AN ABSTRACT OF THE THESIS OF

THEODORE ARTHUR MOLSKNESS for the DOCTOR OF PHILOSOPHY
(Name of student) (Degree)

in Microbiology presented on 5/6/74
(Major) (Date)

Title: β-D-PHOSPHOGALACTOSIDE GALACTOHYDROLASE OF LACTIC STREPTOCOCCI: ENZYME PROPERTIES AND ANALYSIS OF DEFICIENT MUTANTS BY TRANSDUCTION

Abstract approved: Redacted for Privacy
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β-D-phosphogalactoside galactohydrolase (β-Pgal) was examined in a number of lactic streptococci using the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-6-P). Specific activity of β-Pgal ranged from 0.563 units/mg protein in Streptococcus lactis UN, to 0.120 in S. diacetilactis 18-16. Essentially no β-D-galactosidase (β-gal) was found in these organisms when o-nitrophenyl-β-D-galactopyranoside (ONPG) served as the chromogenic substrate. S. lactis 7962 was the one exception found. This organism contained rather high levels of β-gal and very little β-Pgal could be detected. β-Pgal activity was examined in streptococci that differed widely in both their proteolytic ability and rates of lactic acid production during growth in milk. Differences in proteolytic ability did not influence β-Pgal synthesis; also the rate

of lactic acid production was independent of the level of β-Pgal present in the cell since the rate of lactic acid production could be increased approximately four-fold without changing the amount of β-Pgal present in the cell. Various carbohydrates were tested as potential inducers of the enzyme. While galactose, either as the free sugar or combined with glucose in lactose was the only inducer, non-inducing sugars such as mannose or glucose showed some ability to cause fluctuations in the basal level of β-Pgal. Cells growing in mannose or glucose exhibited about 30% of the maximal enzyme levels found in cells growing in lactose or galactose. No gratuitous inducers were found.

A phage-mediated transducing system was used in studying certain physiological characteristics of S. lactis C2 wild type, lactose negative mutants and lactose positive transductants. Lac^- mutants, obtained by acriflavin treatment of the wild type, were similar in all respects to the wild type but lacked β-Pgal and also could not transport ^{14}C lactose. It was subsequently discovered that the lac^- mutants had also lost much of their proteolytic ability. The wild type contained approximately nine times the proteolytic activity observed in the mutant.

The lactose-fermenting characteristic was transduced from the wild type to the lac^- mutant. The lac^+ transductants obtained were

similar to the wild type parent with respect to lactose fermentation and level of β-Pgal activity (0.186 units/mg protein). These transductants, however, had not regained full proteolytic ability and were similar to the lac^- mutant in this respect. Lactic acid production of the transductant in milk was approximately two-thirds that of the wild type. Indications are that both the lactose-fermenting character and certain of the proteolytic enzymes are both carried on extrachromasomal particles (plasmids).

β-D-Phosphogalactoside Galactohydrolase of Lactic Streptococci: Enzyme Properties and Analysis of Deficient Mutants by Transduction

by

Theodore Arthur Molskness

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1974

APPROVED:

Redacted for Privacy

Professor of Microbiology
in charge of major

Redacted for Privacy

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Date thesis is presented 5/6/74

Typed by Velda D. Mullins for Theodore Arthur Molskness

ACKNOWLEDGEMENTS

Sincere appreciation is expressed to Dr. W. E. Sandine for his helpful suggestions, patience, and understanding throughout my years as a graduate student.

The staff members of the Department of Microbiology and all of my fellow graduate students and friends.

My Mother for her thoughtfulness, love and guidance throughout the years.

My Father for his inspiration.

Elaine for her devotion, love and her constant encouragement.

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INTRODUCTION

Rapid lactic acid production, in addition to the formation of certain flavor compounds, is required of bacteria that are utilized in the manufacture of fermented dairy products. Lactic streptococci find use in this regard because they produce large amounts of lactic acid as a fermentation end product from milk lactose. These organisms therefore have been utilized by man for centuries in the manufacture of cheeses, yogurt, buttermilk and other fermented milk products.

The requirement for rapid lactic acid production has a two-fold purpose. First, lactic acid production in milk is needed to lower the pH to a point where milk caseins will precipitate and form the curd. Secondly, a rapid rate of lactic acid can serve to inhibit the growth of potential pathogenic and spoilage organisms that may find their way into the milk during the production process. (Daly, Sandine and Elliker, 1972)

The mechanism of lactose metabolism in the lactic streptococci has been the subject of much research in this laboratory. These studies have involved lactose transport and hydrolysis and the subsequent conversion of the liberated glucose and galactose

to lactic acid.

The research presented here has been concerned with three aspects of lactose hydrolysis. First, some characteristics of the lactose-hydrolyzing enzyme β-D-phosphogalactoside galactohydrolase (β-Pgal), found in the lactic streptococci (McKay et al., 1971) have been examined; temperature and pH optima as well as ionic requirements were determined. The response of intracellular β-Pgal concentration to various inducers also was studied.

The second area of investigation involved a study of the relationship between the levels of β-Pgal found in the cell, the proteolytic activity of the cell, and the rate of lactic acid production. These studies concerned the relationships between levels of β-Pgal in the cell and the rate of lactic acid production by that cell. The relationship between β-Pgal activity and proteolysis was also investigated.

Finally, β-Pgal was used as a marker in a phage-mediated genetic transfer system for the lactic streptococci. This genetic technique was applied to a comprehensive study of lactose metabolism in the lactic streptococci.

LITERATURE REVIEW

Lactose Hydrolyzing Enzymes in Staphylococcus and Streptococcus

β-Galactosidase, the enzyme that hydrolyzes the disaccharide lactose into free glucose and galactose, has been isolated from a number of bacterial and animal sources. Examples include Escherichia coli (Lederberg, 1950), Aeromonas formicans (Rohlfing and Crawford, 1966), Saccharomyces fragilis (Szabo and Davies, 1964) Bacillus subtilis (Anema, 1964) and B. megaterium (Landman, 1947).

In a survey of a number of lactic streptococci including Streptococcus lactis, S. cremoris and S. diacetilactis, however, the classic β-galactosidase (β-gal) could be isolated from only one organism, S. lactis 7962 (Citti, Sandine and Elliker, 1965; McFeters, Sandine and Elliker, 1967, 1971; McFeters, Sandine and Becker, 1969). At first, the inability of the investigators to isolate β-gal was thought to be due to the extremely labile nature of this enzyme in the other strains examined. Eventually, it was determined that, with the exception of S. lactis 7962, all the other lactic streptococci examined contained an enzyme capable of hydrolyzing only a phosphorylated lactose derivative (McKay, Walter, Sandine and Elliker, 1969; McKay, Miller, Sandine and

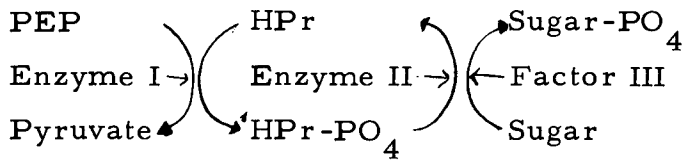
Elliker, 1970). The phosphorylated lactose compound was identified as lactose-6-phosphate with the phosphate group being attached to the sixth position of the galactose moiety. The enzyme responsible for the hydrolysis of this compound was first identified in Staphylococcus aureus and given the name β-D-phosphogalactoside galactohydrolase (Laue and MacDonald, 1968a, 1968b). The production of the β-Pgal substrate (lactose-6-PO_4) is dependent on the action of a phosphoenol pyruvate phosphotransferase system (PTS) first described by Kundig et al. (1964; 1966) in E. coli and later found in both Staphylococcus and Streptococcus organisms. In these genera the PTS is responsible for phosphorylating lactose during its transport into the cell at the expense of phosphoenol pyruvate.

Since the PTS is such an important part of the β-Pgal system in both Staphylococcus and Streptococcus organisms, it is helpful to give a brief description of the system as first described in E. coli. The findings of the studies with E. coli were first applied to S. aureus, in which β-Pgal was discovered, and later to the S. lactis β-Pgal system.

In E. coli, lactose is transported into the cell via an active transport mechanism that requires ATP. During the transport process, lactose combines with an "M" carrier protein and is transported across the cell membrane as an M-lactose complex. Once inside the cell, however, free lactose is liberated along with

an inactive "M" protein which is incapable of combining with more lactose. The "M" protein is converted back to an active carrier only at the expense of ATP (Scarborough et al., 1968; Kennedy and Scarborough, 1967).

A number of carbohydrates, such as glucose, mannose and fructose, were found in E. coli, however, that were transported by a different method. In this transport mechanism a phosphoenol pyruvate-dependent phosphotransferase system was functioning. The net result of this type of transport was the accumulation of phosphorylated derivatives of the carbohydrates being transported. Three papers by Kundig et al. (1964, 1966, 1971) describe the system as follows:



Enzyme I and HPr, (Histidine containing protein) were found to be soluble and constitutive and common to the transport of a number of carbohydrates. Enzyme II (membrane bound) and Factor III (soluble) were found to be carbohydrate specific and inducible. Thus a mutation in Enzyme II, for example, could result in the loss of ability of the cell to ferment one or a few carbohydrates. A mutation in Enzyme I or HPr, however, could cause loss of the ability to ferment a number of carbohydrates. This type of mutation was referred to as a pleiotropic and designated car$^-$. Lactose

metabolism was not affected by car$^-$ mutations in E. coli.

It was in Staphylococcus aureus that the relationship between the phosphotransferase system (PTS), lactose transport and β-Pgal was first demonstrated. S. aureus mutants were isolated that lacked the ability to ferment a number of carbohydrates. These mutants could not ferment mannitol, fructose, galactose, sucrose, lactose, or maltose. In addition, the reversion of a single mutant via transduction to again ferment one carbohydrate, resulted in the ability of the revertant to again ferment all of the carbohydrates it had previously been unable to use (Korman, 1964). This fact, coupled with other data from transduction experiments, indicated that the loss of ability of mutants to ferment all of the above carbohydrates was indeed the result of a single point mutation. Further characterization of these car$^-$ mutants (Egan and Morse, 1965a) indicated that they could still grow on glucose. Thus the glycolytic enzymes were still present. Although the mutants could not ferment sucrose or maltose, the hydrolytic enzymes sucrase and maltase were still present. These results led investigators to believe that a faulty transport mechanism might be involved. Eventually it was determined that the car$^-$ mutants were impermeable to all of the carbohydrates which they could no longer ferment. Uptake of other nutrients was not affected (Egan and Morse, 1965b) and the possibility of a common carrier therefore existed.

Egan and Morse (1966) studied the uptake of carbohydrates in a wild type car$^+$ strain of S. aureus, which was defective in what was then thought to be β-galactosidase. Since this mutant could transport lactose but not hydrolyze the sugar, it was hoped that the nature of the accumulating lactose could be determined. It was shown that lactose as well as sucrose and maltose accumulated as a derivative that no longer chromatographed as the free sugar. Characterization of the lactose derivative revealed a lactose-phosphate compound. This compound was not hydrolyzed by purified E. coli β-galactosidase, but was split by cell-free extracts of S. aureus to yield free glucose and galactose-6-phosphate (Hengstenberg, Egan and Morse, 1967; 1968).

O-nitrophenyl-β-D-galactopyranoside (ONPG), a chromagenic substrate colorless in solution, is hydrolyzed by β-galactosidase to yield free galactose and o-nitrophenyl (ONP) which is yellow in solution. The color development can be measured and the specific activity of β-gal determined. Whole cells of S. aureus were found to hydrolyze ONPG very slowly. Treatment of whole cells with toluene-acetone, which destroys the permeability barrier of the bacterial cell membrane, resulted in the complete loss of ability of S. aureus to hydrolyze ONPG (Egan and Morse, 1965a). It was subsequently discovered that the addition of phosphoenol pyruvate to a suspension of toluene-acetone treated cells restored

the ability of the cells to hydrolyze ONPG. The addition of other high energy compounds did not restore this ability. Sodium fluoride, which inhibits the enolase catalyzed reaction, was found to inhibit ONPG hydrolysis in whole cells of S. aureus, but had no inhibitory effect on toluene-acetone treated cells in the presence of PEP. These results suggested that S. aureus required a supply of PEP in order to hydrolyze lactose. It was at this point that it was suggested that S. aureus possessed a previously unreported enzyme which hydrolyzed ONPG-6-PO_4 instead of ONPG (Hengstenberg and Morse, 1968; Kennedy and Scarborough, 1967). The PEP phosphorylation system previously described in E. coli was thought to be responsible for phosphorylating ONPG. ONPG-6-PO_4 was synthesized and found to be a substrate for the staphylococcus hydrolyzing enzyme but not for the E. coli enzyme (Hengstenberg et al., 1968b). Structures of lactose, ONPG, and ONPG-6-PO_4 are given in Figure 16.

