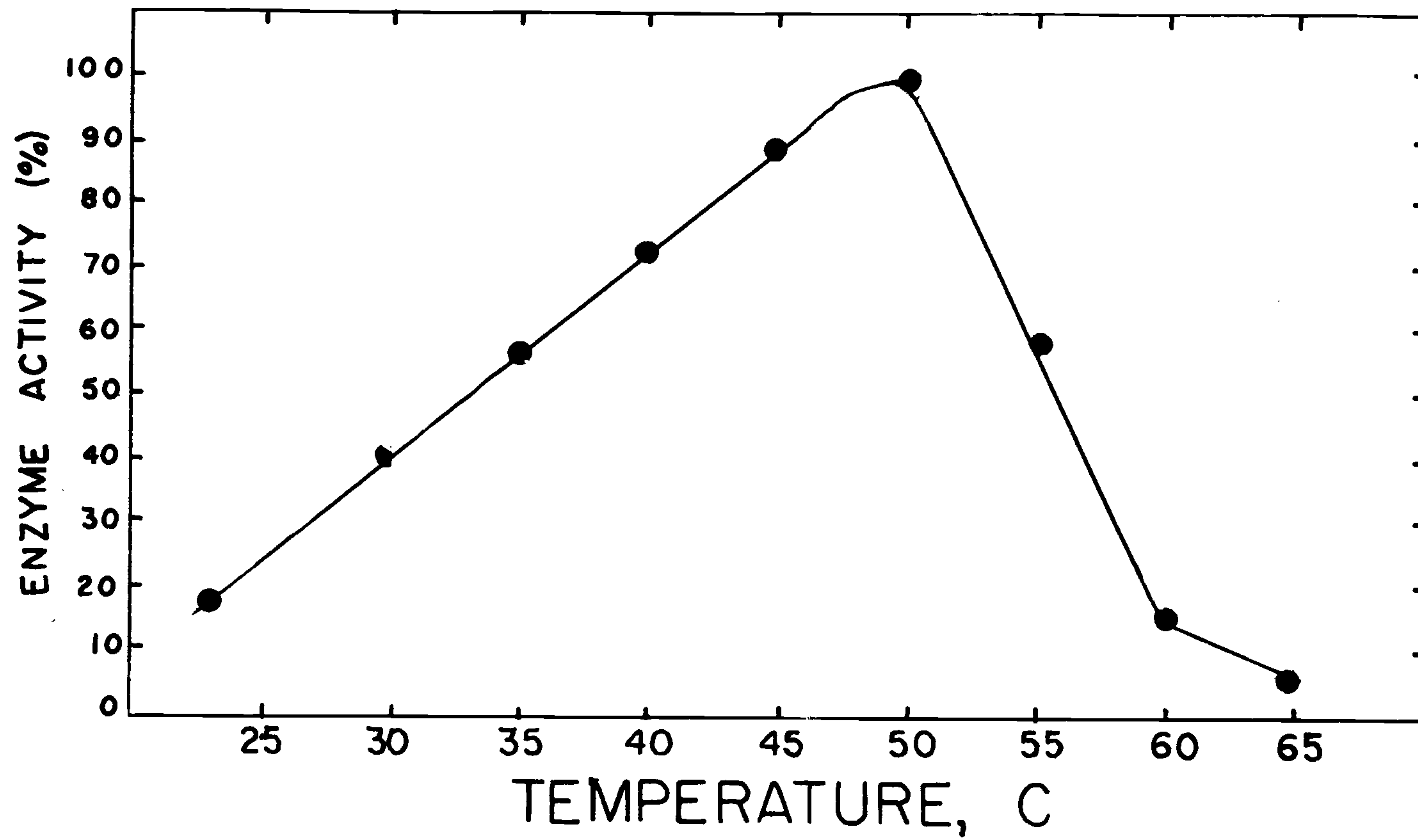


Figure 4. Effect of incubation temperature on the specific activity of β-galactosidase in cell-free extracts of L. helveticus.

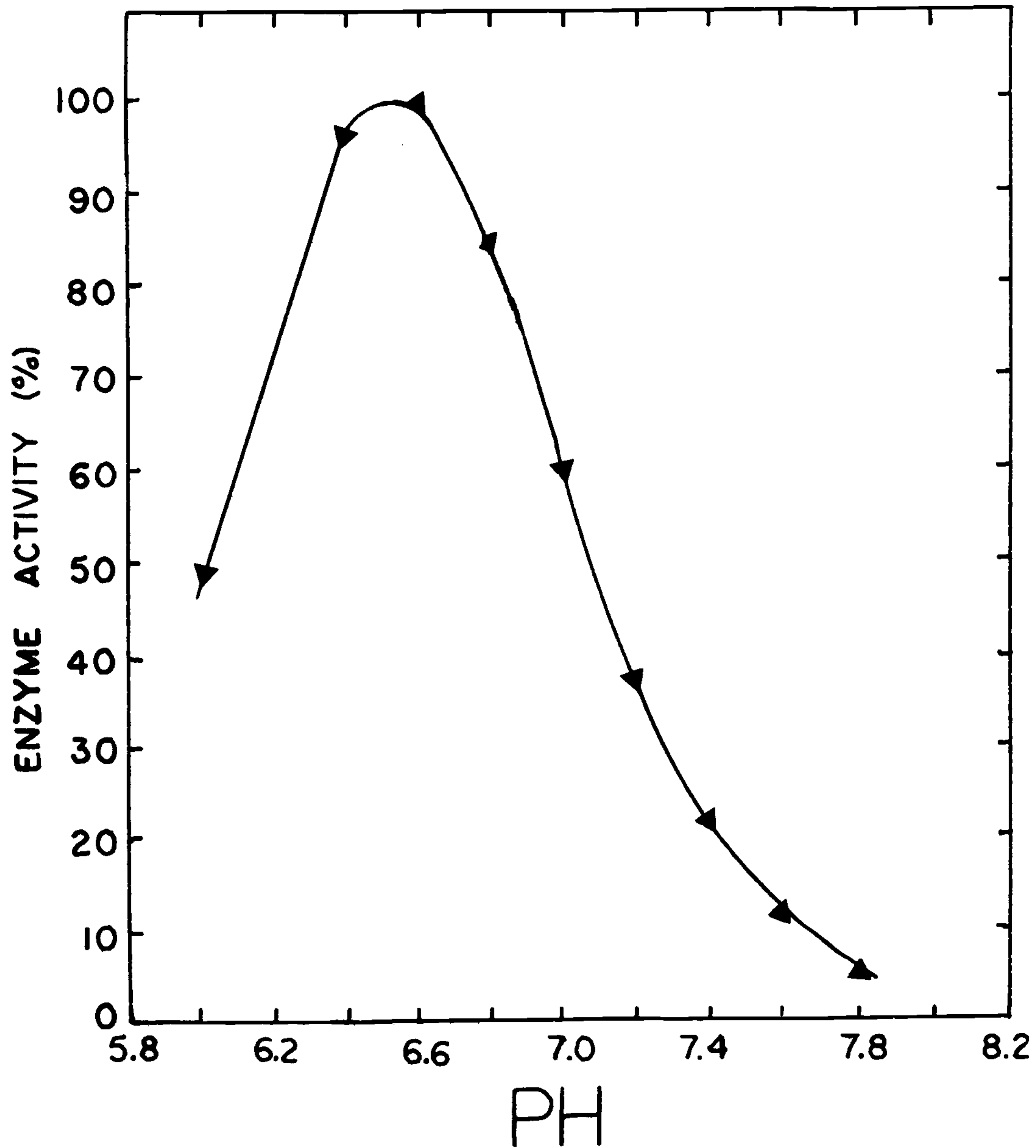


Figure 5. Effect of pH on the specific activity of β-galactosidase in cell-free extracts of L. helveticus. Cell-free extracts were assayed in sodium phosphate buffer at pH 6.6.

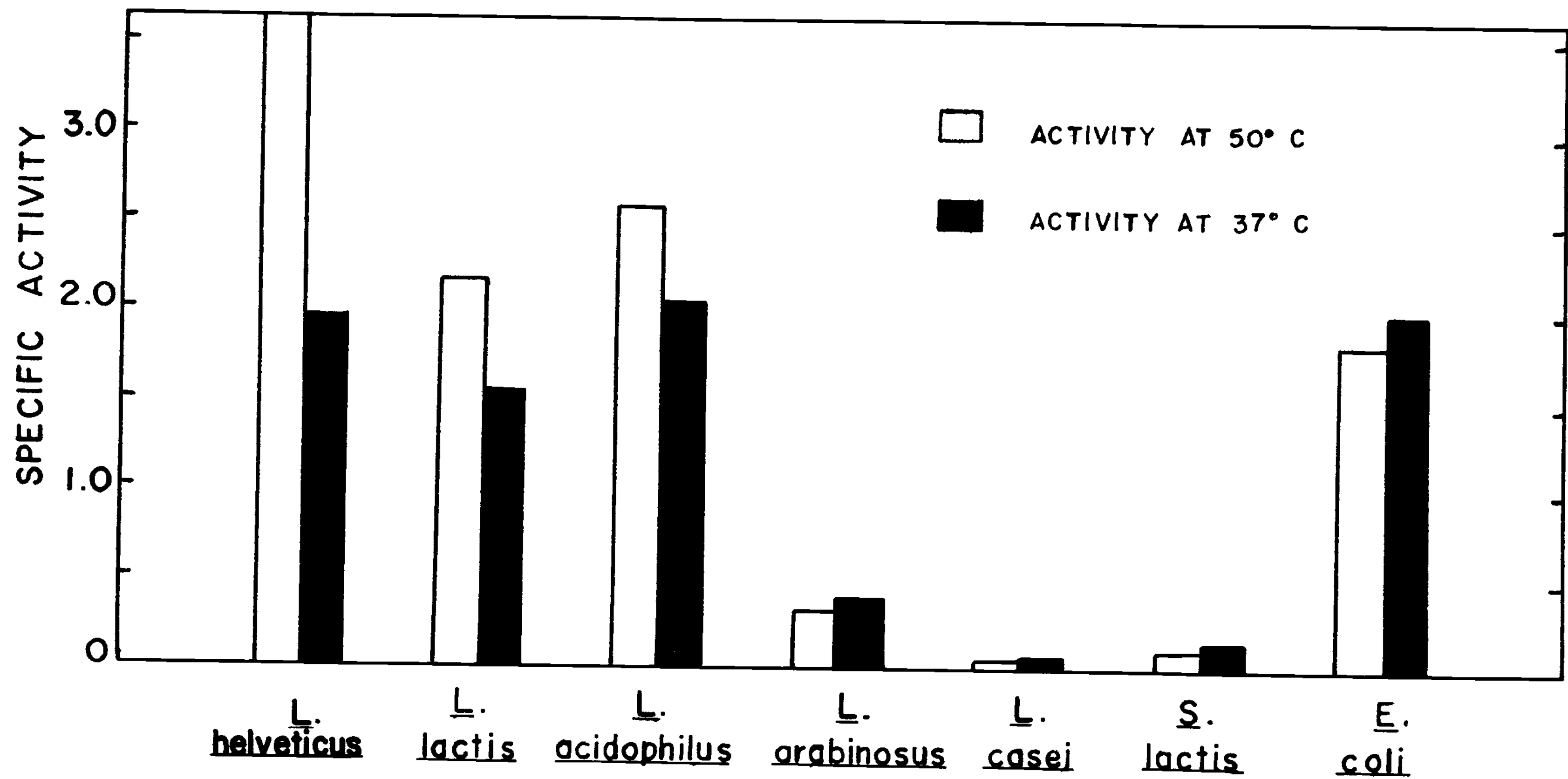


Figure 6. Effect of incubation temperature on the specific activity of β-galactosidase in toluene-treated cells of several lactobacilli. Cells were assayed in sodium phosphate buffer at pH 6.6.

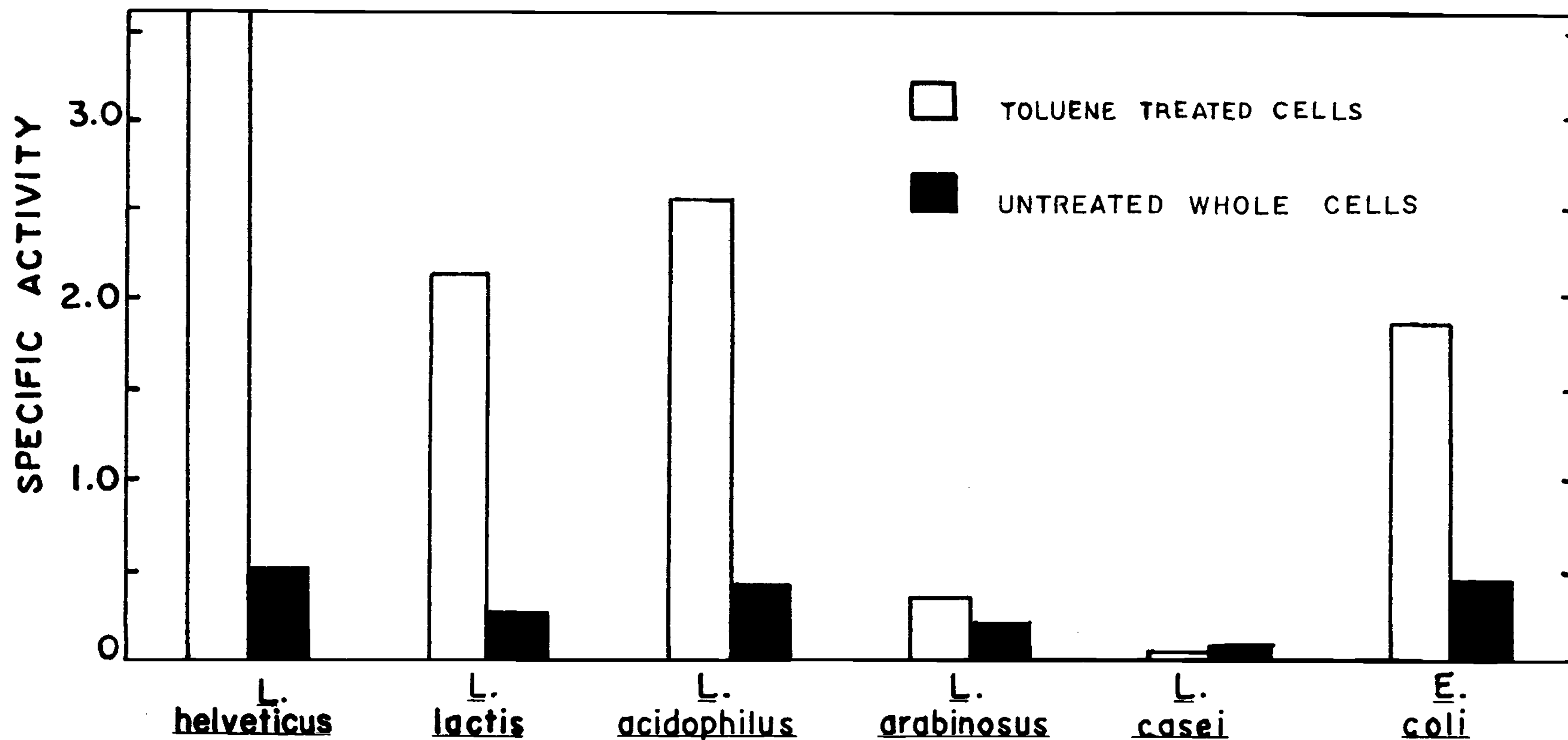


Figure 7. Specific activity of β-galactosidase in untreated whole cells and toluene-treated suspensions of lactobacilli. Assays were conducted in sodium phosphate buffer at pH 6.6 and 50 C.

treatment; and solvent treatment of L. casei seemed to destroy the enzyme activity.

Cells of L. helveticus contained the highest levels of β-galactosidase activity when grown on lactose (Sp. Act.=3.62). Glucose grown cells repressed enzyme activity (Sp. Act. =0.49); whereas maltose and galactose grown cells revealed specific activities of 1.04 and 1.27 respectively.

The specific activities of β-galactosidase from L. bulgaricus in whole cells, toluene treated cells, and cell-free extracts were 0.199, 1.37, and 1.94 respectively at 37 C; whereas at 50 C the specific activities were 0.420, 1.14 and 2.09 respectively.

β-Galactosidase of S. thermophilus

Since several of the lactobacilli appeared to contain a heat stable enzyme, it was of interest to determine β-galactosidase activity in a thermophilic streptococci. Table 2 compares the enzyme activity from three strains of S. thermophilus. Enzyme activity was higher at 50 C than at 37 C and in addition the enzyme from S. thermophilus C 3 appeared to be unstable in cell-free extracts.

Table 2. β -Galactosidase activity from thermophilic streptococci.[1]

Culture	Untreated cells		Toluene treated cells		Cell-Free extracts	
	37 C	50 C	37 C	50 C	37 C	50 C
S. thermophilus Mc	0.36	0.44	1.67	2.46	2.76	3.78
S. thermophilus C3	0.55	1.02	4.62	6.42	0.83	0.99
S. thermophilus S	0.47	1.67	3.40	5.09	2.75	4.23

[1]Assays were conducted in 0.05 M sodium phosphate buffer at pH 7.0.

Effect of β-Galactosidase on Acid Production

Since β-galactosidase may account for six percent or more of the total protein in a cell extract (Zabin, 1963), it was of interest to determine what effect the addition of an extract containing β-galactosidase would have on acid production. The organisms selected were S. lactis C_2F, a fast acid producer, and S. lactis C_2S, a slow acid producer. Table 3 shows that the active enzyme had a marked influence on the ability of the cells to produce acid. The slow strain was stimulated approximately 37 percent, whereas the heat inactivated enzyme afforded only a 12 percent increase in titratable acidity. S. lactis C_2F was also stimulated by the addition of active β-galactosidase.

Lactose Fermentation Studies

The experiments reported in this section were designed to

Table 3. Effect of β-Galactosidase on acid production in milk cultures of *S. lactis*.

System	Percent change in titratable acidity $\times 10^{-3}$	Percent greater than control
S. lactis C_2S	41	----
S. lactis C_2S + Active Enzyme	65	36.9
S. lactis C_2S + Inactive Enzyme	47	12.8
S. lactis C_2F	60	----
S. lactis C_2F + Active Enzyme	93	35.5
S. lactis C_2F + Inactive Enzyme	78	23.1

provide 30 μg of lactose in the anthrone test when no sugar was utilized by the cells. Lactose and glucose grown cells of *S. lactis* 7962 were tested as positive and negative controls respectively. It is apparent from Figure 8 that *S. lactis* 7962, when induced, utilized over 75 percent of the lactose in one hour. The noninduced cell suspension, however, did not take up the sugar. This result suggested the experimental approach could be used to measure lactose transport since only those cells previously induced were capable of utilizing the sugar, and as shown for *E. coli* (Kepes and Cohen, 1962), the rate limiting step in lactose utilization was its rate of transport. The uptake of lactose by the lactobacilli was then studied using this technique.

Figures 9 and 10 show the effect of pH and temperature on lactose uptake by *L. helveticus*. The optimum pH was about 6.6 in 0.05 M sodium phosphate buffer and maximum lactose uptake occurred from 40 to 45 C. Figure 11 compares the rate of lactose uptake and subsequent metabolism by several strains of lactobacilli. *L. helveticus*, *L. lactis*, and *L. acidophilus* rapidly accumulated the sugar, whereas *L. arabinosus* and especially *L. casei* were much slower. *L. bulgaricus* also utilized lactose at a very slow rate.

In later experiments it was shown that 0.03 M NaF did not affect lactose uptake in washed cell suspensions of *L. arabinosus*.

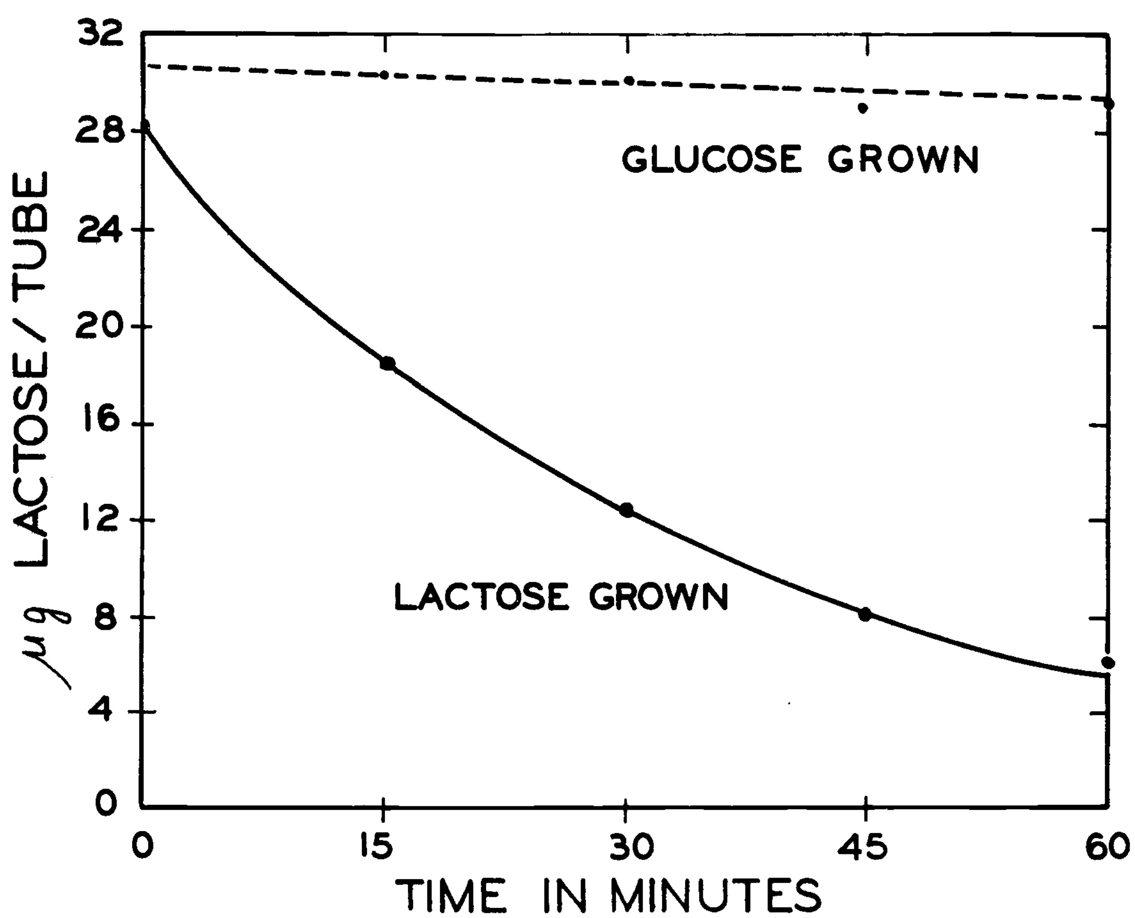


Figure 8. Lactose uptake and subsequent metabolism by lactose and glucose grown cells of S. lactis 7962.

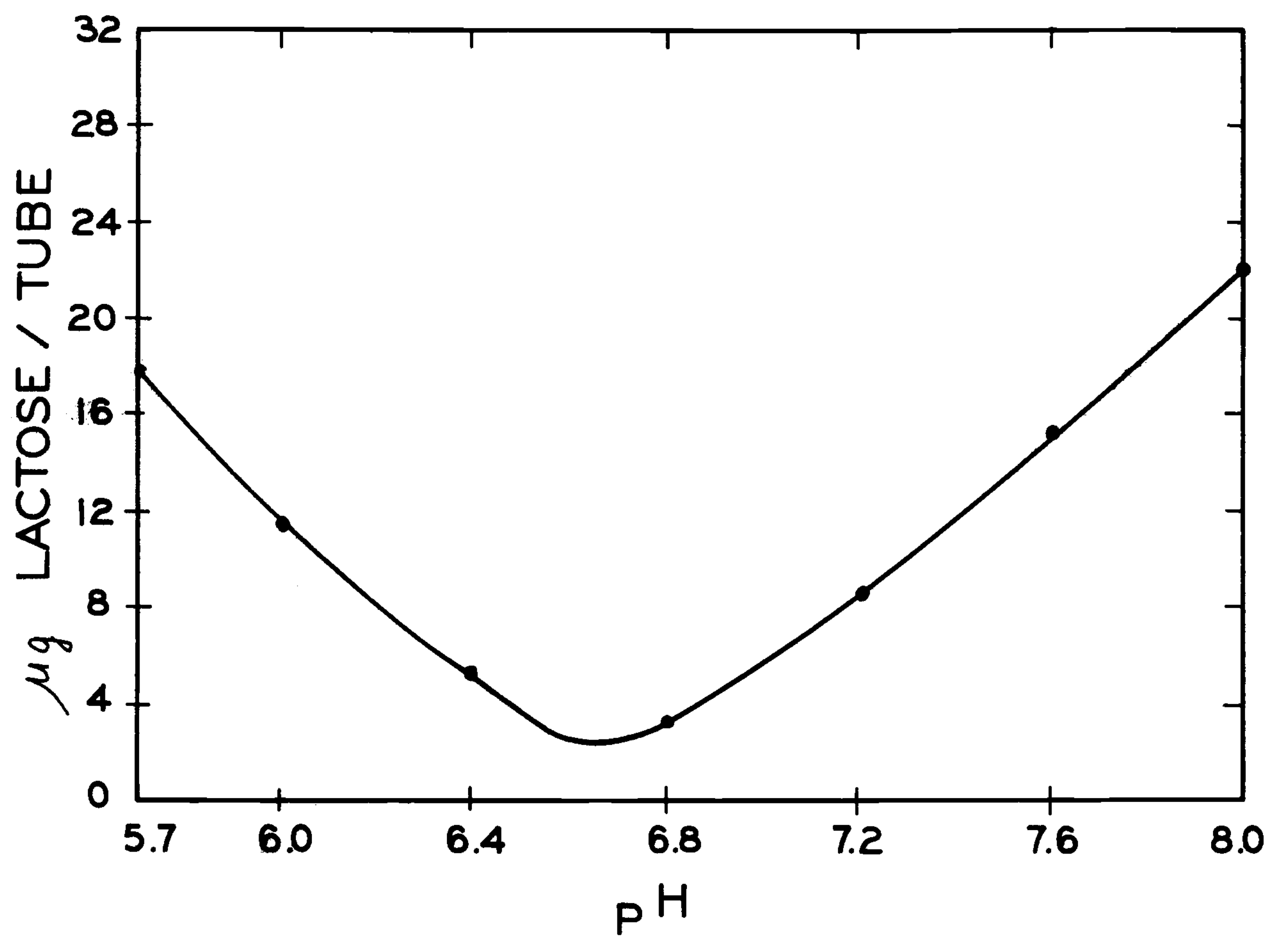


Figure 9. Effect of pH on lactose fermentation by L. helveticus.

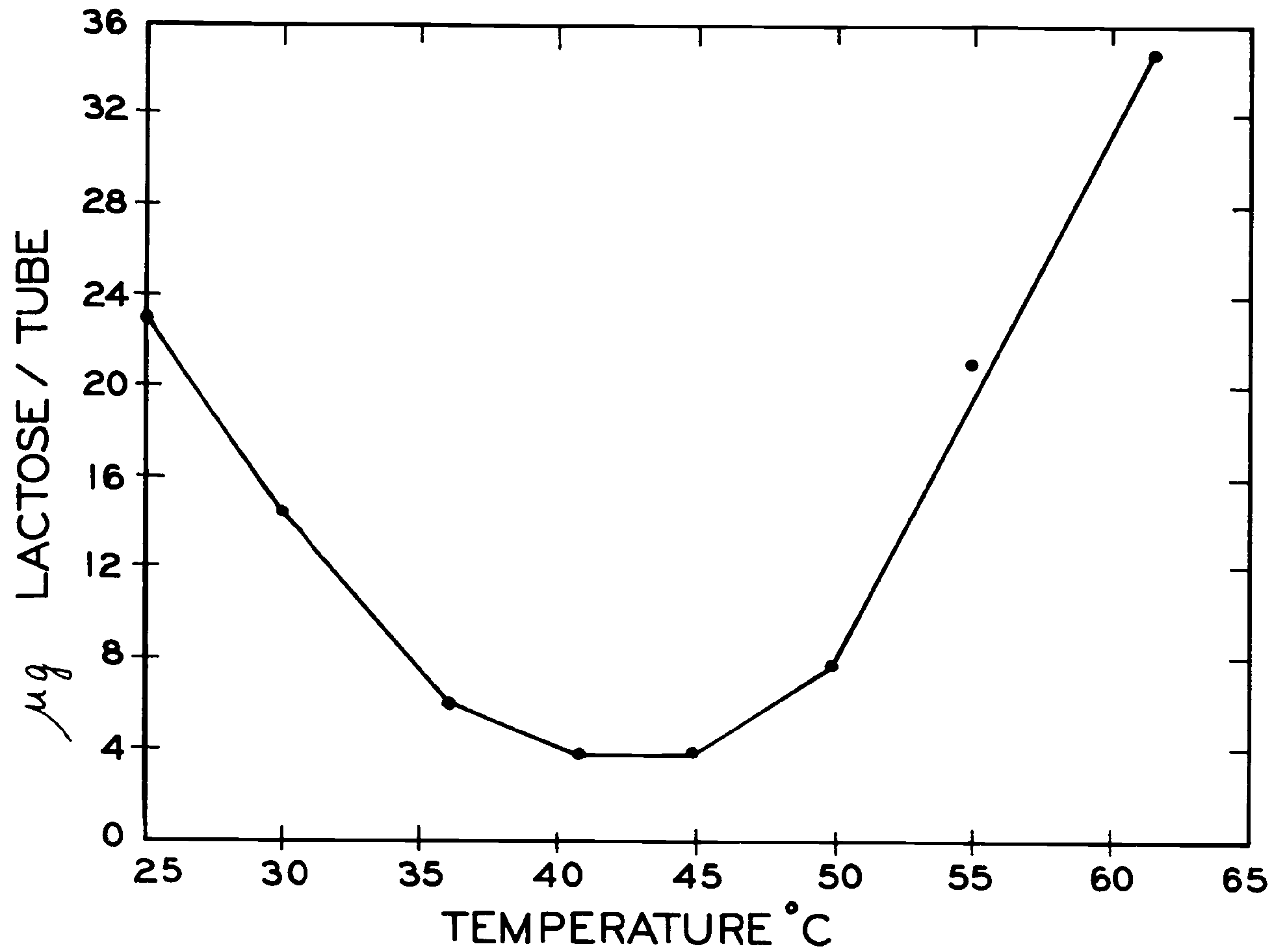


Figure 10. Effect of temperature on lactose fermentation by *L. helveticus*.

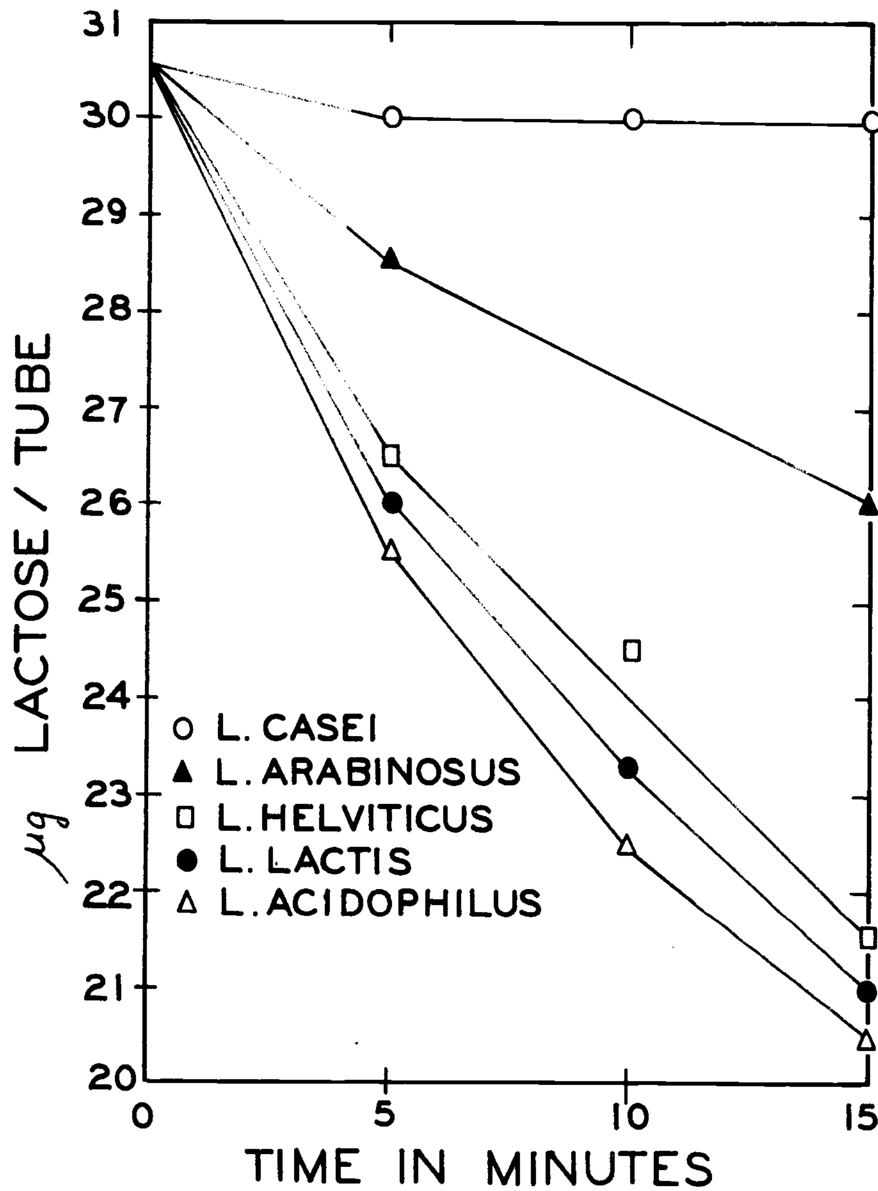


Figure 11. Rate of lactose uptake and subsequent metabolism by several Lactobacilli species.