Further studies showed that while IPTG, an inducer of the E. coli β-gal, was not an inducer of the staphylococcal enzyme, galactose-6-phosphate was a potent inducer, inducing 8-fold higher enzyme levels than did galactose. In mutant strains that were of the lac^- gal^- phenotype, galactose did not induce enzyme synthesis while galactose-6-phosphate did. These results indicated that in the lac^- gal^- mutants, a defect in the PTS resulted in the

cells being unable to phosphorylate free galactose and thus the actual inducer of β-Pgal was not produced (Morse et al., 1968).

Lactose utilization in S. aureus can be summarized as follows: lactose in the medium is transported by a PEP dependent phosphotransferase system into the cell. During the transport process, phosphate is transferred from PEP to the sixth position of the galactose moiety of lactose. The lactose-6-phosphate thus formed is hydrolyzed by the enzyme β-Pgal to yield free glucose and galactose-6-phosphate (Simoni and Roseman, 1973).

As previously stated, it was assumed that the lactic streptococci hydrolyzed lactose by an E. coli-like β-galactosidase. Also the method of lactose transport into the cell was thought to be similar to the E. coli system. Attempts to demonstrate and isolate β-gal from a number of lactic streptococci proved fruitless. Only one member of this group of organisms, S. lactis 7962, could be shown to contain β-gal and the enzyme was isolated and characterized in this organism (Citti et al., 1965; McFeters et al., 1967, 1969 and 1971). The reports of investigators studying lactose metabolism in Staphylococcus, however, prompted workers to examine the possibility that the remaining lactic streptococci contained the enzyme β-Pgal and also a transport system for lactose similar to the PTS found in S. aureus.

Results of a study of lactose metabolism in two lactic

streptococci. S. lactis 7962 and S. lactis C2, suggested that indeed these organisms each contained a different mechanism for lactose utilization. S. lactis C2 was representative of the majority of lactic stretococci that did not contain β-gal. McKay et al. (1969) reported that toluene-acetone treated cells of C2 required the addition of PEP in order to continue to hydrolyze ONPG even in the absence of PEP. Also, cell extracts of C2 could only very slowly hydrolyze ONPG while addition of NaF completely inhibited this hydrolysis. Addition of PEP to C2 extracts in the presence of NaF restored the ability of the preparation to slowly hydrolyze ONPG. The ability of cell extracts of 7962 to hydrolyze ONPG was neither inhibited by NaF, nor stimulated by PEP. The most significant finding, however, was that whole cells of C2 accumulated only a phosphorylated derivative of thiomethyl galactoside (TMG) an analogue of lactose, which behaved chromatographically like TMG-6-phosphate.

The findings of this initial study into lactose utilization by the lactic streptococci prompted further investigation. McKay et al. (1970) reported the following additional information. A number of lactic streptococci were examined for their ability to hydrolyze ONPG-6-phosphate, the substrate for the S. aureus enzyme, as well as ONPG. Both cell-free extracts and toluene-acetone treated cells were used. It was found that S. lactis 7962 could

hydrolyze only ONPG, while the remaining organisms rapidly hydrolyzed ONPG-6-PO_4 and could only hydrolyze ONPG at a slow rate when cell free extracts were used. Solvent treatment, while not changing the pattern of hydrolysis observed in 7962, stopped ONPG hydrolysis by the remaining organisms, but did not impair their ability to hydrolyze ONPG-6-PO_4. The results were consistent with findings of Molskness et al. (1973).

Mutants of S. lactis C2 were isolated which were defective in lactose utilization and all possessed the phenotype lac^- gal^-. These mutants were unable to accumulate TMG, hydrolyze ONPG and to ferment lactose or galactose. A series of complimentation experiments were conducted using S. aureus car^- mutants known to be deficient in one or more of the PTS enzymes (Hengstenberg et al., 1969; Morse et al., 1968). It was found that the C2 mutants contained enzyme I and HPr, but lacked enzyme II and factor III of the PTS system as found in S. aureus (McKay, Miller, Sandine and Elliker, 1970). Thus the mutant's inability to utilize lactose was due to a defect in the phosphorylation-transport step. In the same paper it was also shown that S. lactis 7962 was not dependent on a PTS and transported lactose by a system similar to that found in E. coli. Lee et al. (1973) reported that in lactic streptococci, lactose is the only sugar transported by the PTS. While galactose-6-PO_4 has been shown to be a potent inducer of

β-Pgal in S. aureus (Morse et al., 1968), the actual inducer of this enzyme in the lactic streptococci has not been conclusively shown. Recently, studies have been carried out to determine the fate of galactose-6-phosphate in lactic streptococci. Bissett and Anderson (1973) have shown that in S. aureus galactose-6-phosphate is converted to tagatose-1-6-phosphate and then to fructose-1-6-phosphate. They also showed (Bissett and Anderson, 1974) that the pathway is operative in the lactic streptococci.

β-Pgal is not restricted to Staphylococcus and Streptococcus organisms. Recently a survey was undertaken to determine whether β-gal or β-Pgal was the lactose-hydrolyzing enzyme in Lactobacillus organisms. Results of these studies conducted by Premi et al. (1972) indicated that some species of Lactobacillus contain only β-gal while some contained only β-Pgal, or both. β-Pgal was isolated and purified from L. casei and compared with purified β-gal and L. thermophilus. β-Pgal was slightly less active than β-gal, although both enzymes had similar substrate affinities (Km values). The molecular weight of β-Pgal was 1.3×10^5 while β-gal had a molecular weight of 5.4×10^5. β-Pgal has also recently been partially purified from S. cremoris HP (Johnson and McDonald, 1974), and had a molecular weight of 6.7×10^4.

Slow Acid Production by Lactic Streptococci

Slow lactic acid production by varients of S. lactis was first reported by Harriman and Hammer (1931). In this study it was found that approximately 2% of the colonial isolates of fast lactic acid-producers exhibited the slow character. The reverse case of slow acid producers becoming fast was never observed. Also, it was pointed out that the fast strains produced more soluble nitrogen when growing in milk, and the conclusion was that the proteolytic ability of the cell was somehow related to the rate of lactic acid produced by S. lactis.

A number of interesting aspects of slow acid producing variants has been reported. Garvie and Mabbitt (1956) found that while the generation times and total population of a fast culture S. lactis were the same as that found for a slow variant isolated from the fast culture, the rates of lactose utilization and lactic acid formation were substantially less for the slow variant. These results were confirmed by Citti et al. (1965). Garvie (1959) reported that over an extended period of time the slow variants tend to dominate the culture if proper transferring procedures were not maintained.

The relationship between rate of lactic acid production and proteolytic ability of the culture has been the object of much

investigation. The amino acid and peptide composition of milk is low (Deutsch and Samuelsson, 1959). Thus the lactic streptococci must obtain their amino nitrogen from the milk proteins. If an organism lacked the necessary proteolytic enzymes needed to degrade casein, it would be at a disadvantage when growing in milk. It has been reported that organisms that are slow (acid producers) can be stimulated to fast acid production by the addition of certain additives to the milk. Liver fraction L (Anderson and Elliker, 1953), and yeast extract (Storrs and Anderson, 1949) have been identified as stimulatory agents. Peptides are thought to be the main stimulatory agent in these compounds (Sandine, Speck and Aurand, 1956).

The proteolytic enzymes responsible for rapid lactic acid production of S. lactis growing in milk have been studied. The proteinase system of the lactic streptococci has been shown to contain two elements. S. lactis contains both a membrane associated and an intracellular proteinase (Cowman and Speck, 1967; Cowman, Swaisgood and Speck, 1967; and Cowman, Westhoff, Swaisgood and Speck, 1970). The properties of both of these enzymes in normal and slow variants have been examined. Westoff, Cowman and Swaisgood (1971) found that the intracellular enzyme isolated from a normal and slow variant differed in sensitivity to pH. The wild type enzyme was quite active at pH's

below 6.0, while the slow variant's enzyme was most active at pH's above 6.0. In addition, it was found that the substrate specificity of the intracellular proteinase isolated from the wild type and slow variant differed greatly (Westhoff and Cowman, 1971). In examining the properties of the membrane associated proteinase from the wild type and the slow variant, one important difference was found. Although the physical parameters of the wild type and mutant membrane bound proteinase were similar, the wild type enzyme was found to be more active on milk casein (Westhoff, Cowman, and Speck, 1971). It was proposed then that a mutation in either the intracellular or membrane bound proteinase could account for the slow acid production when the organism was growing in milk. Also such a mutation would limit the organism's ability to obtain nitrogen from milk protein.

Recently it was reported that the genes responsible for proteases in lactic streptococci may be carried on a plasmid. Pearce and Skipper (1971) showed that DNA with a lower average base composition than the chromosomal DNA is present in proteinase positive cells and absent in proteinase negative cells. This would be similar to the proteinase system found in E. coli (Taylor and Trotter, 1972) and Salmonella typhimurium (Sanderson, 1972). Recently Cords et al. (1974) published electronmicrographs of plasmids from group N streptococci.

Transduction in Lactic Streptococci

Bacterial transduction is the process by which a temperate bacteriophage transmits a portion of bacterial chromosome of its host to a recipient bacterium where it may or may not be incorporated into the chromosome (Hays, 1968). Although this phenomenon has been observed in the group A streptococci, which includes S. pyogenes, (Malke and Kohler, 1973; Malke, 1973) very little research has been directed toward transduction in the lactic streptococci (group N) until recently.

The occurrence of lysogenic lactic streptococci, those containing a temperate bacteriophage, has been known for some time (Reiter, 1949). Kozak et al. (1973) tested S. lactis, S. cremois and S. diacetilactis strains for lysogeny and found that only in S. lactis and S. diacetilactis strains could lysogeny be demonstrated. No attempts, however, were made in these studies to utilize the released bacteriophage in transduction. Recently, Lowry (1974) induced the release of temperate phages from S. cremois using UV light or mitomycin C.

The only reported instance of transduction among the lactic streptococci until recently was that of Allen et al. (1965) and Sandine et al. (1962). In these studies, transduction of streptomycin resistance in S. lactis C2 and tryptophan independence in

S. diacetilactis 18-16 was noted. In these experiments, however, the transduction event was brought about by virulent rather than lysogenic bacteriophages.

In a recent paper by McKay, Cords and Baldwin (1973), transduction of the lactose fermentation character in S. lactis C2 was described. The phage used in this transduction were obtained by UV induction of a lysogenic strain of S. lactis C2 (McKay and Baldwin, 1973). This phage was characterized and examined via the electron microscope. It was found that this phage differed morphologically from any lactic phage previously described even in the comprehensive studies by Parmelee et al. (1948) and Bauer et al. (1970). The lac^- mutants that served as recipients in these transduction experiments were obtained by treating S. lactis C2 cells with acriflavin (McKay, Baldwin and Zottola, 1972). Acriflavin treatment greatly increased the occurrence of the lac^- phenotype. Since acriflavin causes mutations by eliminating extra-chromosomal DNA from bacterial cells, it would appear that the lactose-fermenting character is carried on an plasmid-like particle.

MATERIALS AND METHODS

Microorganisms and Media

All of the lactic streptococci used in this study were obtained from the stock culture collection maintained by the Department of Microbiology, Oregon State University. The cultures were regularly maintained in sterile 11 percent nonfat milk. Lactic broth (Elliker, Anderson and Hannessen, 1956) without Tween 80 and with lactose as the sole carbohydrate served as the growth medium for the routine assay of β-Pgal. The survey of organisms, and determination of optimal assay conditions were carried out in this broth. For induction experiments, lactic broth was prepared with a reduced level of yeast extract (1 g/l) and without carbohydrate. After sterilization of the medium, a filter-sterilized solution of the carbohydrate to be tested was added to a final concentration ranging from 0.001-0.005 g/ml. In all experiments involving broth, the organism was transferred 3 times over 36 hr at 32 C in the appropriate broth before beginning the experiment.

In the studies of acid production and proteolysis, cells were grown in 11 percent nonfat milk which had been steamed for 30 minutes or autoclaved for 15 minutes and cooled. Additives, when present, were added to the milk before steaming to a final concentration of 0.25 percent. The following additives were used:

Similac (enzymatic digest of pancreas) Marschall Dairy Laboratory Inc., P. O. Box 592, Madison, Wisconsin, 53701; N-Z Amine Type A (enzymatic digest of casein) and Edamine Type S (enzymatic digest of lactalbumen), both obtained from Sheffield Chemical Co., Norwich, New York. For some studies on growth of lac^- mutants in milk, glucose was added to the milk at a concentration of 1 percent before heat treatment.

Buffer Solutions

Cells were regularly washed, suspended, and assayed in 0.05 M sodium phosphate buffer at pH 7.0. In determining the optimal assay conditions for β-Pgal, 0.05 M potassium phosphate and 0.05 M Tris - HCl at pH 7.0 were also employed.

Preparation of Cell-free Extracts

An 18-24 hr culture was used to inoculate the appropriate broth to the 1 percent level. Cells were grown for 6-7 hr at 32 C at which time they were centrifuged at 3,000 x g for 10 min at 2 C. The pellet was washed twice with the cold phosphate buffer and finally resuspended in 30 ml of buffer. This cell suspension was sonicated in a Raytheon Sonic Ocillator for 20 min at maximum settings. This mixture was then centrifuged at 17,500 x g for 15 min at 2 C. The clear supernatant which contained from 0.5 - 1.8

mg/ml total protein, was assayed for enzyme activity.

Preparation of Toluene-Acetone Treated Cells

Toluene:acetone (1:9) - treated cells were normally used when small numbers of cells were being assayed for enzyme activity. Cells were harvested, washed as above and resuspended to a known optical density (420 nm) and expressed in terms of mg cell dry weight. This insured that a standard amount of cells would be solvent-treated throughout the experiment. The procedure of Citti et al. (1965) was followed when treating the cells with toluene:acetone, but with cells being shaken vigorously for 10 min, instead of 5 min, after addition of the solvent. The resultant suspension was assayed for enzyme activity.

β-Galactosidase and β-Phosphogalactosidase Assays

ONPG and ONPG-6-PO_4 (Fig. 16) were obtained from Sigma Chemical Co. A modification of the procedures for enzyme assays used by Citti et al. (1965) and McKay et al. (1969) was used. A solution containing either 5.0×10^{-3} M ONPG or ONPG-6-PO_4 was prepared in the phosphate buffer. The assay mixture contained 0.5 ml of either the cell-free extract or toluene:acetone-treated cells and 2.0 ml of the chromogenic substrate. Incubation proceeded at 37 C for 1-2 min in the case of the cell-free assay, and 10 min in

the case of solvent-treated cells. The reaction was stopped by the addition of 2.5 ml of 0.5 M sodium carbonate. The release of ONP was measured colorimetrically at 420 nm and mg of ONP released was determined from a standard curve. When using the toluene:acetone assay, cells were removed by centrifugation before absorbancy was measured.

One unit of enzyme was equivalent to 1 μmole of ONP liberated from ONPG or ONPG-6-PO_4 per minute in the case of cell-free extracts, and 1 μmole of ONP liberated from ONPG or ONPG-6-PO_4 per 10 min in the case of the solvent-treated assay. Specific activity was expressed as units/mg protein (Lowry et al., 1951), and units/mg cell dry wt, respectively.

Induction of β-Pgal

The broth with the reduced level of yeast extract (1 gm/l), containing the filter sterilized carbohydrate, was used when examining the inducing ability of a number of carbohydrates. Glucose, mannose, lactose, and galactose were examined along with methyl-β-D-thiogalactopyranoside (TMG), isopropyl-β-D-thiogalactopyranoside (IPTG) and o-nitrophenyl-β-D-galactopyranoside (ONPG). The procedure of Citti et al. (1965) was used with the following modifications. Cells of S. cremoris HP were grown for either 3 hr or 12 hr in 300 ml of the low level (1 g/l) yeast extract

broth containing 0.002 gm/ml mannose. At the appropriate time, cells were harvested, washed once and resuspended in 4-6 ml of the sterile phosphate buffer. This suspension served as inoculum for a series of flasks containing 50 ml of the low level yeast extract broth with the appropriate carbohydrate to be tested. Flasks were incubated for 5 hr at 32 C, with 10 ml aliquots being removed at hourly intervals and prepared for the toluene:acetone enzyme assay as previously described. The point at which the cells were harvested and shifted to the 50 ml of broth was designated as time zero. In some experiments, cells grown either 3 hr or 12 hr in 300 ml of broth containing 0.002 gm/ml lactose or galactose served as the washed and resuspended inoculum of time zero. In experiments where the effect of chloramphenicol on β-Pgal induction was studied, the filter sterilized antibiotic was added at a concentration of 20 μg/ml at the indicated time.

Procedures for Studies of Cells Grown in Milk

In experiments designed to relate β-Pgal activity with lactic acid production, the following protacol was used: a 1 percent inoculum from 18-24 hr milk culture was grown in 200 ml of 11 percent NFM for 13 hr at 32 C. The milk had been previously steamed for 30 min. Cells were then harvested by the method

described by Stadhouders et al. (1969). The pellet was washed 3 times with the phosphate buffer and assayed for β-Pgal activity by the cell-free extract method described above. Lactic acid production expressed as titratable acidity, was determined by titrating a 50-ml aliquot of the 13-hr culture to the phenolphthalein endpoint (pH 8.3) using a Fisher Automatic Titrimeter. Results were expressed as percent titratable acidity. The method of Hull (1947), was used to determine the degree of proteolysis that had occurred in the 13-hr culture. Results were expressed as μg tyrosine/ml of milk. S. lactis C2 was defined as a rapid lactic acid producer, producing greater than 0.7 percent titratable acidity after 13 hr in milk at 32 C; S. cremoris 175 was defined as a slow acid producer, yielding less than 0.2 percent acid under the same conditions.

In experiments designed to measure the lactic acid production of lac^- mutants and lac^+ transformants when growing in milk, the following procedure was used. 18 hr cultures of the organism to be tested were inoculated into 50 ml of 11 percent milk and incubated at 31 C for 6 hr. Samples (10 ml) were taken at 1 hr intervals and pH was determined with a Corning model 12 pH meter. Attempts were made to use a standard number of cells for the initial inoculation where possible.

Isolation of Lactose Negative Mutants

Cultures of S. lactis C2 and 7962 were exposed to acriflavin using a modified procedure of McKay et al. (1972). One drop of a 12 hr old culture of cells grown on lactose only broth was added to fresh lactose only broth containing 0, 1.0, 3.0, 6.0 μg of acriflavin per ml. The acriflavin had been filter sterilized separately. The cultures were incubated at either 21 C or 31 C for 24 hr. After this period, 1-ml samples were removed, and the appropriate dilution spread on indicator plates, incubated for 24-48 hr and examined for the appearance of lactose negative mutants. Two types of indicator plates were employed. Lactic agar with lactose as the sole carbohydrate and 0.004 percent bromcresol purple as the indicator was used. On this agar, lac^+ colonies appeared yellow, while lac^- colonies appeared white. The second indicator medium was a modification of an agar developed by Morse and Alire (1958) referred to as L agar. It consisted of tryptone 20.0g, yeast extract 5.0g, gelatin 2.5g, lactose 10.0g, sodium chloride 4.0g, sodium acetate 1.5g, ascorbic acid 0.5g, tris buffer pH 7-9 1.3g, dihydrocholic acid 1.5g, neutral red 0.075 g, agar 15.0g and water to 1 l. The pH was 7.2. Lac^+ colonies appeared red on this media, while lac^- mutatants were white or clear. Suspected lac^- mutant colonies were picked, inoculated into glucose broth and incubated

at 31 C for 24 hr. These cultures were then checked for their ability to grow in lactose only broth and assayed for β-Pgal activity.

Induction of Prophage in S. lactis C2

Cells of S. lactis C2, 7962 and S. cremois HP were subjected to UV irradiation using a modified procedure of McKay and Baldwin (1973). Cells were grown in lactose only lactic broth at 32 C for 10 hr. The cells were then centrifuged and the pellet resuspended in 0.85 percent saline so that a 1:20 dilution gave an OD of 0.05 at 650 nm. An aliquot (10 ml) of this suspension was placed in a sterile petri dish and irradiated for 25 sec. with a 15W G.E. UV lamp placed 38.5 cm away. After irradiation, 1 ml of this suspension was placed in 9 ml of modified lactic broth (Henning, 1967). An unirradiated suspension served as the control. All samples were incubated at 32 C and turbity followed at 30 min intervals at 650 nm. The irradiation step and initial incubation period after irradiation were carried out in the dark. Lysis was indicated by a drop in OD of the irradiated culture during incubation in Henning's broth.

Transduction

The lysate obtained from UV induction described above was filter sterilized through a 0.45 μ filter. The recipient cells were lac^- mutants obtained from the acriflavin treatment already described. The mutant was grown approximately 4 hr in glucose only lactic broth. The cells were then harvested and resuspended in 2 ml of lactic broth containing no sugar but with 5.0×10^{-3} M calcium carbonate added. This suspension contained approximately 6.8×10^8 CFU's. The lysate was mixed in a 1:1 ratio with the recipient and incubated at 31 C. Samples were removed at intervals and spread on lactose only lactic agar with 0.004 percent bromcresol purple added. Plates were incubated at 31 C for 24-48 hr and examined for the appearance of lac^+ transductants. Possible lac^+ colonies were selected from these plates and examined in further detail.

Characterization of Lac^+ Transductants and Lac^- Mutants

All transductants and mutants were examined for drug resistance patterns, carbohydrate fermentation patterns, β-gal and β-Pgal activity, lactic acid production in lactic broth and milk, and proteolytic activity in milk. Drug resistance patterns were examined by using Difco Dispens-o-disks containing the antibiotic to

be tested. Streptomycin, ampicillan, carbenicillin, gentamicin, kanamycin, chloromycitin, tetracycline and furadantin were the antibiotics employed. The culture to be tested consisted of log phase cells suspended in overlay agar and spread on glucose only lactic agar plates. The disks were applied to the overlay agar after spreading.

Fermentation patterns were determined by using the API System-Lactobacillus, Analytab Products, Inc., New York 10022. More specific patterns were determined by utilizing lactic broth with the appropriate sugar when appropriate.

β-gal and β-Pgal activity were determined as previously described as were lactic acid production and proteolytic activity.

RESULTS

Enzyme Activity of Lactose-Grown Cells

The specific activity of both β-Pgal and β-gal in cell-free extracts and toluene:acetone-treated cells is given in Table 1. β-gal activity was detected in only one lactic strain, S. lactic 7962. This organism also exhibited a low level of β-Pgal activity, which could be the result of a low level of ONPG present in the ONPG-6-PO_4. The rest of the organisms examined contained varying levels of β-Pgal, but essentially no β-gal could be detected by the assay used.

Optimal Enzyme Assay Conditions

Toluene:acetone-treated cells of a 7-hr old culture of S. cremoris HP were used to determine optimal temperature and pH conditions as well as buffer requirements for the assay of β-Pgal. Figure 1 shows the effect on enzyme activity of different assay incubation temperatures. Specific activity of β-Pgal increased with increasing temperature up to about 37 C and decreased sharply at temperatures above 37 C. Figure 2 shows the response of enzyme activity to the pH of the assay solution. Optimal pH was about 7.0 with a sharp decrease above and below pH 7.0. The

Table 1. β-galactosidase and β-D-phosphogalactoside galactohydrolase activities found in lactic streptococci as measured using the substrates ONPG and ONPG-6-P, respectively.