Studies on Lactic Streptococci

Growth Characteristics in Presence of α -MG

The procedure for the enrichment of constitutive mutants depended on the use of the nonmetabolizable analogue of glucose, α -MG, which inhibits adaptation to lactose under conditions where it serves as the sole source of carbon and energy. Cells preadapted to lactose should be relatively insensitive to inhibition. The growth response of induced and uninduced cells of S. lactis 7962 following a 48 hour incubation period in a semi-synthetic medium with and without α -MG is shown in Figure 12. Since S. lactis, like other lactic acid bacteria, does not grow in a basal medium lacking carbohydrate, growth or lack of growth provides a sensitive measure of the ability of the organism to utilize a given carbohydrate as an energy source. No marked increase in turbidity was observed for 18 to 24 hours. The adapted cells were able to utilize the lactose in the presence of α -MG after this lag period as observed by the increase in turbidity. Unadapted cells, on the other hand, failed to induce for lactose utilization after 48 hours and thus no increase in turbidity was observed. The control cultures, lacking α -MG, exhibited the same phenomena in that adapted cells were able to utilize the lactose, but if the cells were not induced, metabolism of lactose could not occur. This

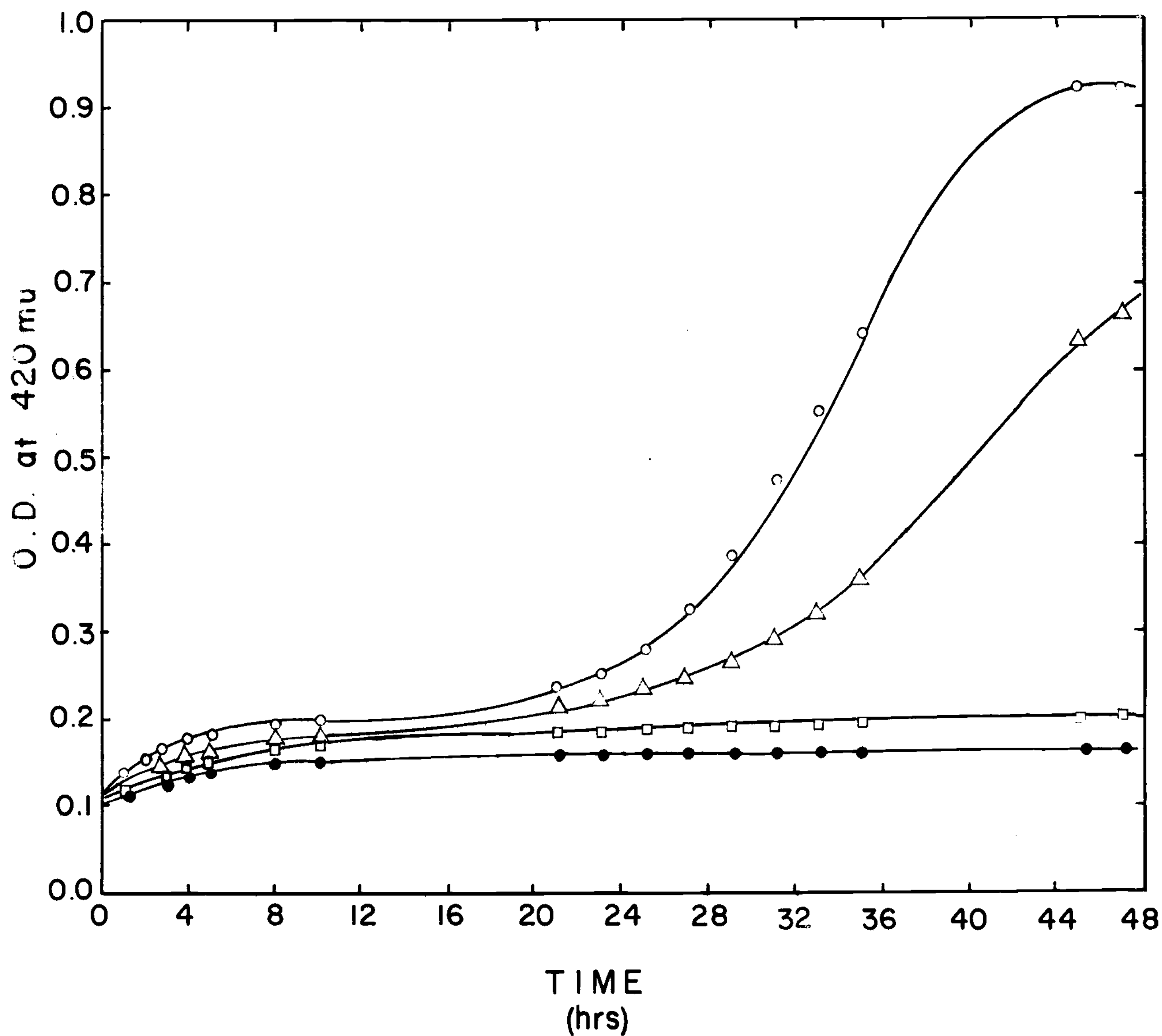


Figure 12. Effect of α-methyl glucoside on adaptation to lactose by glucose or lactose grown cells of S. lactis 7962. Lactose grown cells were inoculated into the semi-synthetic medium with (▲) and without (○) α-MG. Glucose grown cells were also inoculated into the medium with (●) and without (□) α-MG.

observation suggested that the basal medium lacked some component necessary for induction of the lactose enzymes.

This component was not necessary for E. coli since adapted cells and unadapted cells demonstrated rapid growth when inoculated into the basal medium containing lactose.

Growth Characteristics in the Basal Medium

S. lactis 7962 demonstrated rapid growth in the basal medium if the cells were not washed prior to inoculation into the basal medium or if the medium was supplemented with 0.1% yeast extract. The unwashed glucose grown cells, after completion of endogenous metabolism demonstrated a sharp lag period before adaption to lactose, whereas the lactose grown cells grew without the pronounced lag. Figure 13 shows that if the basal medium was enriched with yeast extract not only was the 18 to 24 hour lag eliminated but the unadapted cells become induced and utilization of lactose occurred.

To demonstrate this component was not necessary for growth on other carbon sources, unadapted cells were inoculated into the basal medium containing glucose. Figure 14 shows that no lag period was observed and growth reached a maximum within 18 hours. Likewise, growth occurred without the long lag if galactose served as the sole carbon source. When the basal medium contained glucose plus galactose, growth paralleled that of glucose for 20 hours, but it

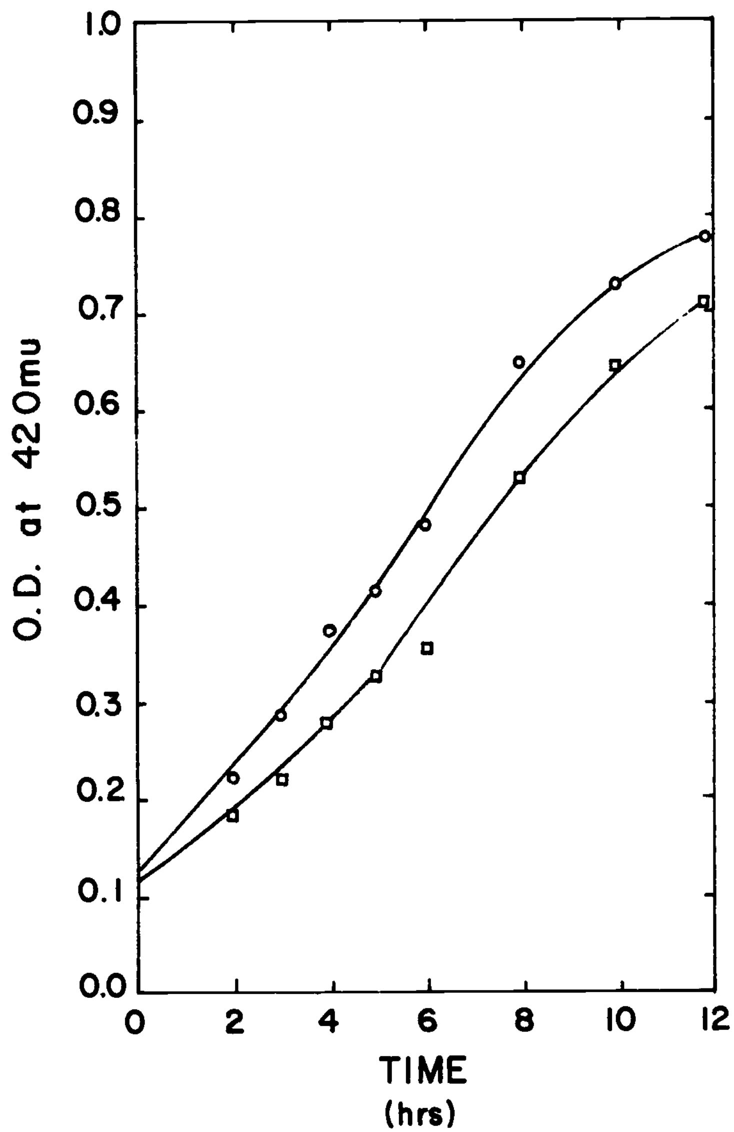


Figure 13. Effect of yeast extract on growth of S. lactis 7962 in the semi-synthetic medium. Cells were previously grown in lactose (O) or glucose (□) broth.

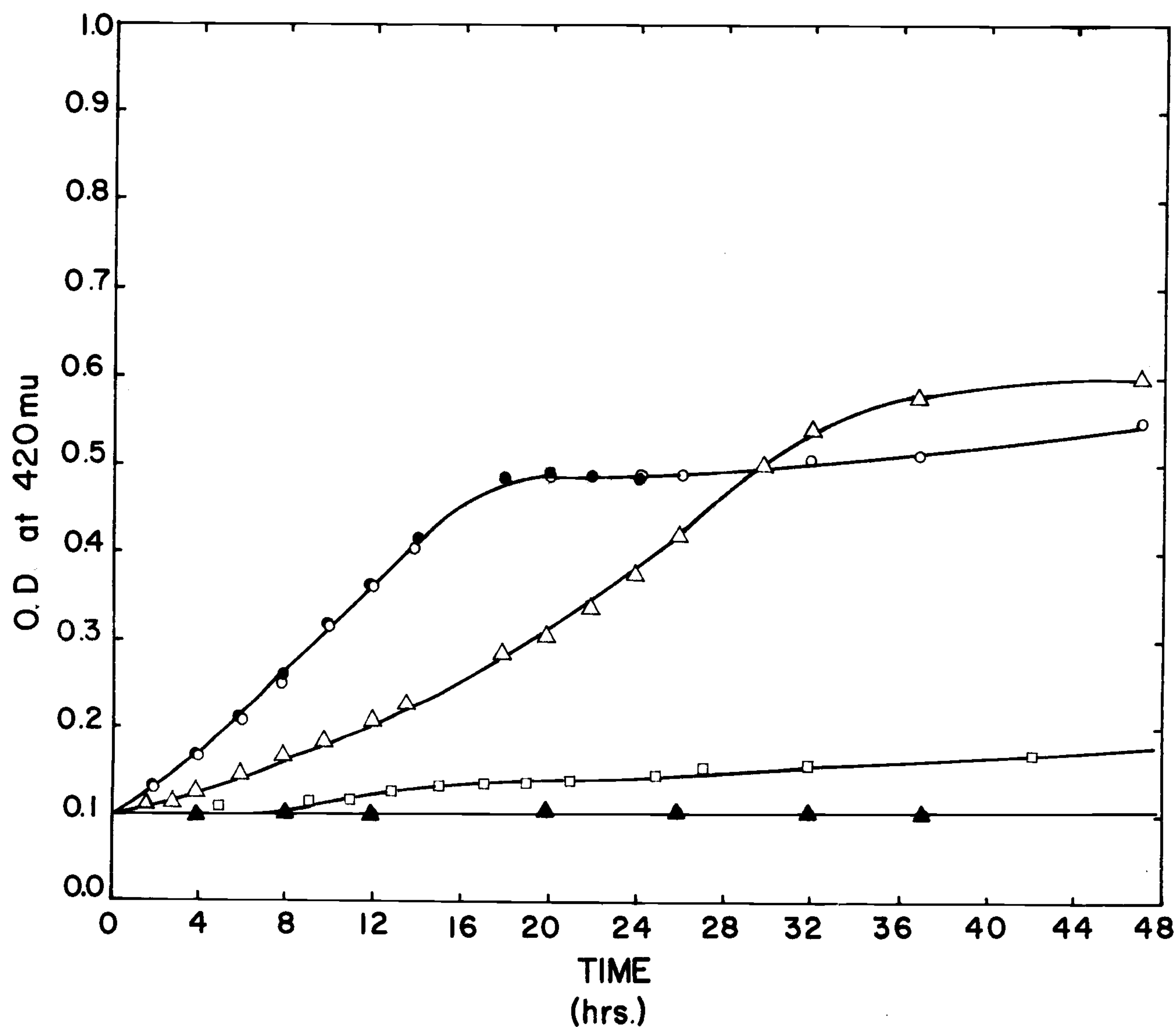


Figure 14. Growth of S. lactis 7962 in the semi-synthetic medium supplemented with glucose (●); galactose (Δ); glucose and galactose, (O); lactose (□); or no carbohydrate (▲).

appeared the cells were unable to utilize the galactose during the extended period of measurement. The controls, containing lactose, and no carbohydrate, exhibited no growth within 48 hours.

Since acid hydrolysis destroys tryptophan, the basal medium containing lactose was supplemented with this amino acid and innoculated with adapted and unadapted cells. Figure 15 shows that even though the 18 to 24 hour lag was still observed, the unadapted cells were able, in the presence of tryptophan, to induce for utilization of lactose.

Results using vitamin-free acid hydrolyzed casamino acids are shown in Figure 16. A lag period from 12 to 16 hours was observed before growth resumed when cells pregrown on lactose were inoculated into the medium. The addition of tryptophan only slightly stimulated growth. Glucose grown cells revealed a 48 hour lag before limited growth was observed. However, as in previous experiments, the addition of tryptophan allowed the cells to utilize lactose and growth approximately paralleled that of preadapted cells.

If Bacto-Casitone, a pancreatic digest of casein, was used in place of the acid hydrolyzed preparations rapid growth occurred for both the induced and uninduced cells of S. lactis 7962 in the basal medium.

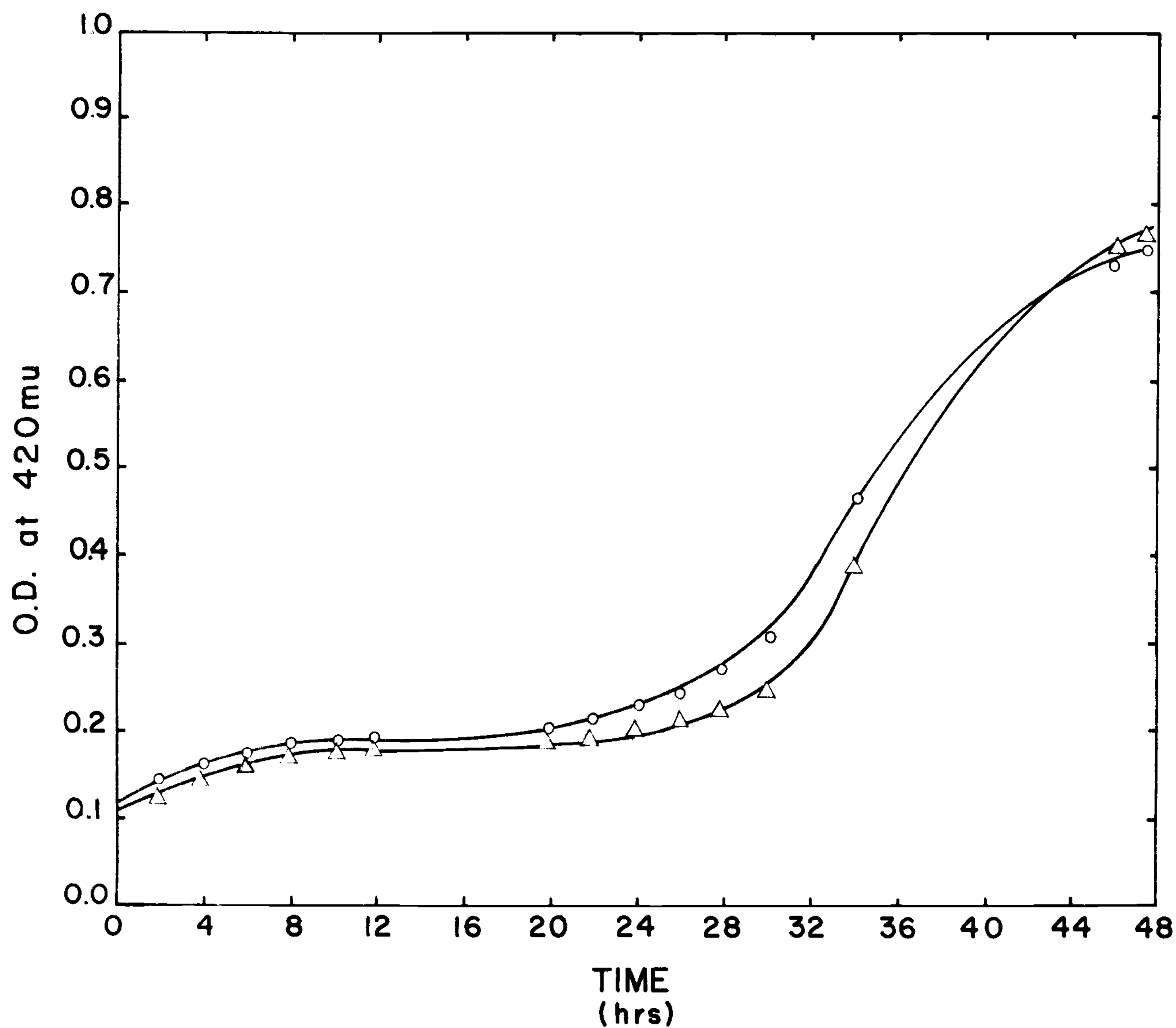


Figure 15. Effect of tryptophan on growth of S. lactis 7962 in the basal medium. Cells were previously grown in lactose (O) or glucose (Δ) broth.