Organism	Specific activity x 10^3			
	Cell-free		Solvent treated cells	
	ONPG-6-P	ONPG	ONPG-6-P	ONPG
Streptococcus lactis				
C 2	367	<1.0	300	<1.0
UN	563	<1.0	-	-
7962	14	259	132	216
Streptococcus cremoris				
HP	263	<1.0	450	<1.0
163	256	<1.0	-	-
459	242	<1.0	-	-
Streptococcus diacetilactis				
18-16	120	<1.0	443	<1.0
DRC-1	151	<1.0	-	-
3D-1	135	<1.0	-	-

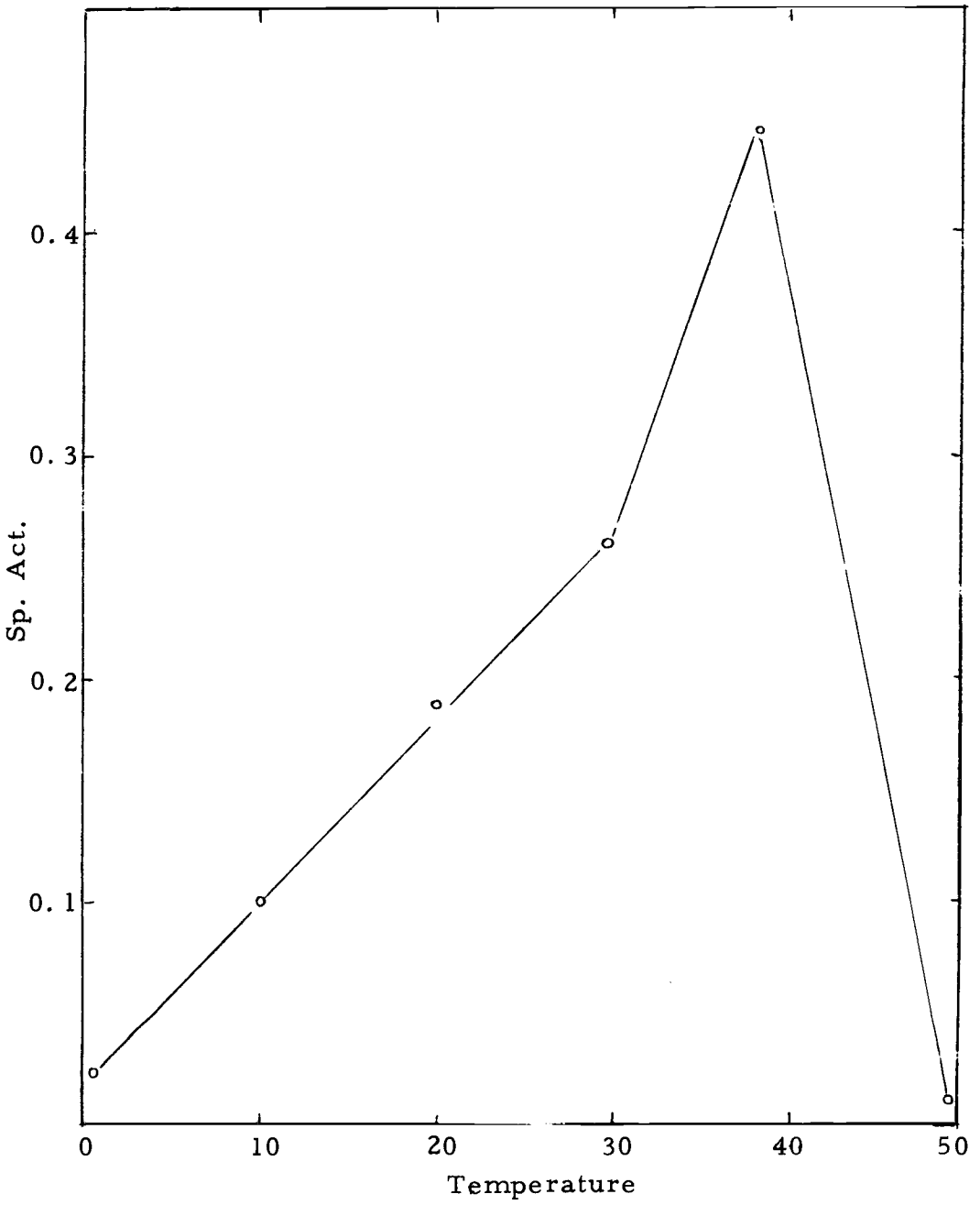


Figure 1. Effect of temperature on specific activity of β-Pgal in toluene acetone-treated cells of S. cremoris HP.

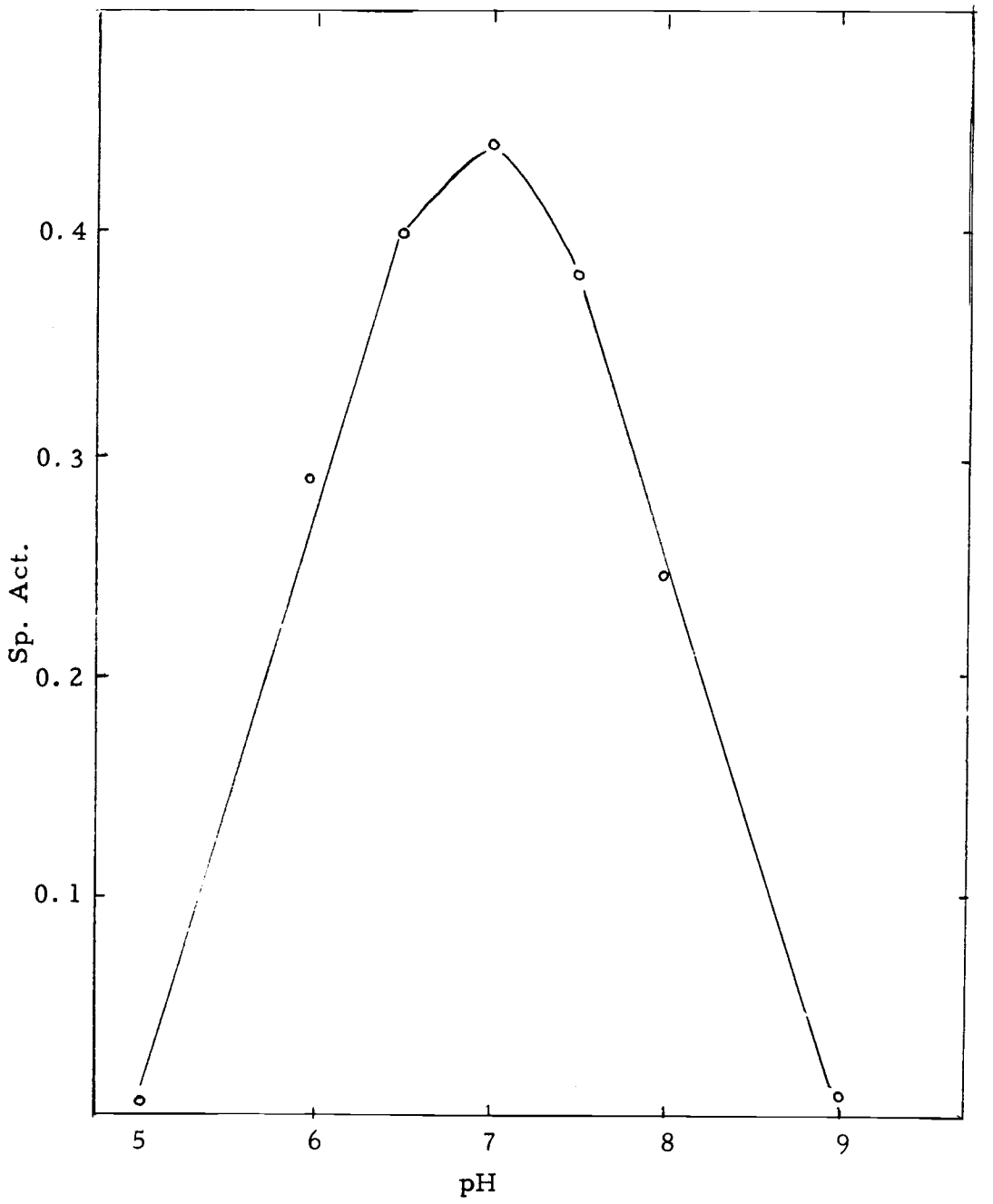


Figure 2. Effect of pH on the specific activity of β-Pgal in toluene acetone-treated cells of S. cremoris HP. Sodium phosphate (0.05M) served as the buffer.

effect of different buffer systems on the activity of β-Pgal is shown in Table 2. Sodium phosphate 0.05 M yielded the highest activity, which was expressed as 100 percent. Potassium phosphate (0.05 M) and 0.05 M Tris (hydroxymethyl aminomethane) buffer gave 84 and 46 percent of the maximal activity, respectively. The addition of 0.05 M NaCl to the Tris buffer had little effect on increasing β-Pgal activity while addition of 0.05 M sodium phosphate to the Tris system, increased β-Pgal activity approximately two-fold.

Enzyme Induction

Figure 3 shows the response of β-Pgal when a 3-hr old culture of S. cremoris HP was shifted at time zero to broth containing either glucose, mannose, lactose or galactose (0.002 g/ml). Doubling time of the organism was approximately 60 min in glucose, mannose, and lactose broth, and 120 min in galactose broth. The initial rate of β-Pgal synthesis was maximal in cells shifted to lactose. The highest level of enzyme activity reached, however, was not maintained but decreased with time. The rate of β-Pgal induction of galactose-grown cells lagged behind that of lactose-grown cells but eventually reached approximately the same level. The maximal level of enzyme attained in galactose-grown cells also started to decline with the 6-hr sample (not shown on the figure). Cells shifted to either mannose

Table 2. Effect of various buffer systems on enzyme activity.

Assay buffer solution	% β-Pgal activity
0.05 M sodium phosphate	100
0.05 M potassium phosphate	84
0.05 M. Tris (hydroxymethyl aminomethane)	36
0.05 M Tris + 0.05 M NaCl	37
0.05 M Tris + .05 M sodium phosphate	77

All assays were performed at 37°C with pH 7.0 with toluene-acetone treated cells of a 10hr lactose induced culture of Streptococcus cremoris HP.

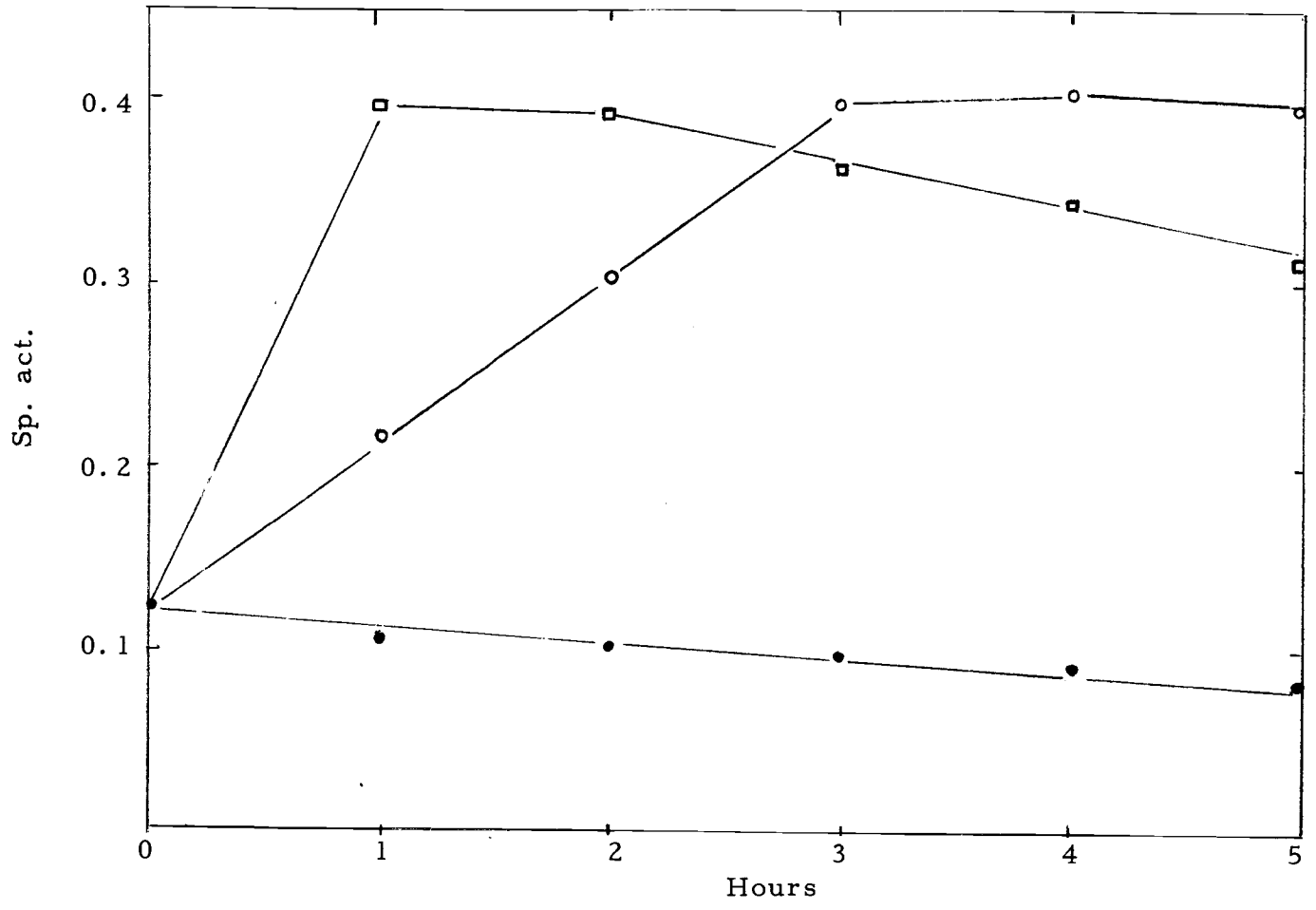


Figure 3. Effect of shifting 3-h-old mannose-grown cells to 0.002g/ml of either glucose or mannose (●), galactose (○), or lactose (□) on the specific activity of β-Pgal in solvent-treated cells of S. cremoris HP.