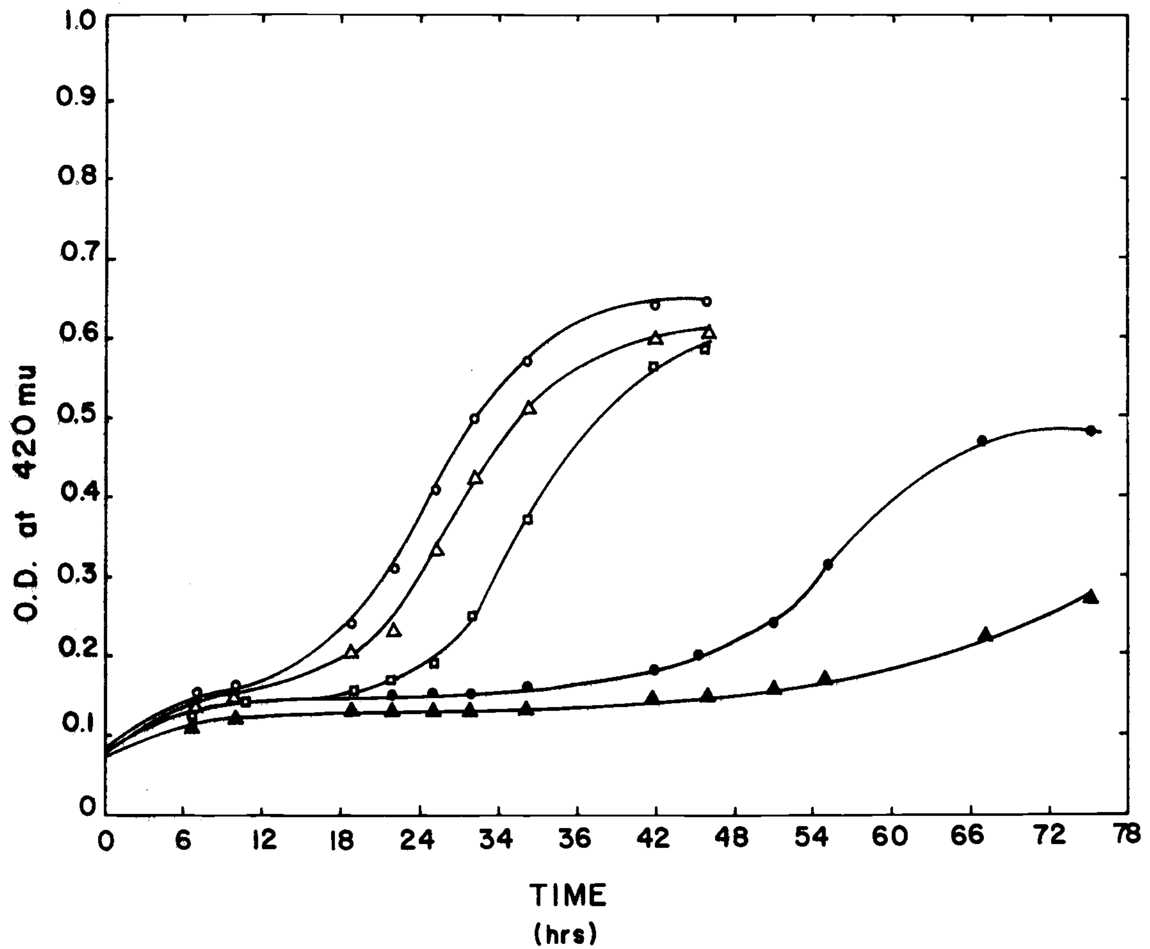


Figure 16. Growth of S. lactis 7962 in the basal medium using vitamin-free acid hydrolyzed casamino acids. Lactose grown cells were inoculated into the medium with (O) and without (Δ) tryptophan. Glucose grown cells were also inoculated into the medium with (□) and without tryptophan (●, ▲).

Selection of Mutants from S. lactis

Table 4 lists the carbohydrate negative mutants isolated from S. lactis 7962 and S. lactis C_2F. Only two mutants were isolated using UV light, however NTG proved to be a very efficient mutagenic agent. Although lac^- mutants could not be isolated from S. lactis 7962 using acriflavin, such mutants were readily isolated from S. lactis C_2F. In addition, a spontaneous lactose negative mutant was isolated from stock cultures of S. lactis C_2F after extended periods of repeated subculturing in lactic broth.

These mutants were purified by repeated subculturing several times in lactic broth and streaking on lactose indicator agar. During this procedure some of the mutants reverted to the wild type phenotype. Two of these revertants were selected for further study.

No constitutive mutants were isolated.

Characterization of S. lactis 7962 Mutants

Table 5 shows the β-galactosidase activity and the ability of the lac^- mutants of S. lactis 7962 to utilize lactose. The latter test was used as a measurement of galactoside permease activity. If no lactose was utilized 30 μg anthrone positive material (as lactose) should be present in the assay solution. Using this technique mutants C, B, 3, 9, 18, and 6 were observed to be defective in lactose

Table 4. Characterization of carbohydrate negative mutants from *S. lactis*.

Strain		Method of isolation	Phenotype[a]
S. lactis 7962-3		NTG	lac^-
"	-C	NTG	lac^-
"	-B	NTG	lac^-
"	-3R	Spontaneous from 3	lac^+
"	-9	NTG	lac^-
"	-18	NTG	lac^-
"	-6	NTG	lac^- gal^-
"	-5	UV	lac^-
"	-16	UV	lac^-
S. lactis C_2F-3		Acriflavin	lac^- gal^-
"	-C_2FW	Spontaneous	lac^- gal^-
"	-F1	NTG	fru^-
"	-F7	NTG	lac^- gal^-
"	-F15	NTG	lac^- gal^-
"	-F22	NTG	lac^- gal^-
"	-F4	NTG	lac^- gal^-
"	-L13	NTG	lac^- gal^-
"	-G1	NTG	lac^- gal^-
"	-G2	NTG	lac^- gal^-
"	-G4	NTG	lac^- gal^-
"	-G6	NTG	lac^- gal^-

[a]The mutants designated lac^- gal^- were unable to ferment lactose and were defective in the fermentation of galactose.

Table 5. Summation of lactose accumulation and β-galactosidase activity of *S. lactis* 7962 and the lac$^-$ mutants.

Organism	Lactose Permease				β-galactosidase	
	Lactose grown		Glucose grown		Lactose grown	Glucose grown
	30 min	60 min.	30 min.	60 min.		
S. lactis 7962	23.5	15	29.5	29.2	0.164	0
S. lactis 7962-C	24.8	19.8	30.5	29.4	0.189	0
S. lactis 7962-B	27.5	19.0	31.5	31.5	0.244	0
S. lactis 7962-3R	22.5	14.8	31.5	29.0	0.191	0
S. lactis 7962-3	31.0	25.0	32.5	29.5	0.186	0
S. lactis 7962-9	29.0	22.3	--	29.5	0.087	0
S. lactis 7962-18	24.8	18.5	28.4	28.0	0.190	0
S. lactis 7962-6	31.0	31.8	31.0	28.5	0	0
S. lactis 7962-49-5	22.0	13	30	27.3	0	0
S. lactis 7962-49-16	22.3	12.5	31.7	28.5	0	0

utilization. This defect could be a permeability problem. S. lactis 7962-6 did not utilize any lactose and mutant 3 utilized very little. When the β-galactosidase activity of these strains were determined, a normal level of enzyme was present except in mutants 9, 6 and B. Mutant 9 contained about half the specific activity as that measured in the wild type; whereas mutant B contained about twice the specific activity as the parent strain. Mutant 6 was devoid of enzyme activity. S. lactis 7962-5 and 16 accumulated lactose as did the parent strain, yet neither mutant was capable of hydrolyzing ONPG.

Characterization of S. lactis C_2F Mutants

All the lac^- mutants isolated from S. lactis C_2F were unable to hydrolyze ONPG and the mutant cells, when suspended in a solution of lactose, were unable to accumulate the sugar. These results suggested the mutants were both permease and β-galactosidase negative. It appeared strange, however, that even though lac^- mutants were randomly isolated, they all appeared to possess the same phenotype.

When S. lactis C_2F was treated with NTG and then screened for the appearance of galactose negative mutants several were found. In contrast to the parent strain, these mutants formed white colonies on galactose indicator agar after 24 hours of incubation at 32 C;

further incubation, however, resulted in acid production. These mutants were also defective in their fermentation of lactose, in fact, they were lactose negative even after prolonged incubation.

Upon this finding the mutants originally isolated as lac$^-$ were reexamined with respect to galactose fermentation using galactose indicator agar instead of the broth. These mutants were also observed to be defective in galactose utilization, although the sugar was fermented within 48 hours. The phenotype of these mutants which are defective in galactose utilization and are lactose negative have been designated lac$^-$ gal$^-$. Since these mutants grew as the wild type on glucose, maltose, mannose, and fructose, it was argued that the defect in the metabolism of lactose and galactose did not involve the glycolytic enzymes.

In an effort to learn the nature of the defective function in the lac$^-$ gal$^-$ mutants, a study of the factors influencing the metabolism of lactose in S. lactis C_2F was initiated. This study was necessary since the instability of β-galactosidase in toluene treated cells or cell-free extracts of S. lactis C_2F precluded any comparison of this enzyme under these conditions.

S. lactis C_2F-Fl (fru$^-$) was initially isolated as a lac$^-$ mutant. However, later tests indicated it could not utilize fructose as a sole carbon source. Growth on lactose, mannose, galactose, maltose, and glucose was also impaired. For example, in one experiment

the doubling time of *S. lactis* C_2F when grown in mannose, galactose, maltose, lactose, or glucose broth was 36, 51, 72, 39, and 36 minutes respectively; whereas with F1 the doubling times were 84, 111, 138, 105, and 81 minutes respectively. In addition, this mutant grew as long chains and the growth in broth rapidly sedimented leaving a clear supernatant fluid. On the other hand, the parent strain exhibited typical morphological and cultural characteristics.

No definite pleiotropic carbohydrate negative mutants were isolated from either *S. lactis* C_2F or *S. lactis* 7962.

Comparison of Strains for Lactose Utilization

Since the method employed for measuring lactose uptake was complicated by intracellular metabolism of the accumulated sugar, the results are a measurement of both entrance of lactose into the cell and of its subsequent metabolism. *S. lactis* C_2F utilized more lactose than *S. lactis* 7962 as shown in Figure 17. After 15 minutes *S. lactis* C_2F had metabolized 2738 μg of lactose as compared to 850 μg of lactose for *S. lactis* 7962; the latter organism was 77% less efficient. Since *S. lactis* appeared to utilize lactose at a much greater rate than *S. lactis* 7962, factors affecting this rate were next studied.

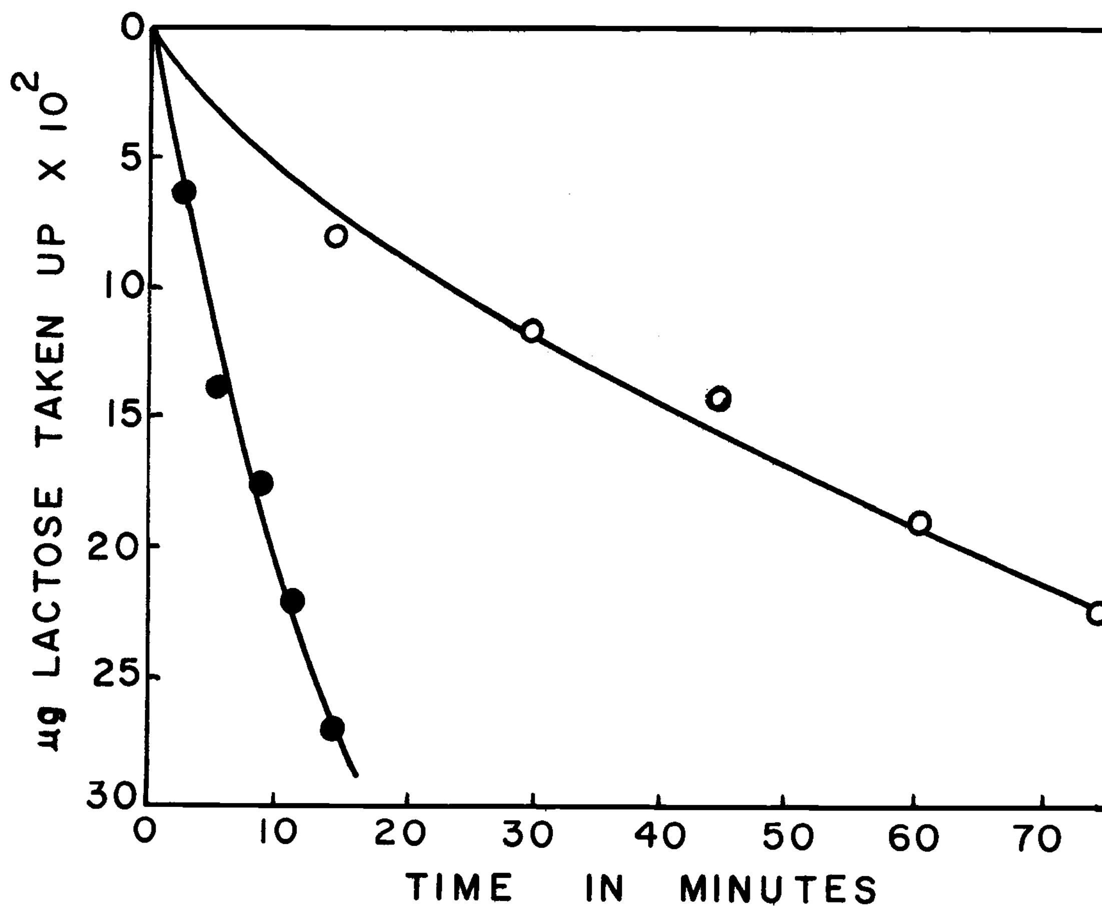


Figure 17. Time course of lactose utilization by S. lactis C_2F (●) and S. lactis 7962 (O). Cells were washed and re-suspended in 0.05 M Na_2HPO_4, pH 7.0. The suspending medium contained 2.4×10^{10} cells and 1 mg lactose per ml in a total volume of 3.0 ml; the uptake was measured by disappearance of substrate from the supernatant fluid. Results represent the total µg lactose taken up. See Materials and Methods for experimental details.

Effect of Suspending Medium

According to Figure 18, *S. lactis* C_2F required phosphate for lactose utilization. This requirement could be replaced by arsenate which is similar in chemical properties to the phosphate ion. KCl or NaCl of equal ionic strength did not allow significant utilization which ruled out the possibility that the effect depended on ionic strength or sodium. *S. lactis* 7962 also metabolized the greatest amount of lactose in the presence of phosphate. In *S. lactis* C_2F arsenate could substitute for phosphate with up to 69% efficiency. These results are shown in Table 6. It should also be pointed out that *S. lactis* C_2F metabolized lactose in the presence of 0.05 M Tris buffer (pH 7.0). The amount utilized was approximated 50% of that observed in sodium phosphate buffer.

Effect of Phosphate Concentration

The effect on lactose metabolism of varying the external phosphate concentration from 0 to 0.2 M is shown in Figure 19. It is apparent that increasing concentrations of phosphate had a stimulatory effect on lactose utilization in *S. lactis* C_2F. This effect was noticed up to 0.05 M and thereafter an inhibitory response was observed.

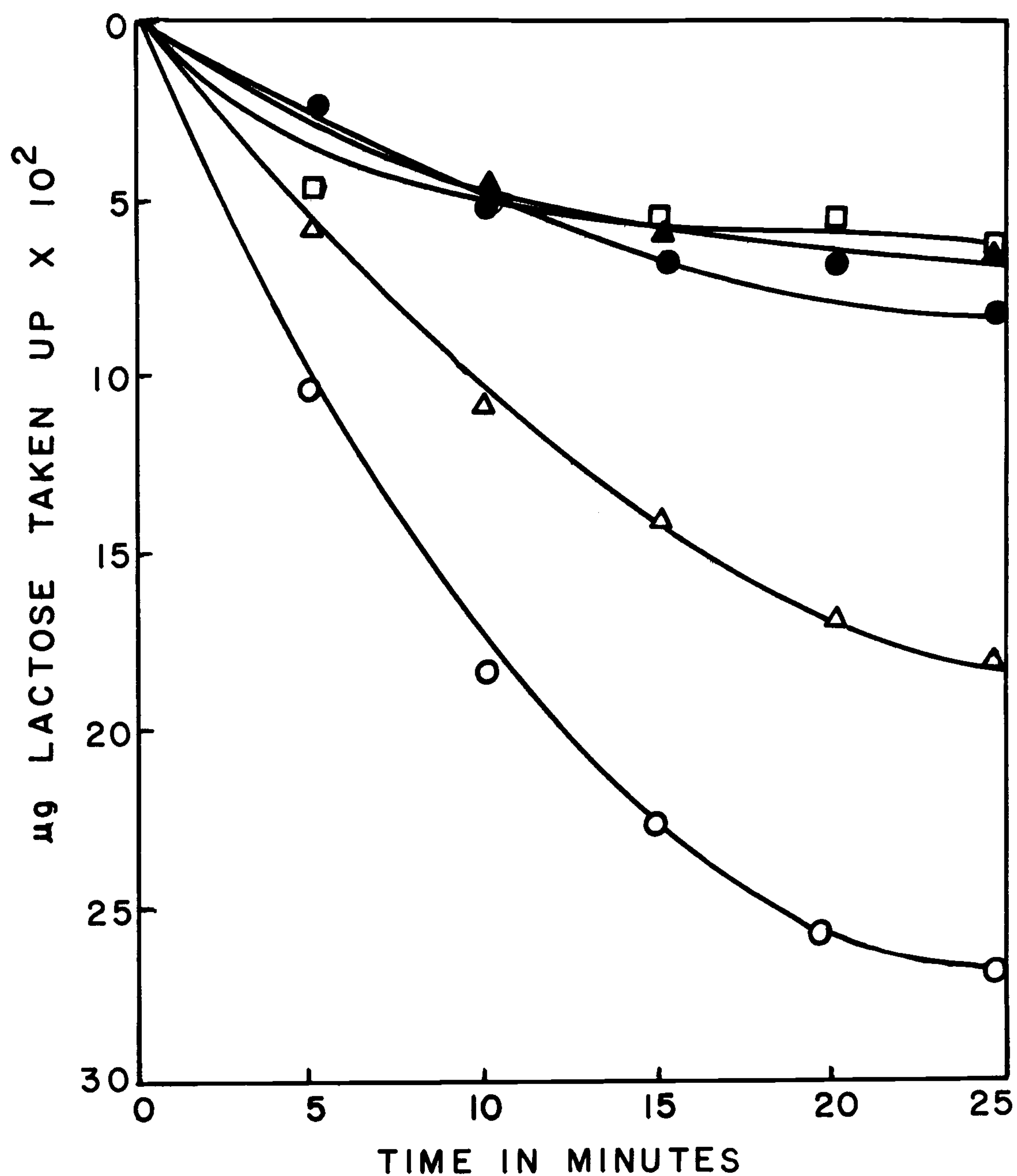


Figure 18. Effect of suspending medium on lactose utilization by *S. lactis* C_2F. Cells were washed and resuspended in 0.05 M Na_2HPO_4, (○); 0.05 M Na_2HAsO_4, (△); 0.15 M NaCl, (●); 0.15 M KCl, (▲); or H_2O, (□), and then tested for lactose uptake as previously described. Results expressed as in Figure 1.