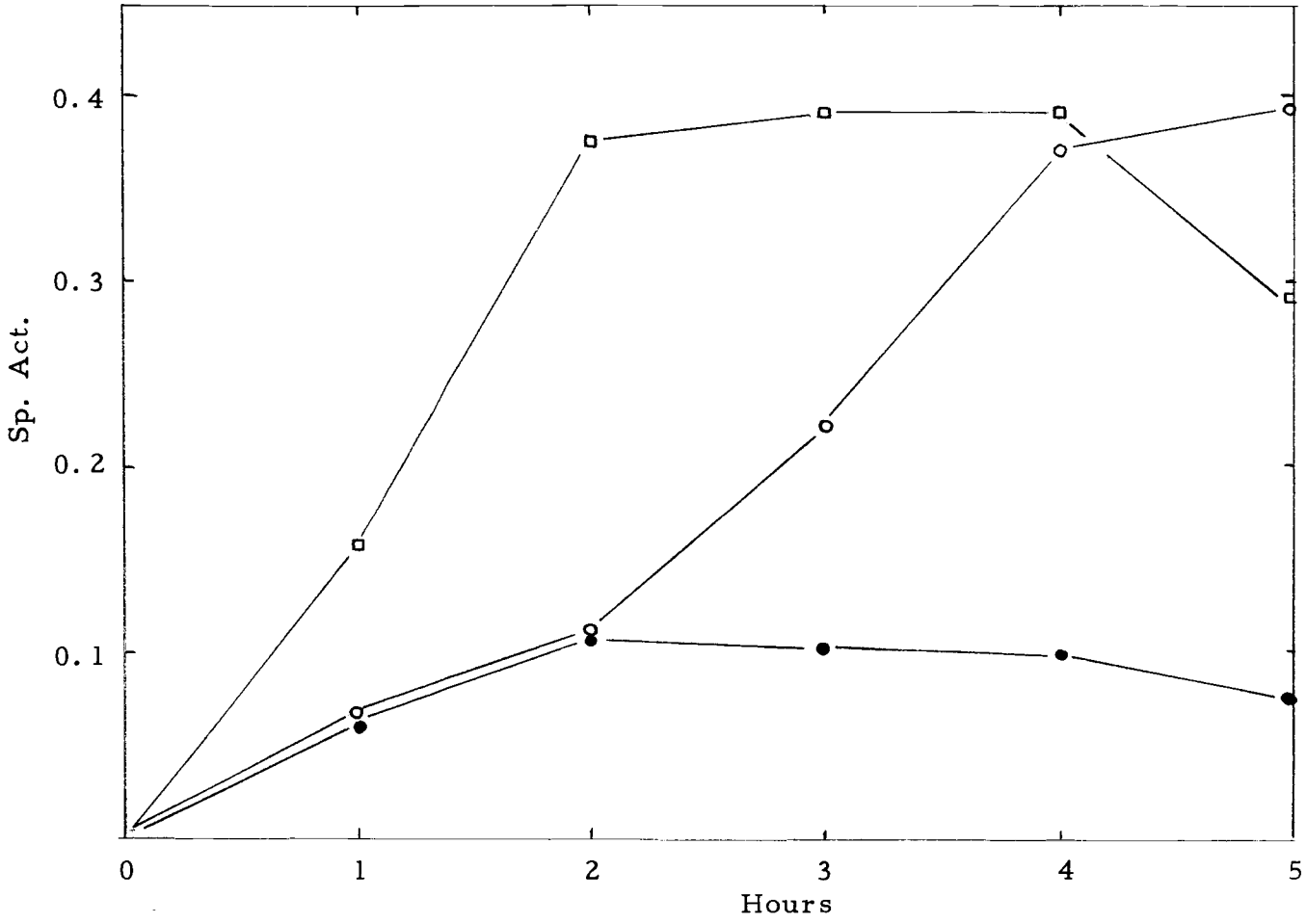


Figure 4. Effect of shifting 12-h-old mannose-grown cells to 0.002 g/ml of either mannose (●), galactose (○) or lactose (□) on the specific activity of β-Pgal in solvent-treated cells of S. cremoris HP.

or glucose did not show any ability to induce enzyme, and gave similar curves.

The relatively high level of enzyme activity of the 3-hr old mannose-grown cells when shifted at time zero, was thought to possibly be interfering with the true nature of β-Pgal induction during the first few hours after shift. Since this basal level did decrease with time in cells shifted to glucose or mannose, it was decided to follow the induction pattern in cells shifted after 12-hr instead of 3 hr.

Figure 4 indicates the response in β-Pgal levels when 12-hr old cells were shifted at time zero from mannose to glucose, mannose, lactose, or galactose (0.002 g/ml). The initial level at time zero of β-Pgal was now observed to be significantly lower than that observed in the 3-hr shift cells (Fig. 3). Again, lactose induced at a maximal rate and the highest level of enzyme reached was not maintained. Induction of β-Pgal, when cells were shifted to galactose, could not be distinguished from an increase that also occurred in cells shifted to either mannose or glucose during the first two hours of sampling after shifting. This increase was reflected in the relatively high initial (time zero) enzyme level found in Figure 3 of cells shifted at 3-hr. The maximal level of enzyme obtained with the galactose-grown cells again decreased at times greater than 6 hr. The initial increase in

enzyme level exhibited by cells grown on carbohydrates presumed to be non-inducers was also observed when 12-hr mannose-grown cells were shifted to broth containing no carbohydrate, TMG, IPTG, or ONPG, although no cell growth was observed. This type of response was taken to be the normal basal levels of β-Pgal in cells grown in a non-inducing medium.

Figure 5 shows the influences on β-Pgal levels in 12-hr cells shifted from galactose to lactose or galactose. The pattern of enzyme induction shown in the figure was the same if the growth carbon source, before shift, was lactose or galactose in that the time zero level was the same for both carbohydrates. Cells shifted to lactose started to induce β-Pgal with little, if any, lag period. Cells shifted to galactose did not show any increase in enzyme levels during the first 2 hours after shift. The maximum level of enzyme found in the cells grown in non-inducing carbohydrates was never observed to be higher than the lowest level of enzyme found when the cells were grown in lactose or galactose. Enzyme levels of cells grown on lactose or galactose fluctuated between specific activities ranging from 0.16-0.40 over a 12-hr period while specific activity of β-Pgal in glucose or mannose grown cells ranged from 0.01-0.12 over the same period (Fig. 5).

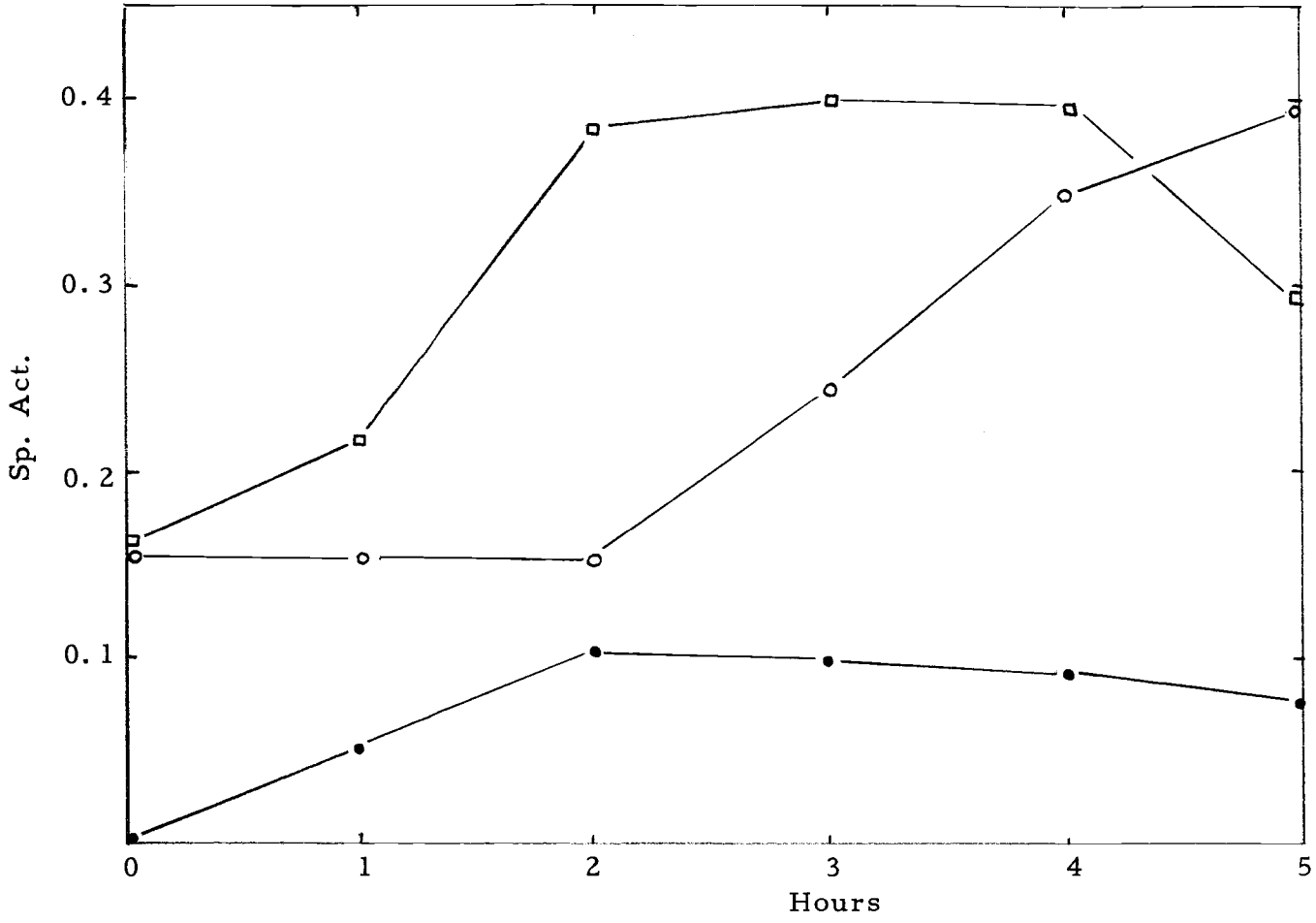


Figure 5. Effect of shifting 12-h-old galactose-grown cells to 0.002g/ml of either galactose (○) or lactose (□) on the specific activity of β-Pgal in solvent-treated cells of S. cremoris HP. Mannose to glucose control (●) is also shown.

The rate of induction of β-Pgal, when 12-hr mannose-grown cells were shifted to lactose, was independent of the concentration of the sugar (0.002 g/ml - 0.005 g/ml.) and yielded essentially the same curve as seen in Figure 6. Increasing concentrations of galactose over the same range, while not changing the induction pattern over the first 2 hr after shift, increased the rate of enzyme induction and the maximal level of enzyme activity attained during the next few hours of growth (Fig. 6).