Table 6. Effect of different suspending media on lactose utilization by two strains of *S. lactis*.

Suspending medium	*S. lactis* C_2F		*S. lactis* 7962	
	μg lactose taken up/2.4 × 10^{10} cells in 25 min.	% of maximum uptake	μg lactose taken up/2.4 × 10^{10} cells in 60 min.	% of maximum uptake
H_2O	630[a]	23.5	220	14.2
0.15 M KCl	780	29.1	100	6.5
0.15 M NaCl	660	24.6	130	8.4
0.05 M Na_2HPO_4	2680	100.0	1550	100.0
0.05 M Na_2HAsO_4	1850	69.0	0	0

[a]Results expressed as in Figure 17.

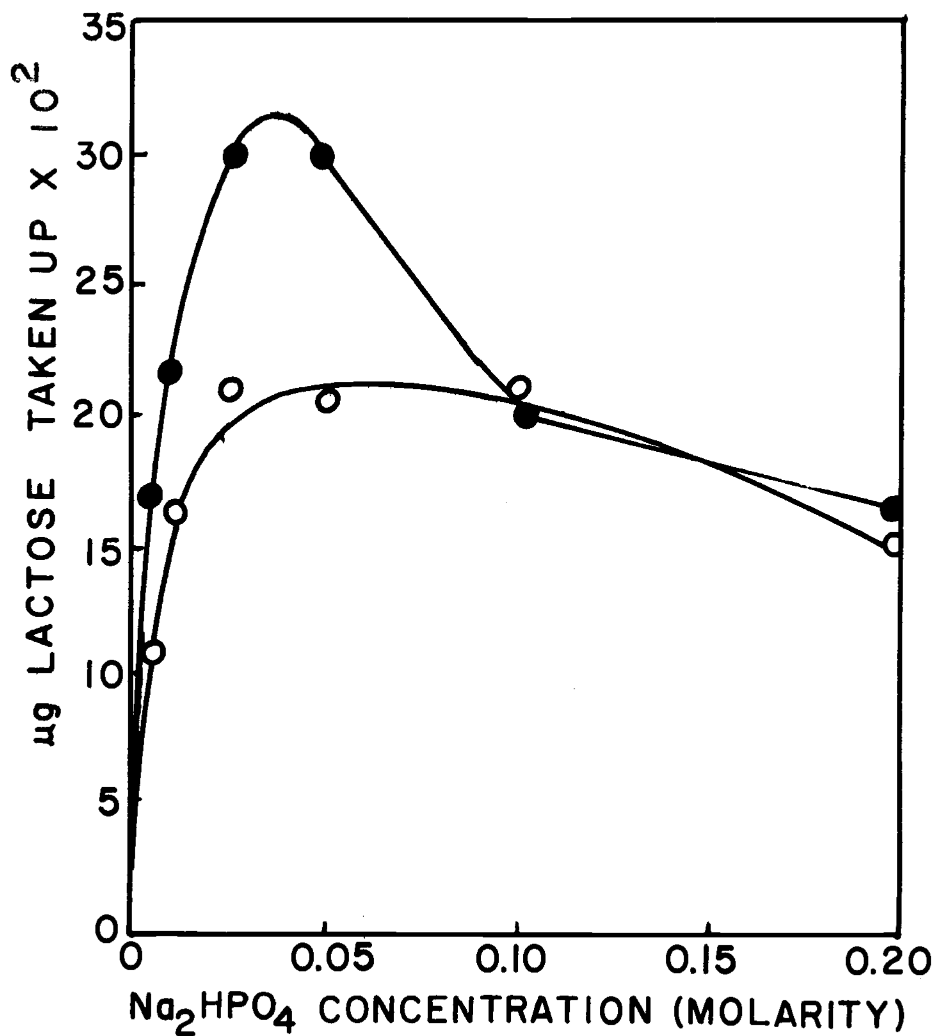


Figure 19. Effect of increasing phosphate on lactose utilization by S. lactis C_2F and S. lactis 7962. Cells were washed and resuspended in various concentrations of Na_2HPO_4 at pH 7.0. Lactose uptake was terminated after 15 min for S. lactis C_2F (●) and 60 min for S. lactis 7962 (O). Results expressed as in Figure 1.

Effect of Inhibitors

Since Na azide is known to inhibit oxidative phosphorylation it was not expected to inhibit lactose utilization by this group of organisms since they lack both a TCA cycle and terminal respiration. As seen from Table 7 there was no significant inhibition.

In the presence of phosphate and NaF *S. lactis* C_2F was unable to utilize lactose, whereas the control cells fermented 2475 μg of substrate in the presence of phosphate alone. *S. lactis* 7962, on the other hand, was not inhibited by NaF. Assuming PEP generation was inhibited by NaF, these results suggested that lactose metabolism in *S. lactis* C_2F was dependent upon its presence, whereas *S. lactis* 7962 was independent of its formation.

Iodacetic acid inhibited lactose utilization in both organisms.

Hydrolysis of Lactose by Crude Cell-Free Extracts

Figure 20 shows typical results from a TLC plate. The extract from *S. lactis* C_2F was unable to hydrolyze the disaccharide under the conditions employed. Paper chromatography also supported the above conclusion; however, glucose and galactose could not be separated by the solven systems employed. Cell-free extracts from each organism in which lactose was not added did not reveal any of the above sugars when chromatographed.

Table 7. Effect of different inhibitors on lactose utilization by two strains of *S. lactis*.

Inhibitor system	μg lactose taken up per 2.4×10^{10} cells	
	S. lactis C_2F[a]	*S. lactis* 7962[b]
0.05 M Na_2HPO_4 (control)	2475[c]	1250
0.05 M Na_2HPO_4 + 0.01 M NaN_3	2213	1500
0.05 M Na_2HPO_4 + 0.03 M NaF	0	1000
0.05 M Na_2HPO_4 + 0.001 M IAA	0	0

[a]After 15 min incubation.

[b]After 60 min incubation.

[c]Results expressed as explained in Figure 17.

Figure 20. Effect of cell-free extracts on the hydrolysis of lactose by lactic streptococci. The figure is a reproduction of a typical thin layer chromatogram resulting when cell-free extracts were incubated with lactose for 1 hr at 37 C chromatographed and tested for hydrolytic breakdown products. As shown, the spots from left to right represent (a) control mixture of glucose, galactose and lactose; (b) lactose alone; (c) galactose alone; (d) glucose alone; (e) spots resulting when lactose was incubated with 7962 extract; and (f) spot resulting when lactose was incubated with C_2F extract.

Induction of Lactose Utilization

Lactose utilization in *S. lactis* 7962 was previously shown to be preferentially induced by lactose; galactose was only a partial inducer. However, in the present study when whole cells of *S. lactis* C_2F were tested, it was found that galactose was a better inducer than lactose. Several other "β-galactosidase-less" lactic streptococci were also examined and found to be preferentially induced by galactose; lactose was only a partial inducer. Table 8 indicates that in *S. lactis* C_2F, 7963, and b ONPG hydrolysis was approximately doubled when the cells were grown on galactose. In *S. lactis* E the stimulation was almost tripled by growing the cells on galactose. ONPG hydrolysis by lactose grown cells of *S. lactis* 7962, however, was almost two fold higher than that observed in galactose grown cells. The thio-galactosides TMG and IPTG were ineffective as inducers of β-galactosidase synthesis in *S. lactis* C_2F, in fact, the maximum induction observed for galactose was decreased by the presence of IPTG.

Studies Using Labeled Lactose and TMG

If *S. lactis* C_2F metabolized lactose via the PEP-dependent phosphotransferase system, the inability to demonstrate lactose or ONPG hydrolysis in dilute cell-free extracts would be expected since the proper substrate for the "β-galactosidase" would be the

Table 8. Effect of inducer on ONPG hydrolysis in the lactic streptococci.

Organism	Inducer	
	Lactose	Galactose
S. lactis C_2F	63	122
S. lactis 11454	174	254
S. lactis 7963	56	114
S. lactis E	70	200
S. lactis b	63	128
S. cremoris 144F	210	224
S. lactis C10	72	101
S. lactis 7962	476	249

[a] Results are expressed as μmoles ONP released per 30 min per g cell dry weight. Assay system consisted of 1.0 ml whole cells, 1.0 ml sodium phosphate buffer pH 7.0, and 1.0 ml 0.033 M ONPG. Tubes were incubated at 37 C for 30 min and reaction stopped by addition of 3.0 ml 0.5 M sodium carbonate. Cells were removed by centrifugation before reading absorbancy at 420 mμ.

phosphorylated derivative. To determine whether or not lactose-P accumulated in whole cells of S. lactis C_2F the uptake of lactose-1-^{14}C was measured. The results suggested the sugar was being metabolized, since the labeled substrate did not accumulate as a distinct peak but rather as several peaks along the chromatogram (Figure 21). Although lactose negative mutants have been isolated from both strains (C_2F and 7962), a mutant which accumulated lactose without subsequent metabolism of the sugar, i.e., lacking β-galactosidase, has not been isolated. To overcome this difficulty the accumulation of TMG, a non-metabolizable structural analogue of lactose was examined. Figure 22 illustrates the time course of ^{14}C-TMG uptake in S. lactis C_2F and 7962. S. lactis C_2F accumulated the analogue at a rapid rate, in comparison to the slow uptake by S. lactis 7962. S. lactis C_2F had accumulated over 10,000 cpm in 20 minutes; whereas only 500 cpm had been accumulated by S. lactis 7962 after 60 minutes exposure to the labeled ^{14}C-TMG. Since both strains accumulated TMG it was necessary to determine if ^{14}C-TMG accumulated as a phosphate derivative or as free TMG.

Figure 21 indicates the chromatographic behavior of radioactive compounds extracted from cells of S. lactis C_2F previously exposed to ^{14}C-TMG. The figure shows that a labeled derivative traveled as a peak distinct from TMG; the labeled material (small peak) corresponding to TMG probably resulted from carry over of substrate from

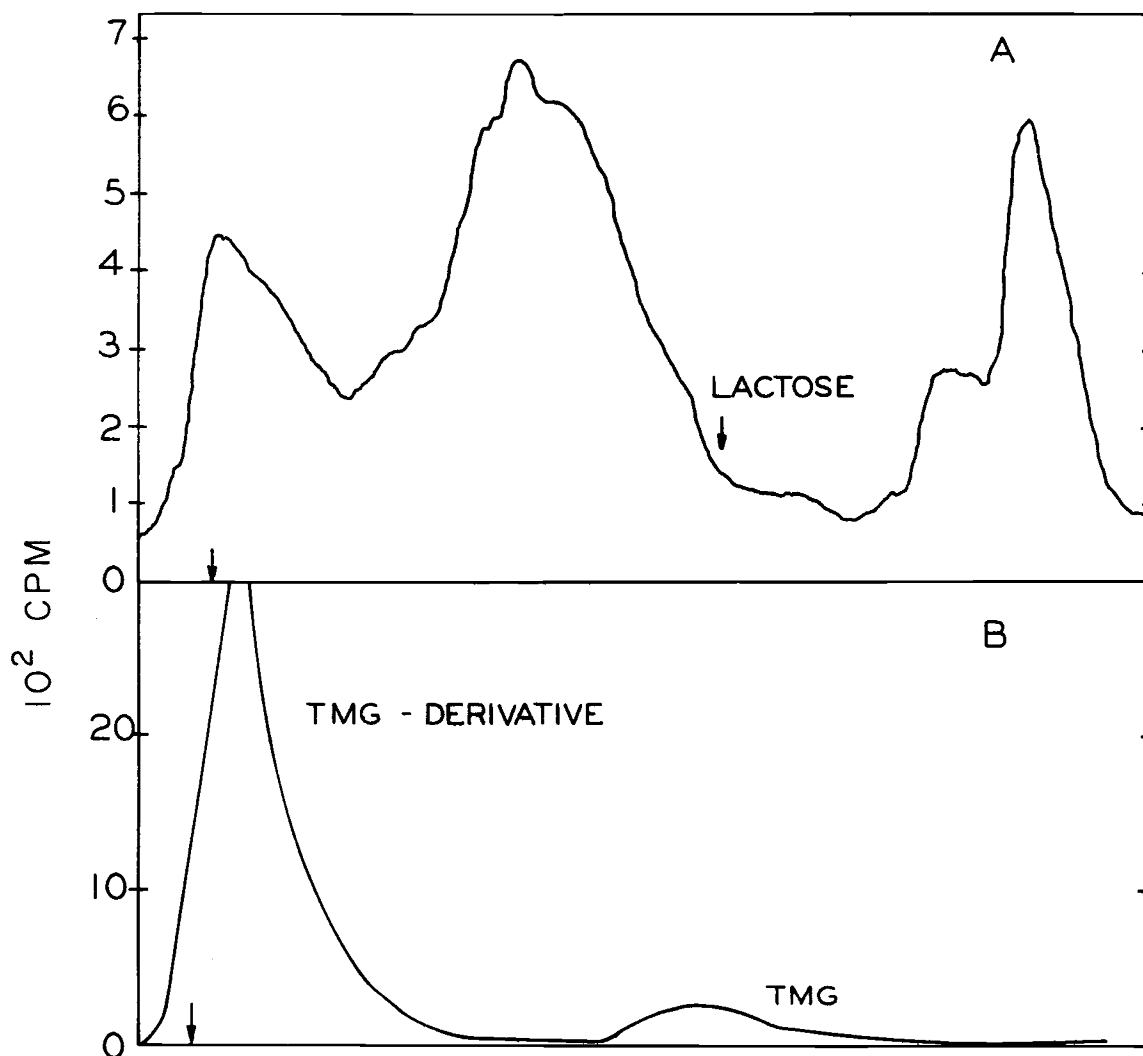


Figure 21. Accumulation of lactose-1-^{14}C and ^{14}C-TMG by S. lactis C_2F. Figure 21A represents the distribution of the labeled material extracted from whole cells previously exposed to lactose-1-^{14}C. The position marked lactose indicates the location of lactose-1-^{14}C when chromatographed with the extract. Figure 21B represents the distribution of the labeled material extracted from whole cells previously exposed to ^{14}C-TMG. The arrows mark the origin of the chromatogram.

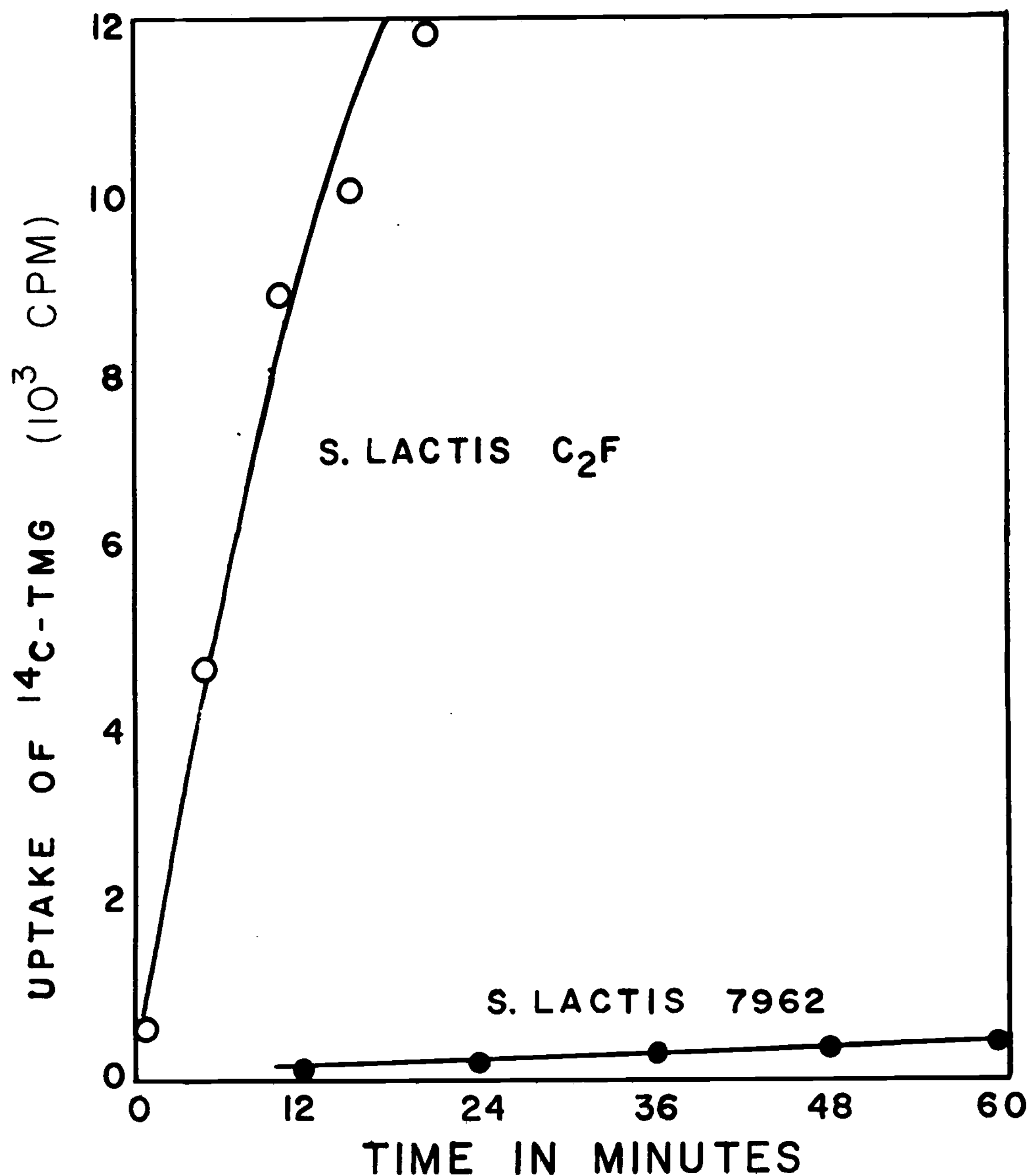


Figure 22. Intracellular accumulation of ^{14}C-TMG by resting cell suspensions of S. lactis C_2F and S. lactis 7962 previously induced for lactose utilization. Incubation mixture consisted of about 0.92 mg cell dry weight per ml (S. lactis C_2F) or about 1.39 mg cell dry weight per ml in the case of S. lactis 7962 and approximately 33.3 μM ^{14}C-TMG in a total volume of 4.0 ml.

the supernatant fluid.

Electrophoretic treatment (Figure 23) of portions of the extract revealed that the derivative was more acidic and moved toward the anode at a faster rate than TMG. This result suggested that S. lactis C_2F may accumulate TMG by phosphorylation. To obtain proof of this possibility, the derivative was purified, concentrated, and treated with alkaline and acid phosphatase. Figure 24 illustrates that alkaline phosphatase hydrolyzed the derivative liberating a compound which corresponded to TMG on the chromatograph. Acid phosphatase also hydrolyzed the derivative in a similar manner and these data indicated that the derivative was TMG-P.

Surprisingly, when S. lactis 7962 was examined for the intracellular state of the accumulated ^{14}C-TMG it also was found to accumulate as a derivative. Experiments performed at OC did not reveal any TMG- derivative or TMG when the extracts were chromatographed (Figure 25).

Effect of Cofactors on ONPG Hydrolysis

In confirmation of previous reports from our laboratory, cell-free extracts of S. lactis C_2F were defective in their ability to hydrolyze ONPG. However, such preparations did hydrolyze ONPG, if highly concentrated extracts (5. 7 mg protein/ml) were used (Table 9). This basal level of enzyme activity was only enhanced

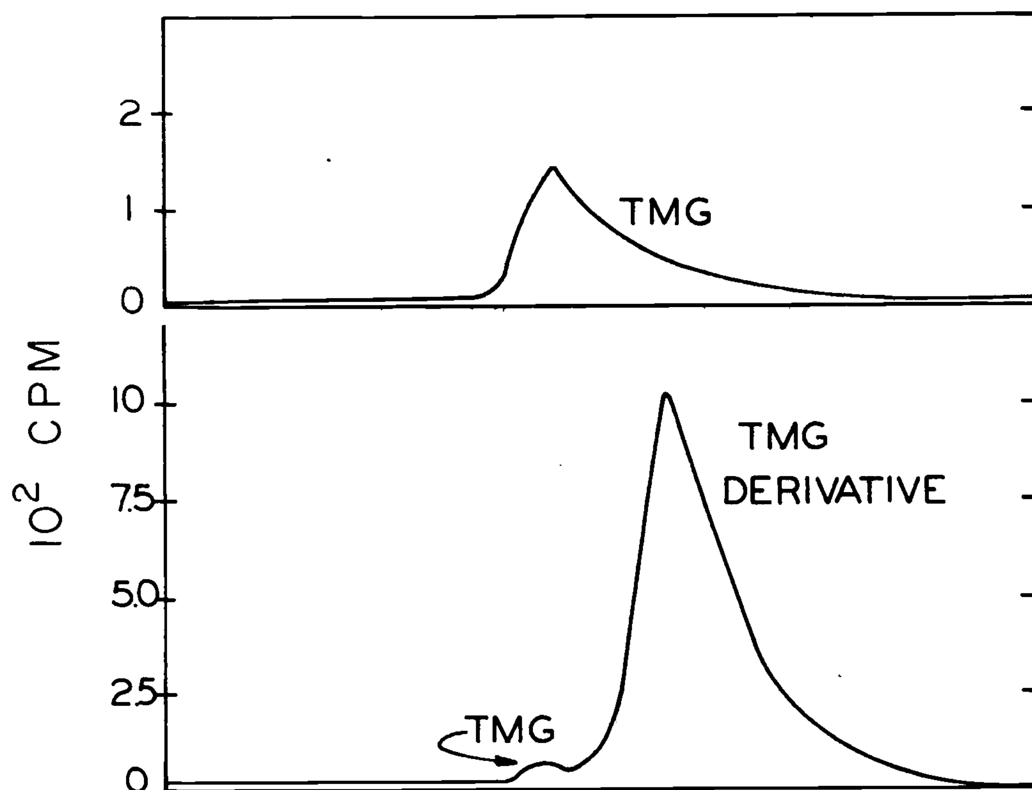


Figure 23. Electrophoresis of TMG and TMG derivative. Labeled TMG and ethanol extracts of *S. lactis* C_2F cells previously exposed to ^{14}C-TMG were spotted on cellulose acetate strips. Electrophoretic mobilities of the labeled compounds were determined at a voltage of 300 and amperage of 15 ma for 20 min. The distribution of the label was determined by the Radiochromatogram Scanner. The anode was located to the right of the figure.

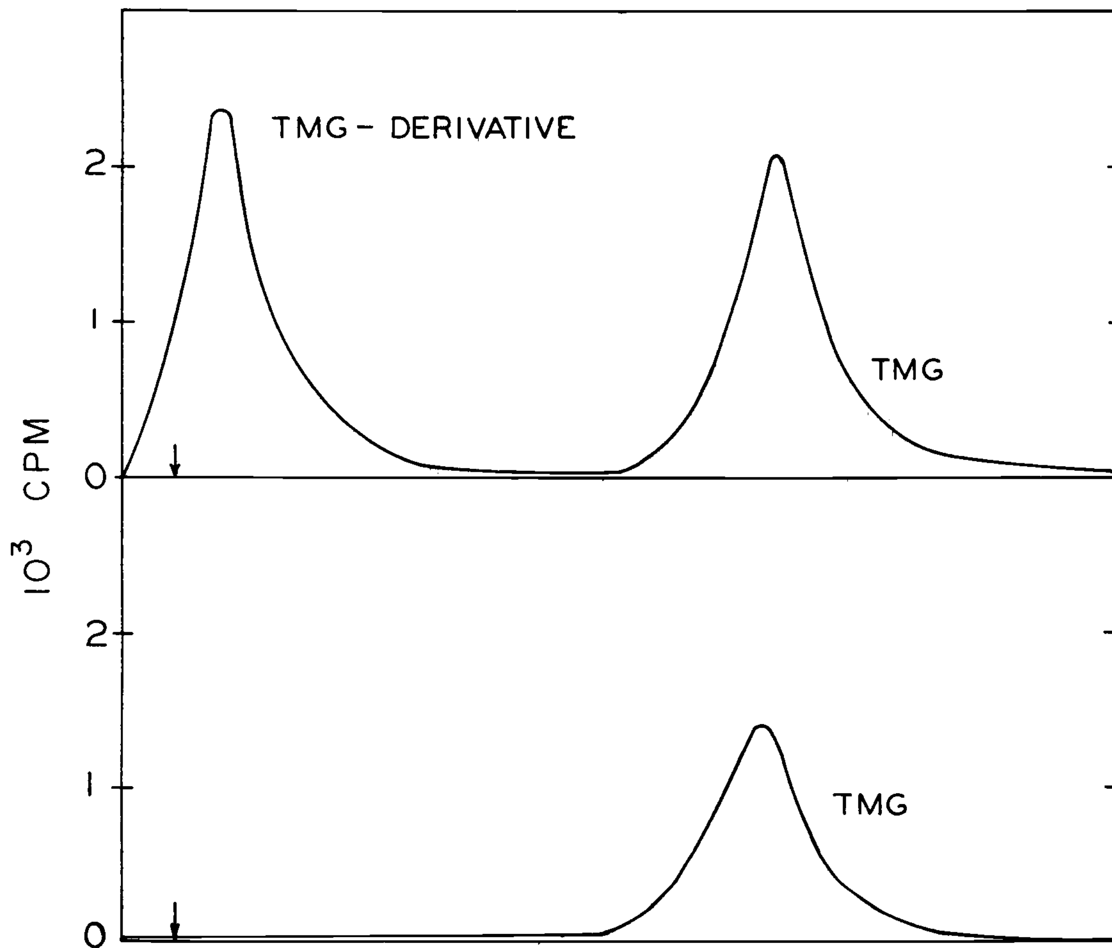


Figure 24. Treatment of the TMG derivative with alkaline phosphatase. The upper curve represents chromatography of ^{14}C-TMG and ^{14}C-TMG derivative. The bottom curve represents the location of the label from TMG after treatment of the ^{14}C-TMG derivative with alkaline phosphatase. The arrows mark the origin of the chromatograms.

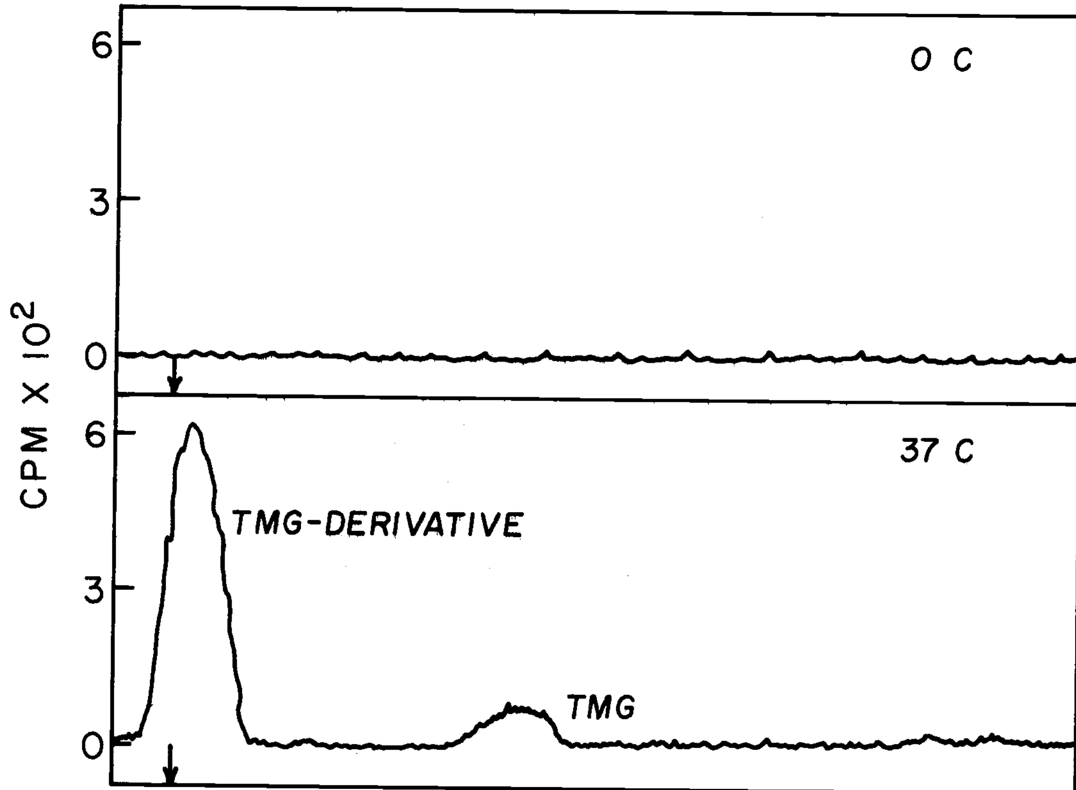


Figure 25. Accumulation of ^{14}C-TMG by resting cell suspensions of S. lactis 7962.

Table 9. Effect of NaF on ONPG hydrolysis in cell free extracts of *S. lactis* C_2F and *S. lactis* 7962.[a]

Cofactor	*S. lactis* C_2F -NaF	*S. lactis* C_2F +NaF	*S. lactis* 7962 -NaF	*S. lactis* 7962 +NaF
None	573[b]	168	1131	1083
Acetyl-P	558	171	1065	1080
Carbamyl-P	579	168	1086	1092
ATP	516	162	1005	762
PEP	840	885	1083	1104

[a]The assay mixture contained 11.4 mg soluble protein for *S. lactis* C_2F and 128 μg protein in the case of *S. lactis* 7962, 10 μmoles ONPG, 20 μmoles cofactor, and, when used, 20 μmoles NaF in a total volume of 2.0 ml. After incubation at 37 C for 10 min the reaction was stopped by the addition of 2.0 ml 0.5 M Na_2CO_3.

[b]All results are expressed as mμmoles ONP formed in 30 min.

by the addition of PEP. In addition, this PEP-dependent elevated ONPG hydrolysis was not affected by the presence of 0.01 M NaF; however, in the absence of PEP, NaF inhibited ONPG hydrolysis. Guanosine-5-triphosphate (GTP) and uridine-5-triphosphate (UTP) were also unable to stimulate ONPG hydrolysis. When the above experiments were conducted employing cell-free extracts from S. lactis 7962 the addition of cofactors did not stimulate ONPG hydrolysis, nor did NaF inhibit the reaction (Table 9).

Induction of ^{14}C-TMG Transport

Figure 26 illustrates a number of characteristics of ^{14}C-TMG uptake by S. lactis C_2F. The uptake of radioactive label was inducible since galactose grown cells readily accumulated the label whereas glucose grown cells were repressed in ^{14}C-TMG uptake. It also appeared that all of the TMG was accumulated by the galactose grown cells after a 20 minute exposure to the labeled compound, thus establishing an apparently infinite concentration gradient. In addition, the labeled TMG accumulated by the glucose and maltose grown cells occurred as the TMG derivative.

S. lactis 7962 accumulated a TMG derivative if the cells were previously grown in lactose, however, no derivative could be detected if the cells were grown on glucose. This indicates the galactoside permease is also inducible in S. lactis 7962.

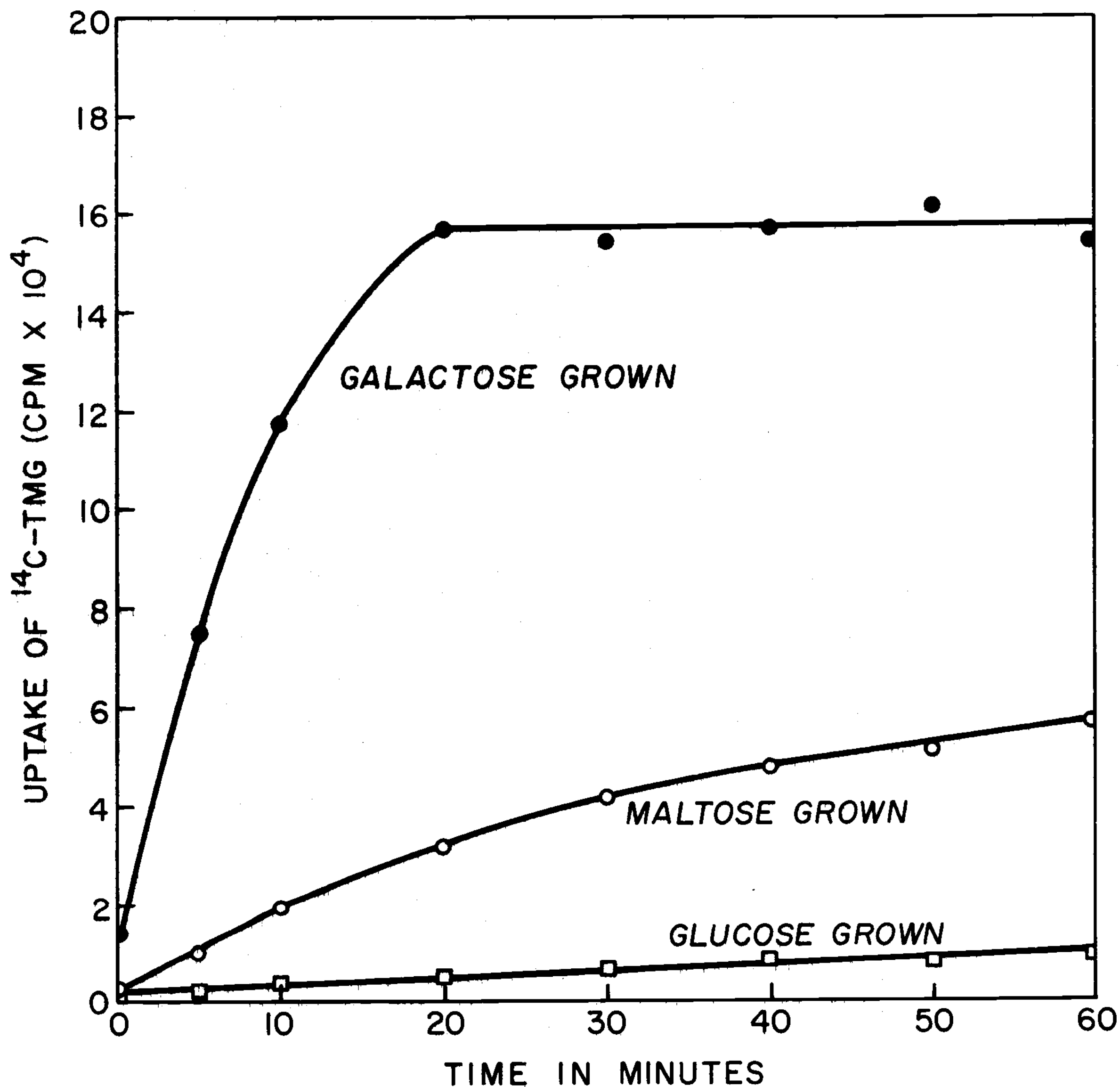


Figure 26. Intracellular accumulation of ^{14}C-TMG by resting cell suspensions of S. lactis C_2F grown in galactose, maltose, or glucose broth. Incubation mixtures consisted of about 1.66 mg cell dry weight per ml and approximately 88 μM ^{14}C-TMG in a total volume of 6.0 ml.

TMG Transport by the Lactose Negative Mutants

These experiments were designed to supplement the previous studies in which the disappearance of lactose from the supernatant fluid was used to measure galactoside permease activity. ^{14}C-labeled TMG was added to cell suspensions and the uptake of counts into the cells followed as a function of time. Figure 27 indicates the transport of TMG in S. lactis C_2F and the lac^- gal^- mutants F15, L13, C_2FW, F4, 3, F22, F7, G2, G4, G6, and G10. It can be seen that the uptake of TMG by the mutants is negligible compared to the uptake of this compound by the parent strain.