Figure 7 shows the response of β-Pgal levels when 12-hr mannose-grown cells were shifted into broth containing galactose or lactose (0.005 gm/ml) and also containing 0.001 gm/ml of glucose. Under these conditions, the inducing ability of galactose was almost totally inhibited, while the inducing ability of lactose was only partially inhibited, when compared with the normal induction curves shown in Figure 4. Figure 8 plots the response of β-Pgal levels when 12-hr mannose-grown cells were shifted to broth containing galactose or lactose 0.005 gm/ml. Glucose (0.001 gm/ml) was added at the time indicated by the arrow. Glucose added at 1-hr to cells growing on lactose did decrease the inducing ability of lactose somewhat. Glucose added at 2-hr to galactose-growing cells almost totally inhibited the inducing ability of galactose.

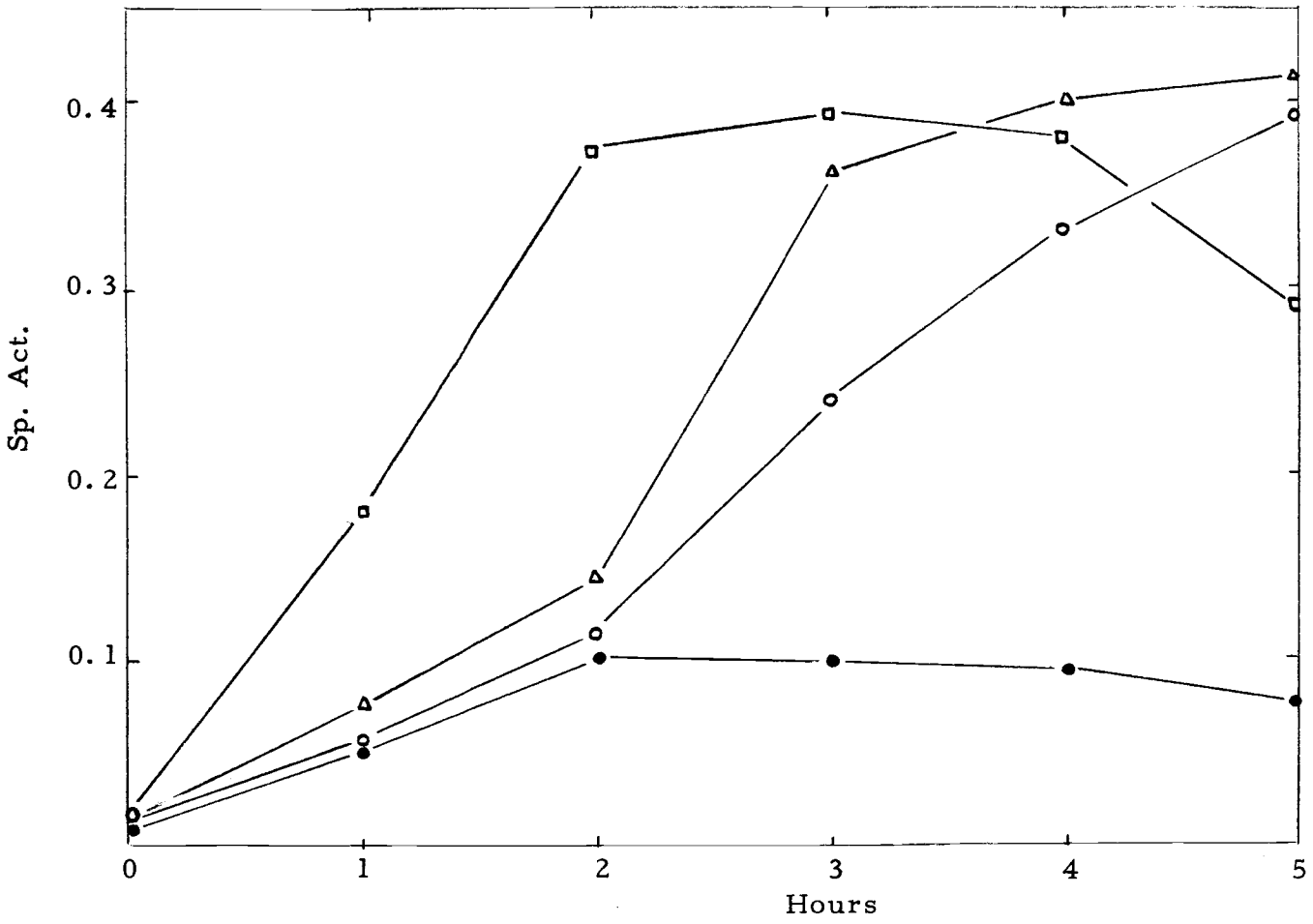


Figure 6. Effect of shifting 12-h-old mannose-grown cells to either glucose (●) 0.002g/ml (○) or 0.005g/ml (△) galactose, and 0.002 to 0.005g/ml lactose (□) on the specific activity of β-Pgal in solvent-treated cells of S. cremoris HP.

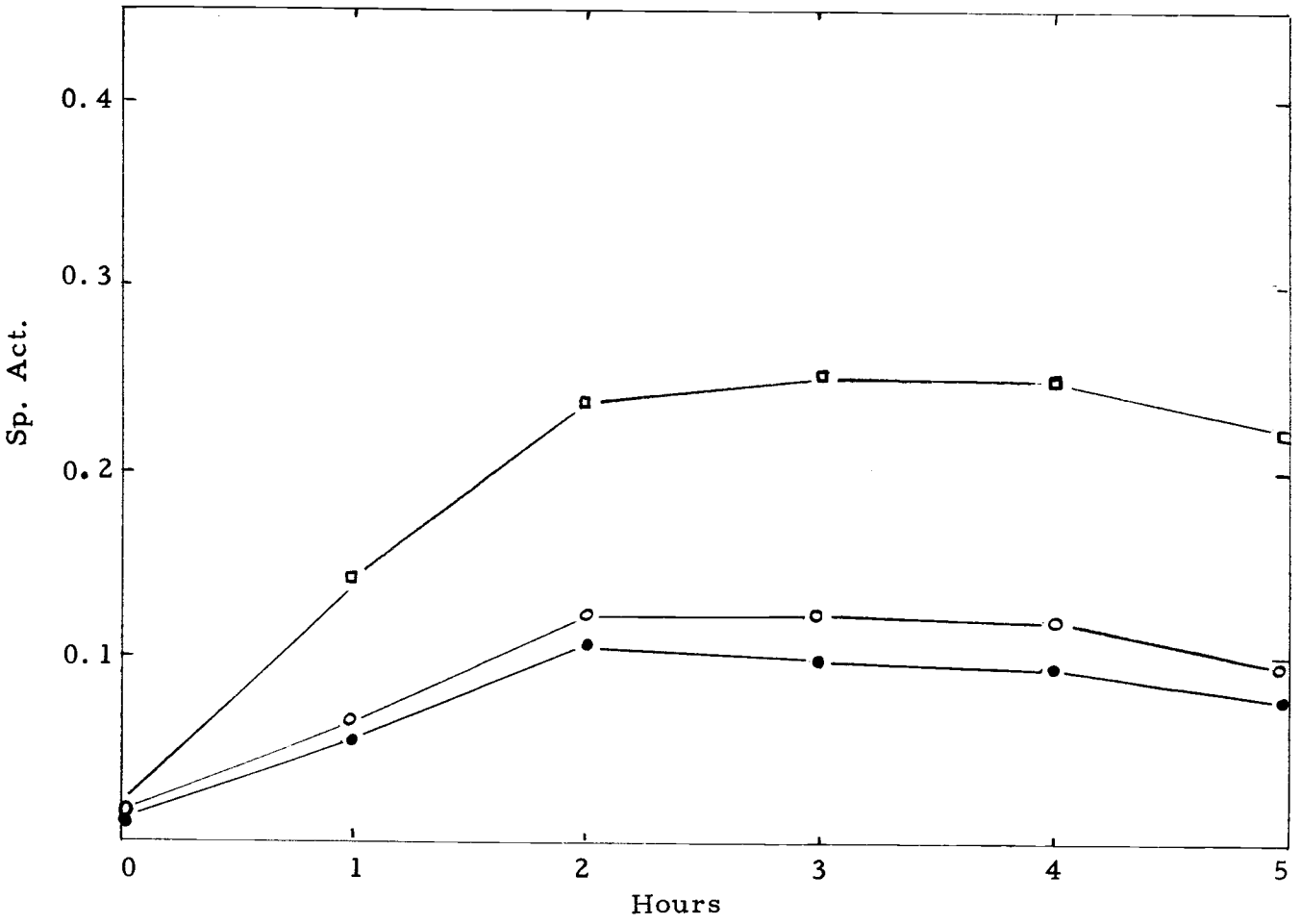


Figure 7. Effect of shifting mannose-grown cells to glucose (●), glucose 0.001g/ml and lactose (□) 0.002g/ml, or glucose 0.001g/ml and galactose 0.002g/ml (○) on specific activity of β-Pgal in solvent-treated cells of S. cremoris HP.

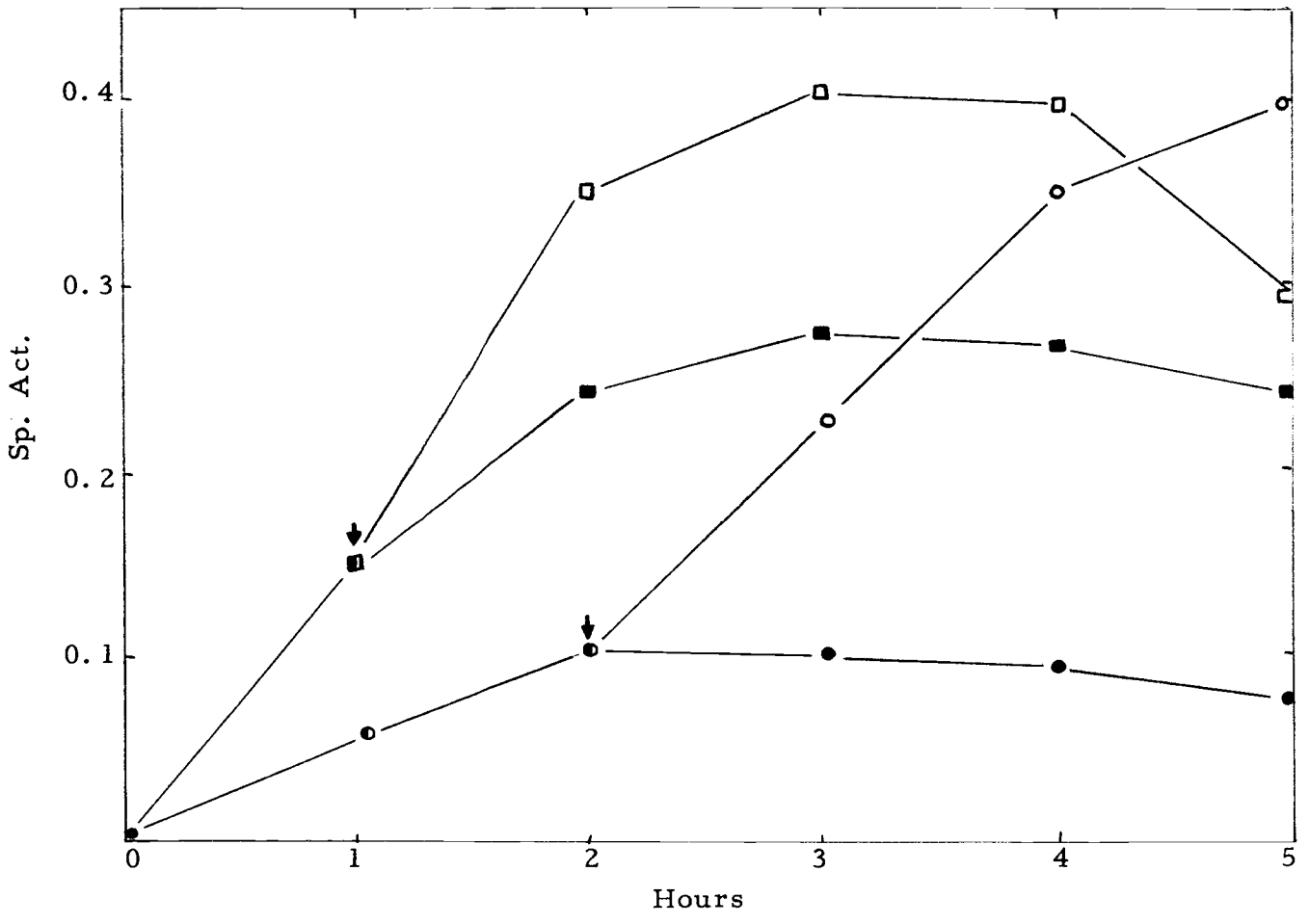


Figure 8. Effect on specific activity of β-Pgal when glucose 0.001g/ml is added ↓ to cells of S.cremoris HP growing in lactose 0.002g/ml (□, ■) or galactose (○, ●).

When the relationship between induction of β-Pgal was plotted against lactic acid production in cells transferred from mannose to lactose broth, the following results were obtained. (Fig. 9) Lactic acid production lagged for the first two hours after shift, or until β-Pgal levels reached a high level. Maximal enzyme induction occurred during the lactic acid production lag period. Results were similar in cells transferred from lactose only broth to fresh lactose broth at time zero. This lag in acid production was not as long when cells were inoculated into glucose broth.

In order to determine whether the increase in β-Pgal levels in cells grown in a "non-inducing" carbohydrate (glucose or mannose) represented an actual synthesis of new enzyme, or an activation of already existing inactive basal enzyme, two approaches were used. Cells of S. cremoris HP were grown in both lactose and glucose broth for four hours at 31 C. Cell free extracts were then made of both cultures. Cell free extracts of lactose-grown cells were mixed with cell free extracts of the glucose-grown cells and incubated at 31 C for various periods of time. β-Pgal activity was then examined in these mixtures to determine if the glucose-grown cell-free extracts had any inhibitory effect on the normally high level of β-Pgal activity in cell-free extracts of lactose-grown cells. No inhibition was observed when the lactose cell-free extracts were

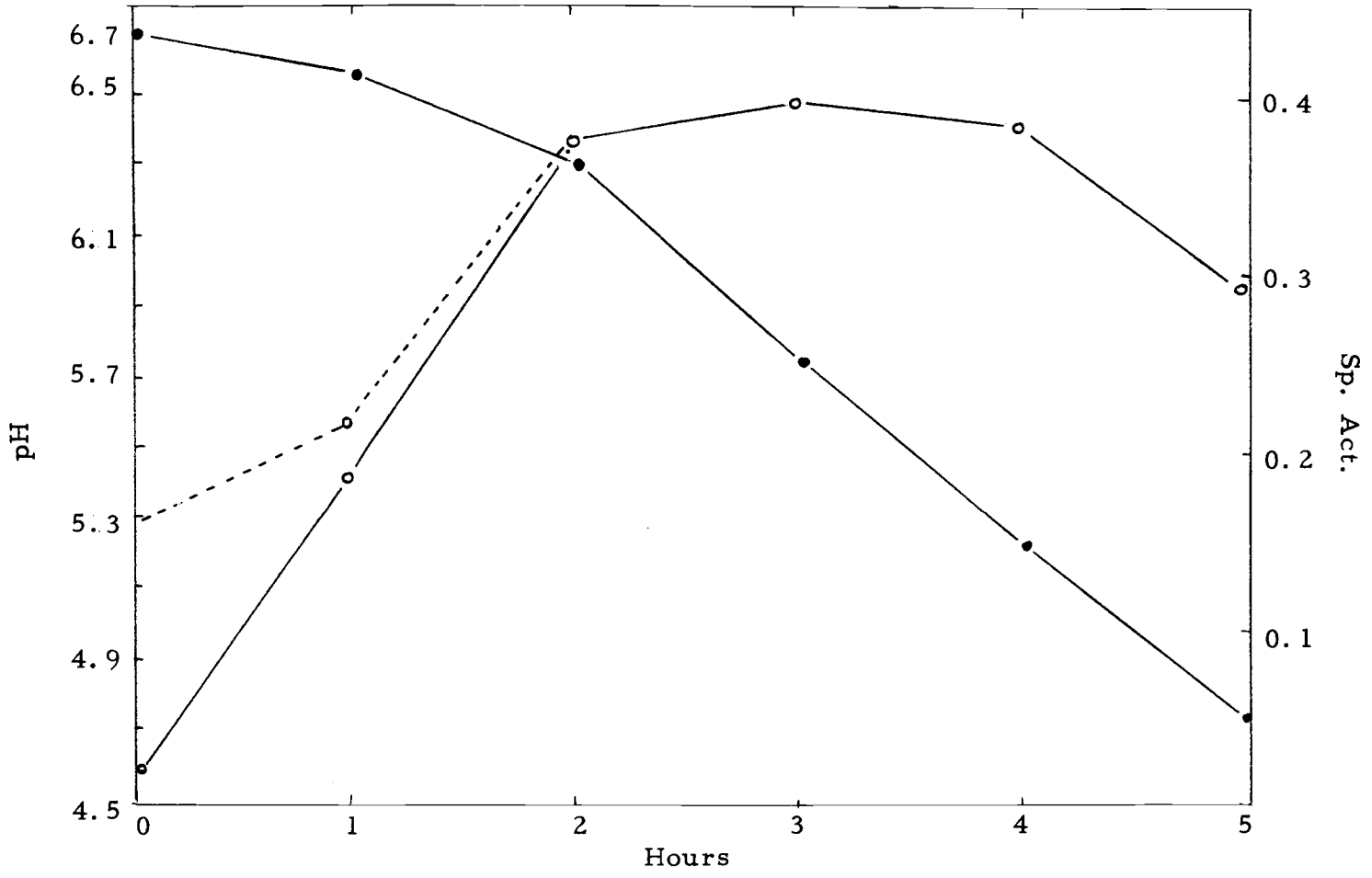


Figure 9. Lactic acid production (●) compared with β-Pgal induction (o) of cells of S. cremoris HP growing on lactose. Cells pregrown in mannose (——) and pregrown in lactose (---).

mixed with extracts prepared from glucose-grown cells, even when the glucose extract was present in a ten-fold excess.

The effect of chloramphenicol on β-Pgal induction was examined using the induction methods previously described. Figure 10 shows the effect of 20 μg of chloramphenicol added at time zero of the induction experiment. Chloramphenicol completely stopped the induction of β-Pgal in cells growing on lactose and galactose, and also stopped the basal increase observed in glucose or mannose growing cells. When chloramphenicol was added to cells where induction of β-Pgal was already underway, further induction was stopped after the point of addition of the drug. (Fig. 11)

Enzyme Activity of Cells Grown in Milk

β-Pgal activity, proteolysis and titratable acidity were examined in a fast lactic acid-producer, S. lactis C2, and a slow lactic acid-producer, S. cremoris 175. The results are given in Table 3. In the case of S. lactis C2, the amount of lactic acid produced, and the specific activity of β-Pgal, in plain milk, did not change significantly when protein hydrolysates were added to the milk. There also was a considerable amount of tyrosine liberated under all conditions, due to the proteolytic activity of the culture. The slow lactic acid producer S. cremoris 175, exhibited a different pattern. In plain milk, very little acid was produced. Upon

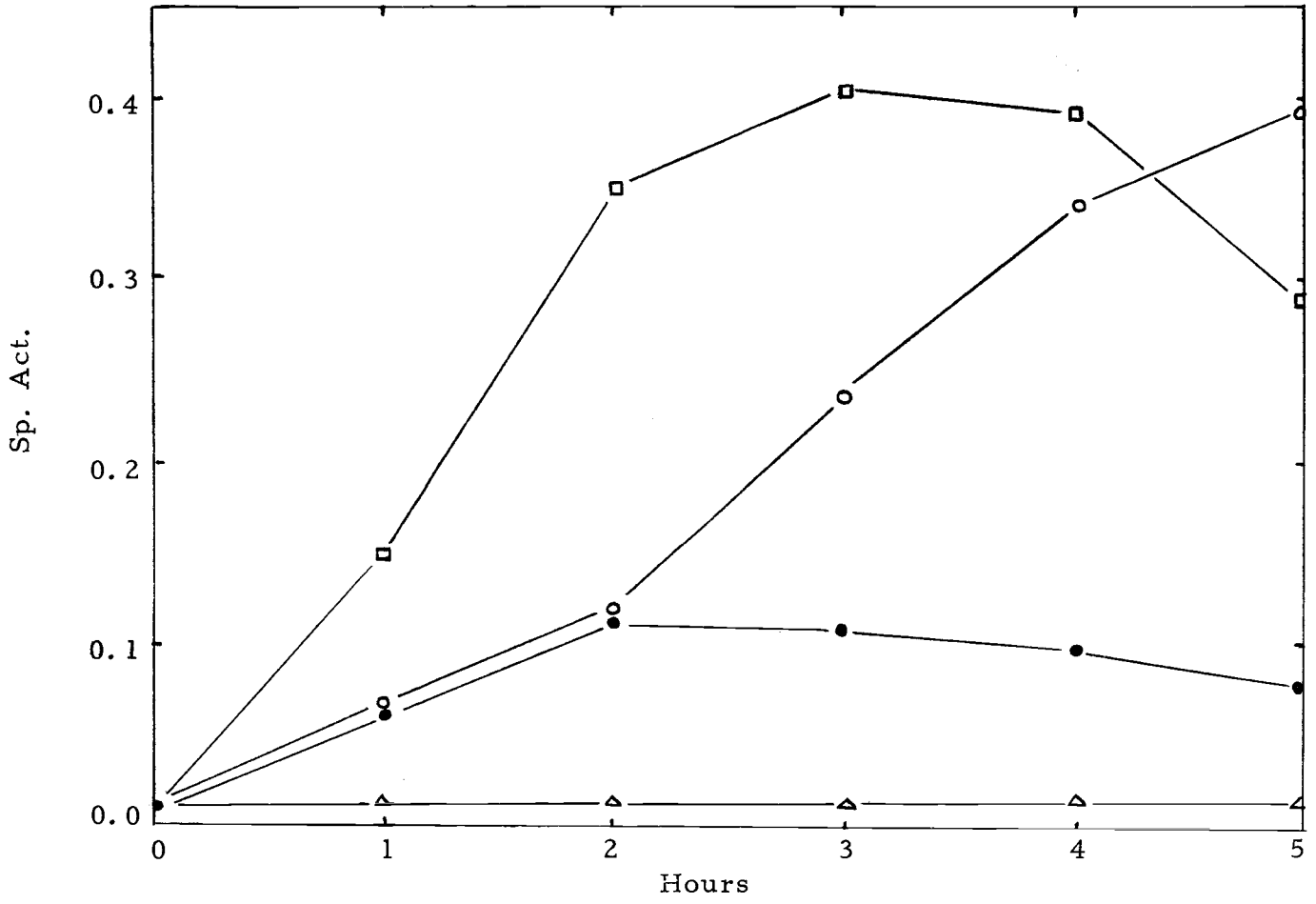


Figure 10. Effect on specific activity of β-Pgal when chloramphenicol (20 ug/ml) was added at time zero to cells of S. cremoris HP grown in either lactose, galactose or glucose (△). Control curves without the addition are also shown: lactose (□), galactose (○) and glucose (●) (solvent treated cells).