Figure 28 illustrates the transport of ^{14}C-TMG by S. lactis 7962 and the lac^- mutants 5, C, and 3. Again the uptake of TMG by the mutants is defective in comparison to the wild type. S. lactis 7962-5, however, did accumulate some TMG. The uptake of TMG by mutants 3R, 6, and 9 resembled that observed for 3 and C.

Serological Identification of S. lactis

Extracts of S. lactis C_2F and S. lactis C_2FW gave positive Group N precipitin reactions, whereas extracts from S. lactis 7962 were negative not only for Group N but also for the remainder of the streptococcus serological groups. Repeated efforts to demonstrate a positive Group N precipitin reaction in S. lactis 7962 were negative.

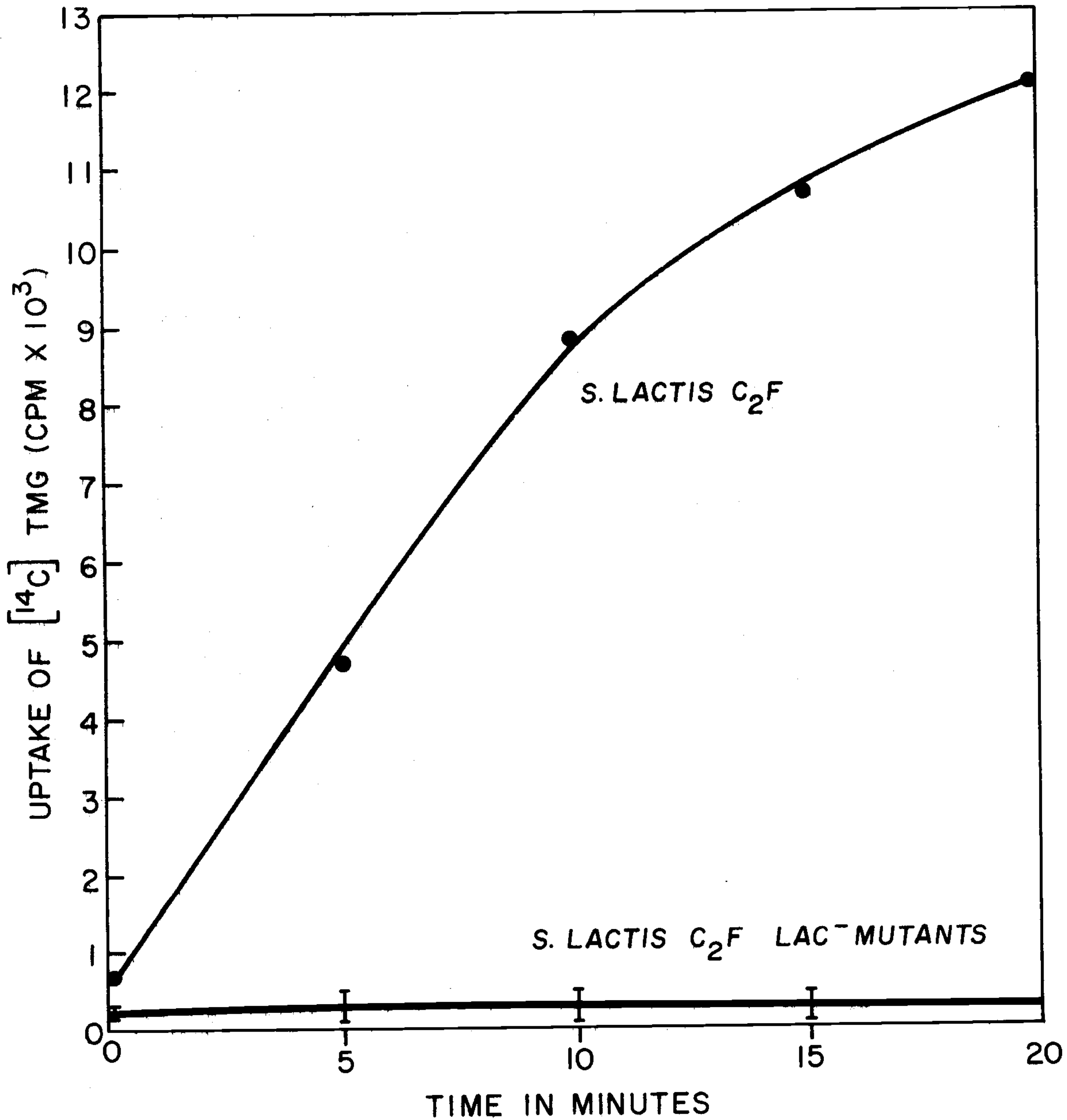


Figure 27. Uptake of ^{14}C-TMG by S. lactis C_2F and the lac$^-$ gal$^-$ mutants. Incubation mixtures consisted of about 0.92 mg cell dry weight per ml and approximately 33.3 μM ^{14}C-TMG in a total volume of 4.0 ml.

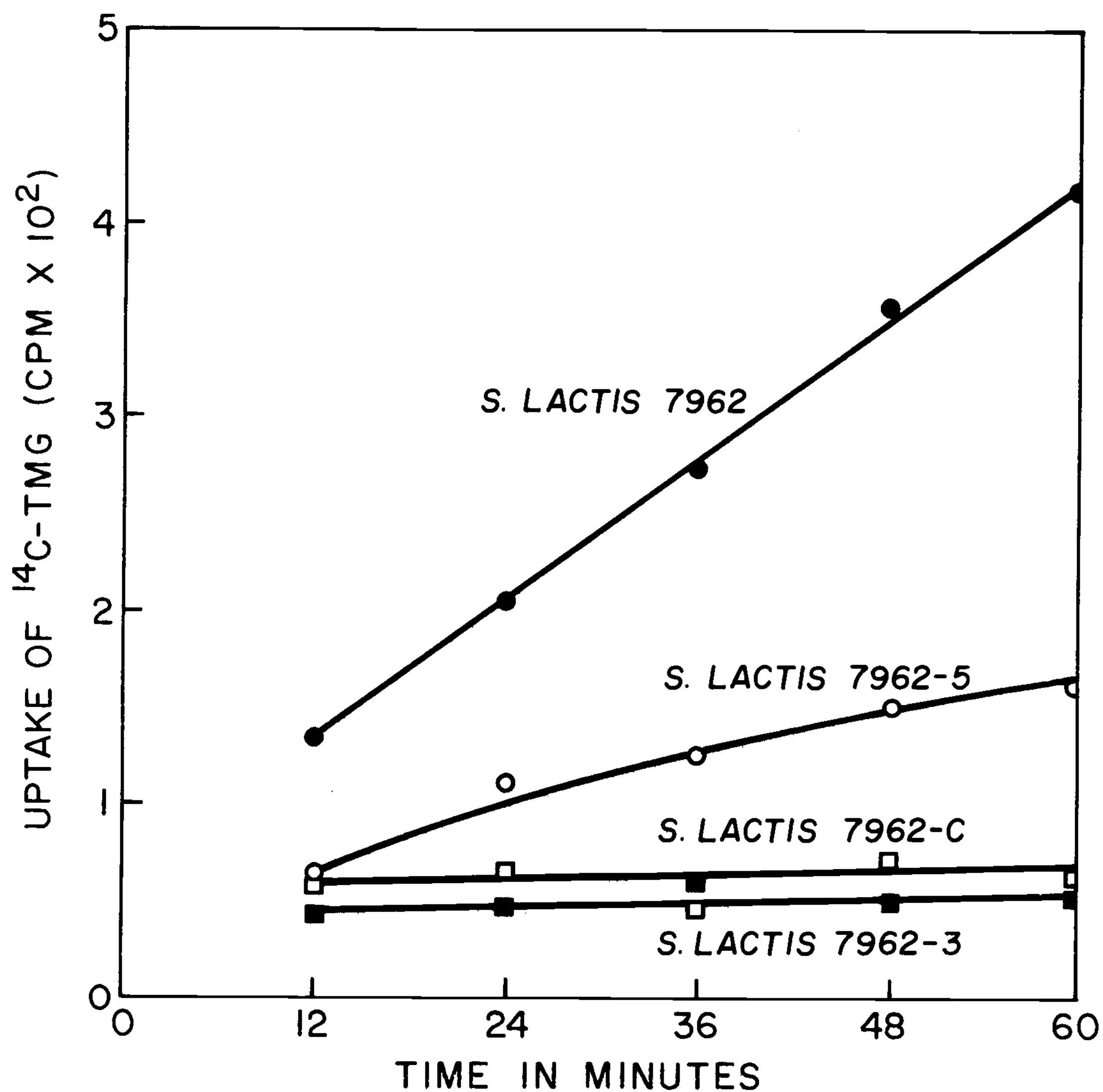


Figure 28. Uptake of ^{14}C-TMG by S. lactis 7962 and the lac$^-$ mutants. Incubation mixtures consisted of about 1.39 mg cell dry weight per ml and approximately 33.3 μM ^{14}C-TMG in a total volume of 4.0 ml.

DISCUSSION

The purpose of this study was to examine several lactobacilli for β-galactosidase activity and to investigate the factors involved in the fermentation of lactose by lactic streptococci. The latter study was approached by the isolation of lactose negative mutants from the strain (7962) containing β-galactosidase and S. lactis C_2F a strain chosen to represent those lactic streptococci unable to hydrolyze ONPG in cell free extracts. It was anticipated that the isolation of these mutants would provide further information with respect to the mechanism of lactose metabolism by this unique group of micro-organisms. The significance of these findings is now discussed.

Studies on Lactobacilli

The production of lactic acid by homofermentative lactic streptococci is of considerable importance to the dairy industry. For example, S. lactis is a constituent of starter cultures that are used in the dairy industry to produce many fermented milk products. This production of lactic acid is essential to achieve proper keeping quality, consistency, and flavor of the final product.

Growth of lactic streptococci in milk, their natural habitat, depends on their ability to hydrolyze lactose. This function requires the biosynthesis of β-galactosidase. In 1965 Citti, Sandine, and

Elliker suggested that the more proteolytic nature of S. lactis C_2F may allow more rapid synthesis of β-galactosidase by providing a greater quantity of amino acids to the cell than is true for the less proteolytic C_2S strain. The loss of proteolytic ability which occurs during the fast to slow mutation could then result in an impaired ability to synthesize β-galactosidase in the slow acid producers. Therefore, it would seem fruitful to study the relationship between proteolysis and β-galactosidase. Experiments of this nature in other organisms have been performed. For example, Fox (1961) measured the incorporation of ^{14}C-peptides into M-protein, streptolysin O, and proteinase of a group A Streptococcus. Incorporation of these peptides was dependent on proteolytic activity.

A study of this type would require developing a procedure for the purification of β-galactosidase from S. lactis. Unfortunately, S. lactis 7962 was the only strain among over 40 lactic streptococci tested by Citti, Sandine, and Elliker (1965) which produced an enzyme that was stable in either toluene-treated suspensions or cell-free extracts. All attempts to stabilize the enzyme in the other strains with conventional procedures were unsuccessful. The enzyme from S. lactis 7962, however, was further studied by McFeters, Sandine, and Elliker (1967) and by McFeters et al. (1969). They extracted and purified the β-galactosidase from this strain and some of its enzymatic and chemical properties were determined. The enzyme proved to

be quite unstable in buffer solutions. This observation and the fact that this enzyme could not be detected in cell free extracts of over 40 strains of lactic streptococci led the author to examine several lactobacilli for β-galactosidase activity.

During studies on the properties of enzymes in microorganisms, it is generally desirable to measure enzyme activity in the presence of substrate under conditions in which the permeability of the cell is not a limiting factor. To attain this, cell-free preparations or toluene treated cell suspensions have been used. For example, Koppel, Porter, and Crocker (1953) observed that toluene-treated cells of E. coli could be used for the assay of β-galactosidase. In the present study, toluene treatment was very effective in the "activation" of β-galactosidase from L. helveticus, L. lactis, L. acidolphilus, and L. bulgaricus. The enzyme activity from L. arabinosus was only slightly enhanced by toluene treatment, whereas the β-galactosidase from L. casei was partially destroyed upon solvent treatment. The latter observation is similar to that noted by Anderson and Rickenberg (1960) for certain strains of Paracolobactrum aerogenoides in which the original, intact-cell β-galactosidase activity remaining after treatment with toluene was partially destroyed. However, in this case, unlike the S. lactis strains, crude extracts of P. aerogenoides did possess enzyme activity. It is also apparent that, unlike the majority of the lactic streptococci, the

lactobacilli do possess a β-galactosidase which is not destroyed upon toluene treatment, in fact, in many cases toluene treatment greatly enhances the activity.

The optimal conditions for the assay of the β-galactosidase in L. helveticus were in contrast to those for other microorganisms in which the enzyme has been studied. Maximal enzyme activity was observed with cell-free extracts and toluene treated cell suspensions which were assayed at about 50 C in sodium phosphate buffer at pH 6.6. The requirement for sodium ion to maximally activate the enzyme when ONPG was the substrate was evident; similar results were obtained with E. coli (Cohn and Monod, 1951), P. aerogenoides (Anderson and Rickenberg, 1960), and with S. lactis 7962 (Citti, Sandine and Elliker, 1965). L. lactis, L. acidophilus, and L. bulgaricus also revealed greater enzyme activity at 50 C than at 37 C; whereas L. arabinosus and L. casei revealed slightly higher activities at 37 C. It is interesting to note that those organisms showing greater enzyme activity at 50 C also had an optimum growth temperature above 37 C while those organisms having higher enzyme activity at 37 C had an optimum growth temperature at or below 37 C. This would suggest that some of the lactobacilli may possess a fairly heat stable β-galactosidase, although such an assumption must be made with caution since a high temperature optimum for enzyme activity does not necessarily imply the presence of a heat stable enzyme

per se, but may reflect an elevated heat of activation for the substrate or cofactor-stabilized enzyme complex. Nevertheless, the fact that the lactobacilli possessing the high optimum growth temperature also possessed a high optimum temperature for β-galactosidase activity, may reflect the presence of a more heat stable enzyme in these microorganisms. In this regard, *S. thermophilus*, which has an optimal growth temperature of 45 C, also exhibited greater enzyme activity at 50 C than at 37 C.

Further experiments with respect to the presence of heat stable enzymes among the lactic acid bacteria would be interesting. For example, the optimum growth temperature of these organisms range from 30 C in the case of *L. arabinosis* to 60 C in *L. thermophilus*. The amino acid composition data from several β-galactosidase enzymes isolated from lactobacilli over this temperature range could provide further information with respect to the ability of these organisms to proliferate at the higher temperatures. In this respect, Campbell and Manning (1961) demonstrated that the amino acid composition data of α-amylase from *Bacillus stearothermophilus* which has an optimum growth temperature of about 60 C contained an unusually high proline content. The suggestion was then made that the thermostability of the protein molecule may be as a consequence of the fact that the enzyme exists in an unfolded configuration in the native state, and therefore heat has little further denaturation effect

(Manning, Campbell, and Foster, 1961). Since the β-galactosidase enzymes would catalyze the same reaction (hydrolysis of lactose), any differences observed with respect to temperature could reflect a change in amino acid composition.

The increase in β-galactosidase activity observed in L. helveticus when cells were disrupted by toluene treatment and in the preparation of cell free extracts suggested that transport into the cell may be a rate limiting factor in lactose fermentation. The effect of pH and temperature on fermentation of lactose by L. helveticus agreed quite well with its optimum growth temperature and the fact that the growth of lactobacilli is not adversely affected by low acidity. In comparing the rate of lactose fermentation by several strains of lactobacilli it was observed that the rate was correlated with their ability to coagulate milk. In other words, L. lactis, L. helveticus, and L. acidophilus would coagulate milk within 12 hours at 37 C, whereas L. arabinosus was much slower and L. casei required two to three days to coagulate milk when grown near its optimum growth temperature. One exception existed however, L. bulgaricus which coagulated milk as rapidly as L. helviticus was defective in lactose utilization when washed cell suspensions were suspended in a solution of the sugar. These same cells however contained high levels of β-galactosidase activity. Since L. bulgaricus would not grow in lactic broth supplemented with carbohydrates under normal

conditions, it is tempting to suggest that this inability could be related to the transport of energy sources into the cell. Even when grown under CO_2 tension, repeated transfers in the broth would eventually result in no growth, yet in milk this organism grew quite readily.

From this study we have learned that the lactobacilli possess β-galactosidase activity which can be measured in toluene-treated or cell-free extracts. The enzyme from these organisms may be stable enough to be purified and later used to determine the relationship between proteolysis and β-galactosidase synthesis. Although the experiments concerned with the addition of crude extracts containing β-galactosidase to milk cultures of S. lactis are of preliminary nature, the active enzyme had a marked influence on the ability of the cells to produce acid. The heat-inactivated enzyme however, afforded less stimulation of acid production, the increase that was observed with the inactivated enzyme could have been due to the presence of nucleic acid or protein components which are known to stimulate acid production (Koburger, Speck, and Wilbur, 1958). Experiments to determine the effect of adding β-galactosidase to milk on the hydrolysis of the lactose could be performed by the recently described procedure of Miah et al., 1968.

Studies on Lactic Streptococci

During the isolation of lactose mutants from *S. lactis* 7962, it was noted that a semi-synthetic medium, containing lactose as the sole carbon source, would not support growth when the cells were previously grown on glucose. Glucose-grown cells did not increase in population within 48 hours, whereas the lactose-grown cells attained their maximal number at the end of the same period. When glucose or galactose served as the sole carbon source, rapid growth occurred. Likewise, if the medium contained lactose but was supplemented with yeast extract, the lactose and glucose grown cells reached the stationary phase of growth after a 12 hour incubation period. Addition of 0.2 g of tryptophan per liter to the basal medium permitted the glucose-grown cells to utilize lactose and maximal growth occurred within 48 hours. Thus, tryptophan was stimulatory for growth of *S. lactis* 7962 on lactose but was not required for growth on glucose or galactose. However, even in the absence of tryptophan, the unadapted cells did eventually develop limited growth. This may be due to cryptic growth in that members of the initial population may be dying and therefore the survivors would no longer be starved since leakage and lysis products from the dead organisms may be supporting growth of the survivors (Ryan, 1959).

The meaning of the above findings are not clear at the present

time and further experimentation is necessary to elucidate the role of tryptophan in lactose utilization by S. lactis 7962. This role may be direct in that tryptophan could be an important factor in the adaptation of these cells for lactose utilization or indirect in that metabolites of tryptophan decomposition could be responsible for the adaptation to lactose.

The results discussed throughout the remainder of this thesis indicate that lactic streptococci may possess different mechanisms for lactose utilization, functioning separately or together in a given strain. One involves a PEP-dependent phosphotransferase system, while another appears to be dependent on β-galactosidase.

When measuring lactose uptake from aqueous solutions by washed S. lactis cells, it was found that there was no detectable sugar utilization by any of the S. lactis C_2F lac^- gal^- mutants. This result, coupled with the observation that whole cells of the same mutants were unable to hydrolyze ONPG, suggested that these mutants were both permease and β-galactosidase negative. On the other hand, lac^- mutants from S. lactis 7962 could be differentiated into several classes depending upon their rate of lactose fermentation. The slow rate observed in many of the mutants suggested a defect in permease function, especially since the same mutants possessed full β-galactosidase activity. One mutant appeared to lack both permease and β-galactosidase activity, whereas a third phenotype

isolated was characterized by its inability to hydrolyze ONPG. The latter mutant, however, still fermented lactose, suggesting the substrate specificity of this enzyme for ONPG was altered. In an effort to further characterize the C_2F mutants, factors affecting the fermentation of lactose in the wild type were examined.

The ability to ferment lactose was much greater in S. lactis C_2F than in S. lactis 7962. Next, the factors influencing the disappearance of lactose from the suspending medium were examined. It should again be mentioned, however, that the method employed for the measurement of lactose fermentation does not only quantitate permease activity, but also the metabolism of the sugar. Therefore the results obtained could be affecting permease activity, subsequent metabolism of lactose, or both.

The results demonstrated what appeared to be a specific requirement of phosphate for the fermentation of lactose. Neither sodium chloride nor potassium chloride replaced phosphate, which ruled out the possibility that the effect depended on ionic strength. Similar conclusions were reported for glucose transport in Nocardia species (Cerbon and Ortigoza-Ferado, 1968). However, since lactose fermentation also occurred in the presence of Tris-buffer, the effect of pH cannot be ruled out. Nevertheless, maximal lactose fermentation occurred in the presence of phosphate buffer.

Sodium arsenate inhibited lactose fermentation in S. lactis 7962

whereas in S. lactis C_2F only a slight inhibitory response was observed. The effect of arsenate on these cells is not clear; however, by analogy with other systems, wherein arsenate replaces phosphate, any combination involving arsenate in the present system would probably also be unstable and decompose spontaneously (Korzenovsky and Werkman, 1953). Although the effect of arsenate on the utilization of other carbohydrates was not tested, the results suggested that the two organisms may ferment lactose via different mechanisms.

This conclusion was supported by the observations that NaF prevented lactose fermentation and ONPG hydrolysis in whole cells of S. lactis C_2F, but had no effect on S. lactis 7962. Moreover, only the addition of PEP to toluene treated cells of strain C_2F restored its ability to hydrolyze ONPG (Walter, 1969). In addition neither strain was affected by NaN_3. The hydrolysis of ONPG by intact cells of E. coli displays a very different pattern of response to these inhibitors, being unaffected by NaF, and somewhat inhibited by azide (Kennedy and Scarborough, 1967). The above results suggested that a continuous supply of PEP via the enolase reaction was needed for lactose utilization in S. lactis C_2F. It thus appeared this strain metabolized lactose via the PEP-dependent phosphotransferase system.

In agreement with the results of Citti, Sandine, and Elliker (1965) cell-free extracts of S. lactis C_2F were unable to hydrolyze

ONPG. However, concentrated cell-free extracts from this organism, which presumably would contain the three soluble components of the phosphotransferase system in addition to the membrane bound enzyme (Hengstenbert et al., 1968; Simoni, Smith, and Roseman, 1968) revealed ONPG hydrolytic activity stimulated only by PEP. NaF inhibited the hydrolysis of ONPG, yet only the addition of the PEP in the presence of NaF restored maximal activity. This indicated that the lactose hydrolyzing enzyme of S. lactis C_2F was not the fluoride sensitive component of the system, since maximum ONGP hydrolysis occurred in the presence of NaF if PEP was present.

ONPG hydrolysis by strain 7962 was not stimulated by the addition of cofactors nor did NaF inhibit the process. In this respect, S. lactis 7962 resembles E. coli, since the latter organism hydrolyzed ONPG in the presence of NaF and did not appear to phosphorylate lactose (Kennedy and Scarborough, 1967). S. lactis 7962 also contained a typical β-galactosidase as measured by its ability to cleave lactose to glucose and galactose, whereas S. lactis C_2F did not contain an enzyme capable of hydrolyzing free lactose under the conditions employed. It therefore appeared that the appropriate substrate for the lactose catabolizing enzyme from the C_2F strain would be a phosphorylated derivative of lactose. Experiments using concentrated cell extracts should yield fruitful results in future

studies since the extracts apparently contain the components of the phosphotransferase system.

Iodoacetic acid is a general inhibitor of enzymes because of its reaction with sulfhydryl groups. At low concentrations, this inhibitor appears to be relatively specific for glycerolphosphate dehydrogenase (Green, Needham, and Dewan, 1937). It should therefore inhibit the production of PEP in whole cells and thus limit lactose fermentation. Lactose fermentation was inhibited by IAA in S. lactis C_2F and also in S. lactis 7962. The former could be due to the lack of PEP formation, whereas the latter could reflect the necessity of an energy source in the fermentation of lactose. In vitro assays on the hydrolysis of ONPG in the presence of IAA should also be performed to insure that the efect of the inhibitor was not on the hydrolyzing system.

Experiments testing for the presence of phosphorylated lactose in S. lactis C_2F using the labeled carbohydrate were unsuccessful due to rapid metabolism of the sugar. However, when a non-metabolizable analogue of lactose (TMG) was used, a derivative (TMG-PO_4) was shown to accumulate, supporting the hypothesis that lactose fermentation, in S. lactis C_2F requires substrate phosphorylation by the PEP-dependent system. S. aureus which ferments lactose via the PEP-dependent system, has also been shown to accumulate TMG within the cell as TMG-PO_4 (Laue and MacDonald, 1968).

S. lactis 7962 also accumulated TMG as a derivative, which was unexpected since the organism hydrolyzed lactose to glucose and galactose. However, this result could explain the inability of TMG, a potent inducer of β-galactosidase in E. coli, to induce the enzyme in S. lactis 7962 (Citti, Sandine, and Elliker, 1965). Since TMG appeared to accumulate as the phosphate derivative, it may no longer function as inducer. The possibility that lactose is accumulated via the PEP-dependent system in S. lactis 7962 as lactose-P and then cleaved by a specific phosphatase cannot be ruled out. This would imply that the specific phosphatase had little affinity for the phosphate group of TMG, and thus it would accumulate as observed. On the other hand, the inability of TMG to efficiently induce S. lactis C_2F, could reflect the inability of the organism to hydrolyze the TMG-P to galactose-6-P, which may function as the true inducer as occurs in S. aureus (Morse et al., 1968; Simoni, Smith, and Roseman, 1968).

It was found that the rate of TMG uptake into cells of S. lactis 7962 was slow in comparison to the rapid rate observed in cells of S. lactis C_2F. The slow rate observed for S. lactis 7962 confirms a previous report from this laboratory (Citti, Sandine, and Elliker, 1965). Subsequent experiments on the transport system of S. lactis C_2F indicated the uptake of radioactive lable was inducible and also suggested that all of the added TMG was accumulated by the cells, rapidly establishing a concentration gradient. Similar observations were made by Laue and MacDonald (1968) on TMG uptake by S. aureus, in this

case, the total amount of TMG taken up reached 2% of the cell dry weight and at low external concentrations of TMG, all of the TMG was accumulated by the cells. The galactoside permease, as measured by the accumulation of ^{14}C-TMG, was also inducible in S. lactis 7962. These results suggest that both organisms contain specific mechanisms for the transport of galactosides into the cell.

The rapid rate of TMG uptake by whole cells of S. lactis C_2F raises the questions of the possible energy sources for this purpose. Since these organisms lack both a TCA cycle and terminal respiration, it was assumed that anaerobic endogenous metabolism was supplying the necessary energy. In this regard, endogenous metabolism of S. faecalis has been reported to provide necessary energy to maintain a constant level of activity in the glycolytic enzymes for several hours after the removal of exogenous energy sources (Forrest and Walker, 1963; Forrest and Walker, 1965). Since the TMG uptake experiments were conducted with freshly washed cells, endogenous metabolism could account for the necessary energy.

It was also interesting to note that lactose was better than galactose for induction of lactose utilization in S. lactis 7962. In S. lactis C_2F and the other lactic strains tested, however, galactose was the better inducer. This is identical to findings with S. aureus where galactose was shown to be the inducer for lactose utilization; later experiments have shown, however, that galactose-6-P is the

true inducer (Creaser, 1955; Morse et al., 1968). This may also be true for S. lactis C_2F and other strains of lactic streptococci lacking β-galactosidase.

The importance of galactose in the metabolism of lactose by S. lactis C_2F was shown by the isolation of lactose negative mutants. These mutants were unable to hydrolyze ONPG, transport TMG, and were also defective in galactose metabolism. Transport mutants isolated from S. lactis 7962 were capable of hydrolyzing ONPG and the metabolism of galactose was not altered. These observations also confirmed the previous conclusions in that the mutants from S. lactis C_2F and S. lactis 7962 were unable to transport galactosides. The mutants from C_2F could not be induced for lactose utilization by growing the cells in the presence of lactose or galactose, yet galactose was eventually utilized as a carbon source. These observations, coupled with the fact that the C_2F mutants were unable to accumulate ^{14}C-TMG suggested that the mutants were defective in the phosphorylation step. Thus if these mutants were unable to phosphorylate lactose or galactose and if galactose-6-P is the true inducer as in S. aureus, then the defect in the induction of lactose metabolism in the S. lactis C_2F mutants is explained.

The fact that S. lactis 7962 was only one of many strains of lactic streptococci examined in our laboratory which possessed the classical β-galactosidase raises doubt regarding its classification.

This organism was first described by Rahn, Hegarty, and Dewel (1938) as an isolate from milk typical for the species. Hegarty (1939) used the strain in certain physiological studies and presumably deposited the bacterium with the American Type Culture Collection (ATCC). Repeated attempts over the past two years in our laboratory to confirm the positive precipitin reaction between commercially available group N antiserum and extracts of *S. lactis* 7962 as reported earlier (Sandine, Elliker and Anderson, 1959) have been negative; *S. lactis* C_2F gave a positive reaction. The response of *S. lactis* 7962 to other streptococcal antisera also gave negative results. When this finding was brought to the attention of the ATCC they confirmed that *S. lactis* 7962 did not give a positive Group N precipitin reaction, however, physiological tests carried out by them indicated it was a lactic streptococcus (C. K. Mills, personal communication). Previous physiological tests carried out in our laboratory also indicated it was a lactic streptococcus (Sandine, Elliker, and Anderson, 1959; Sandine, Elliker, Hays, 1962).

Apparently, *S. lactis* 7962 is not the only lactic streptococcus containing β-galactosidase. Vakil and Shahani (1969) reported that *S. lactis* UN possessed β-galactosidase which was preferentially induced by lactose. Novikova (1956) also reported on three strains which were assumed to contain β-galactosidase. It would be interesting to determine if these strains, like *S. lactis* 7962 also fail to

give a positive Group N precipitin reaction.

Although preliminary in nature, the experiments reported here support the view that a phosphotransferase in S. lactis C_2F is an important part of the permease system at least insofar as TMG is concerned. Furthermore, from the above results it may be concluded that the apparent lability of β-galactosidase in S. lactis C_2F and other strains tested is due to the absence of this enzyme and the presence of a PEP-dependent system in which lactose, like TMG, would accumulate as the phosphorylated derivative; the lactose-PO_4 is then hydrolyzed by a different enzyme. This enzyme may be analogous to the one found in staphylococci where the apparent in vitro lability of the β-galactosidase also was shown to be an artifact since the proper substrate was lactose-PO_4 and not the free disaccharide (Hengstenberg, Egan, and Morse, 1967, 1968; Kennedy and Scarborough, 1967). The derivative would then be hydrolyzed to form glucose and galactose-6-P by the new streptococcal "β-galactosidase." If this is the case, it will be interesting to learn how galactose-6-P is further metabolized in S. lactis C_2F and the remaining strains lacking β-galactosidase.

The above studies have only touched on the many aspects of the enzymic and genetic regulation of lactose metabolism in the lactic streptococci. It has been shown, however, that at least two different mechanisms of lactose utilization exist in S. lactis C_2F

and *S. lactis* 7962 functioning separately or together in the same strain. Numerous areas of research remain to be explored; some were mentioned throughout this discussion.

The further isolation and characterization of mutants from lactic streptococci affecting lactose metabolism will undoubtedly lead to a further understanding of the genetic control governing lactose metabolism in this group of microorganisms. Since it was observed that concentrated cell-free extracts of *S. lactis* hydrolyzed ONPG in the presence of PEP, the fractionation of these extracts into cell membranes and soluble constituents of the PEP-dependent system will also clarify the enzymatic steps involved in the hydrolysis of lactose. Once these enzymes are identified, it will permit the fractionation of extracts from the *S. lactis* C_2F mutants into the various components of the phosphotransferase system and an identity of the specific mutation. Such a procedure may show that the lac$^-$ gal$^-$ mutants isolated from *S. lactis* C_2F can be differentiated into several classes. Complementation experiments using extracts from the various mutants would also reveal the presence of more than one type of mutation if ONPG hydrolysis was restored. Such a procedure was utilized to characterize lac$^-$ mutants from *S. aureus* (Morse *et al.*, 1968; Hengstenberg *et al.*, 1968).

SUMMARY

Synthesis of β-galactosidase by several strains of lactobacilli were examined. Among those tested *L. helveticus* possessed the greatest activity and properties of the enzyme in this species were examined. Treatment of whole cells with acetone-toluene (9:1) resulted in the greatest enzyme activity. The optimum temperature for the enzyme in a cell-free extract prepared by sonication was 50 C and the optimum pH was found to be about 6.6 when using sodium phosphate buffer (0.05 M) at 50 C; potassium phosphate buffer afforded 30% less activity. Of the inducers tested, lactose was the most effective inducer of enzyme synthesis. Galactose was also an inducer of the enzyme but not as effective as lactose. The optimum pH and temperature for fermentation of lactose by *L. helveticus* agreed quite well with the optimum growth conditions for the organism. Rates of lactose fermentation by several strains of lactobacilli was related to the times the organisms required to coagulate milk.

During the isolation of lactose mutants from *S. lactis* 7962 it was noted that a semi-synthetic medium, containing lactose as the sole carbon source would not support growth if inoculated with cells previously grown on glucose. When glucose or galactose served as the sole carbon source, rapid growth by the glucose grown cells

occurred. Likewise, if the medium contained lactose, but was supplemented with yeast extract, the lactose and glucose grown cells reached the stationary phase of growth after a 12 hour incubation period. Addition of tryptophan to the medium permitted the glucose-grown cells to utilize the lactose. Thus tryptophan was stimulatory for growth of S. lactis 7962 on lactose, but was not required for rapid growth on glucose.

The apparent instability of "β-galactosidase" in toluene-treated cells or cell-free extracts of over 40 lactic streptococci was explained. These organisms did not contain the enzyme. Sodium fluoride prevented lactose utilization by whole cells of S. lactis C_2F but had no effect on S. lactis 7962. Sodium arsenate prevented lactose metabolism in S. lactis 7962, but had only a slight inhibitory response on S. lactis C_2F. Concentrated cell extracts of S. lactis C_2F hydrolyzed ONPG; this hydrolysis was inhibited by NaF, yet the addition of PEP, in the presence of NaF, restored maximal activity. Addition of acetyl-P, carbamyl-P, ATP, GTP, or UTP did not stimulate activity. The presence of cofactors did not stimulate nor did NaF inhibit the hydrolysis in extracts of S. lactis 7962. To confirm the absence of β-galactosidase, S. lactis 7962 was shown to hydrolyze lactose to glucose and galactose, whereas S. lactis C_2F was unable to split the disaccharide. In addition, whole cells of S. lactis C_2F rapidly accumulated a derivative of TMG. This derivative was negatively charged, and after treatment with

phosphatase, chromatographed as TMG. In addition, galactose was a better inducer than lactose in the "β-galactosidase-less" lactic streptococci. Therefore, these results suggest that the β-galactosidase-less lactic streptococci utilize lactose via the PEP-dependent system as occurs in S. aureus, in which lactose would accumulate as lactose-P and consequently be split by a different enzyme. Unexpectedly, S. lactis 7962 also accumulated TMG as a derivative, although the rate was extremely slow.

The lactose negative mutants isolated from S. lactis C_2F all possessed the phenotype lac^- gal^-. These mutants were unable to transport TMG, hydrolyze ONPG, or utilize lactose if the cells were previously grown in the presence of lactose or galactose. This suggested the defect was in the phosphorylation step. Lactose negative mutants from S. lactis 7962 could be separated into several classes such as z^+y^-, z^-y^- (this mutant was also gal^-) and z^-y^+. Although lactose was utilized, the latter mutant could not hydrolyze ONPG which suggested the β-galactosidase has lost affinity for ONPG but not for the natural substrate lactose.

Lactose metabolism was inducible in S. lactis C_2F and S. lactis 7962. Furthermore, both strains possessed an inducible transport system for the accumulation of ^{14}C-TMG. The means of control of induced β-galactosidase and the TMG transport system of S. lactis 7962 appears to be similar but is not identical to that in E. coli.

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