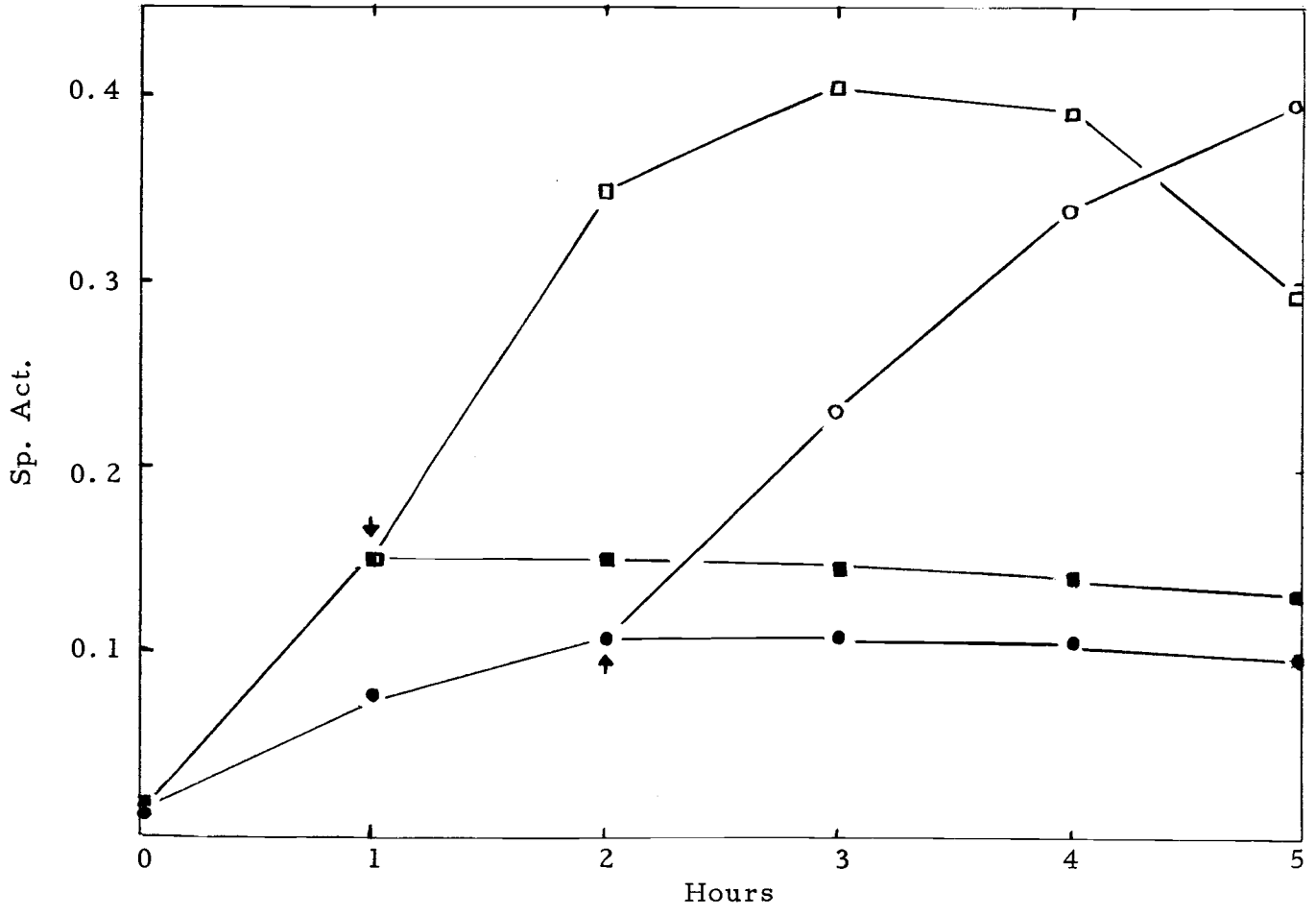


Figure 11. Effect on specific activity of β-Pgal when chloramphenicol (20ug/ml) was added ↓ to cells of S. cremoris HP growing in lactose (□, ■) and galactose (○, ●). Solid figures indicate inhibition pattern (solvent treated cells).

Table 3. Effect of added protein hydrolysate products (0.25%) on acid production, proteolysis and β-D-phosphogalactoside galactohydrolase activity in a fast S. lactis C2 and slow S. cremoris 175 lactic streptococcus.

Medium	Tit. Acid (Net %)		Tyrosine (Net μg/ml)		β-Pgal (Sp. Act. x 10^3)	
	S. lactis C2	S. cremoris 175	S. lactis C2	S. cremoris 175	S. lactis C2	S. cremoris 175
11% nonfat milk (NFM)	0.74	0.18	28	0.0	68	93
11% NFM + Similac	0.78	0.65	28	6.0	71	85
11% NFM + NZ Amine	0.74	0.72	62	12.0	65	81
11% NFM & Edamin	0.74	0.61	32	0.0	65	85

addition of protein hydrolysates, however, the amount of acid produced was increased approximately four-fold. β-Pgal activity remained about the same whether protein hydrolysates were present or not. The amount of free tyrosine remained low in all cases, indicating poor proteolytic ability.

Effect of Acriflavin Treatment on S. lactis

Cells of S. lactis C2 and 7962 were treated with acriflavin (1.0 - 6.0 μg/ml) and examined for the appearance of lac$^-$ variants. The results are shown in Table 4. S. lactis C2, when treated with acriflavin, yielded lac$^-$ variants at a rate of approximately 3%; S. lactis 7962 when treated with acriflavin however, did not yield any lac$^-$ variants. S. lactis 7962 did appear to be more sensitive to acriflavin with respect to the number of surviving cells when compared to S. lactis C2 however.

Comparison of S. lactis C2 Wild Type and Lac$^-$ Mutants

Lac$^-$ mutants isolated from S. lactis C2 by acriflavin, were compared with the parent strain for a number of characteristics. Table 5 lists the results of this comparison. Lac$^-$ mutants differed from the wild type by being unable to transport and ferment lactose. The ability of the mutant to ferment galactose was only slightly

Table 4. Effect of acriflavin on Streptococcus lactis C2 and 7962 and the appearance of lac$^-$ variants.

Organism	Acriflavin (μg/ml)	Temperature: 21C CFU/ml	21C Number of lac$^-$ variants	31C CFU/ml	31C Number of lac$^-$ variants
C2	0.0	6.8×10^8	0	6.9×10^8	0
	1.0	1.9×10^6	4 of 190	6.4×10^7	2 of 64
	3.0	7.0×10^3	2 of 70	1.0×10^1	0
	6.0	1.0×10^5	3 of 100	2.4×10^2	4 of 240
7962	0.0	3.3×10^9	0	3.0×10^9	0
	1.0	1.5×10^5	0	1.0×10^2	0
	3.0	5.0×10^5	0	1.2×10^3	0
	6.0	3.5×10^4	0	no growth	0

Table 5. Comparison of physiological characteristics of S. lactis C2 wild type with a lac$^-$ mutant.

Characteristic	C2 wild type	C2 lac$^-$ mutants
Ferments		
lactose	+	-
galactose	+	+ (-)
glucose	+	+
fructose	+	+
mannose	+	+
maltose	+	+
Arginine hydrolysis	+	+
Diacetyl production	-	-
β-Pgal activity	+	-
β-gal activity	-	-
Lysogenic	+	+
Uptake of C^{14} lactose	+	-

impaired. The lac$^-$ mutant also lacked even a basal level of β-Pgal activity, mutant cells growing on galactose did not contain any β-Pgal. The wild type and mutant were similar in all the other comparisons made. This included the resistance-sensitivity pattern for the eight antibiotics examined. Both the wild type and the lac$^-$ mutants were sensitive to ampicillan, carbenicillan, gentamicin, kanamycin, chloromycetin, tetracycline and furadantin. The wild type and the lac$^-$ mutants were both resistant to streptomycin. Both organisms gave a positive arginine hydrolysis reaction and did not produce diacetyl, typical of S. lactis organisms. Acriflavin treatment had not cured the wild type with respect to lysogeny; UV-treatment of the lac$^-$ mutant resulted in lysis of the organism, the lysate however did not yield lac$^+$ organisms when added back to another lac$^-$ host.

Prophage Induction by UV Irradiation

When cells of S. lactis C2 were irradiated with UV-light and inoculated into Henning's broth, the cells lysed after approximately 2 hr. Figure 12 shows the pattern of lysis when OD was followed at 650 nm. Unirradiated cells served as the control and continued to grow in Henning's broth without any indication of lysis. Results of subjecting other strains to UV irradiation are given in Table 6. While both S. lactis C2 wild type and lac$^-$ mutant could be induced

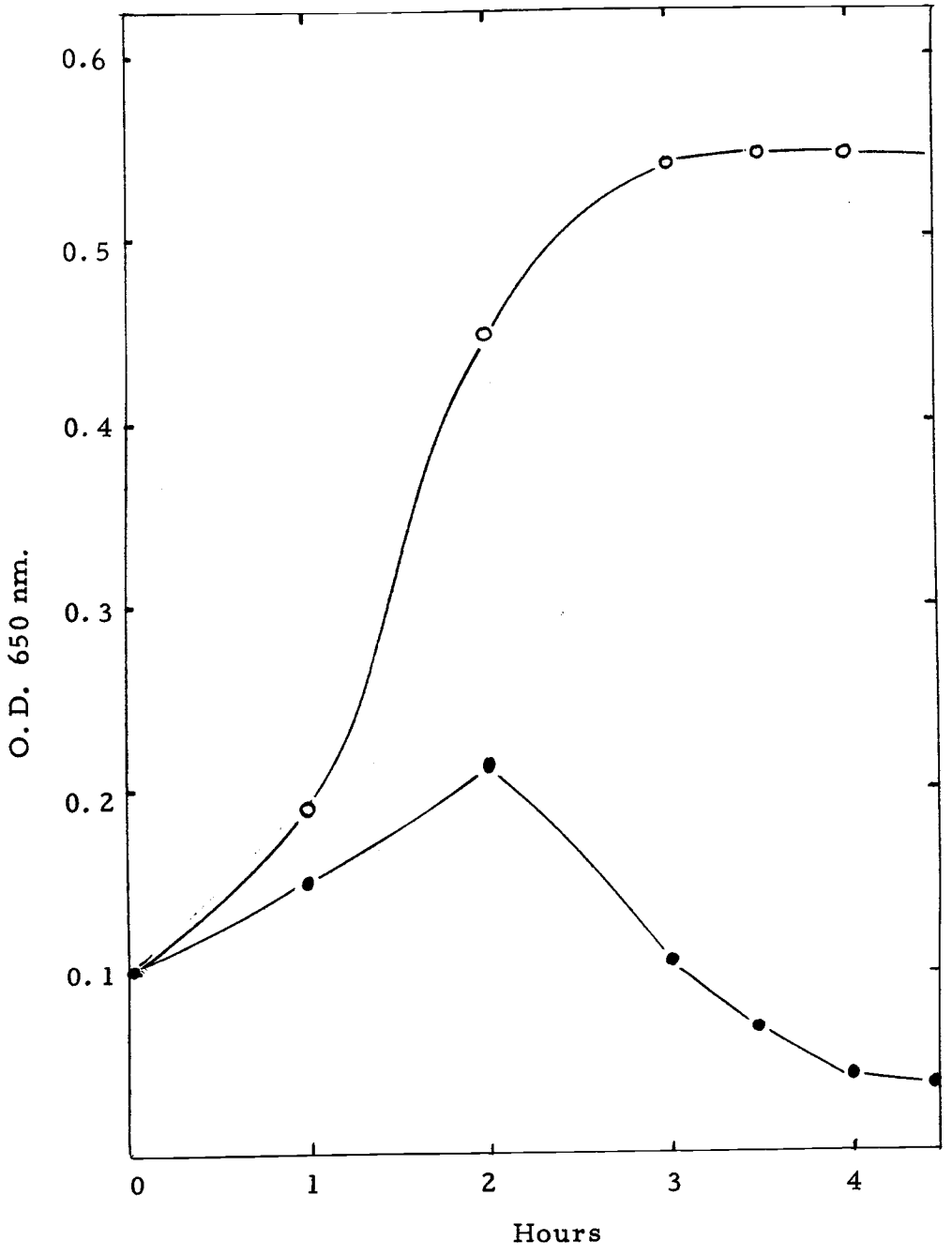


Figure 12. Change in absorbancy of UV irradiated cells of S. lactis C2 (●) inoculated into Henning's broth. Unirradiated control (o).

to lyse, S. lactis 7962 and S. cremoris HP could not. In the case of S. lactis C2, once lysis had occurred, no further growth was observed in the culture even after 24 hr of further incubation. The lysate, when plated out however still contained approximately 1×10^2 viable cells per ml.

Table 6. Susceptibility of some lactic streptococci to lysis by UV irradiation.

Organism	Lysis
S. lactis C2	+
S. lactis C2 lac^- mutant	+
S. cremoris HP	-
S. lactis 7962	-

Transduction of the Lactose Fermenting Characteristic

Results of mixing the phage lysate obtained from UV irradiation of S. lactis C2 (lac^+) with lac^- mutant recipient cells are shown in Table 7. Lac^+ cells could only be demonstrated when the phage lysate was mixed with the lac^- recipient. The phage lysate and lac^- mutant alone did not yield any lac^+ cells upon plating. DNAse controls of the phage lysate were also negative. The number of lac^+ transductants obtained from the mixture decreased as the length of incubation of the mixture before plating

Table 7. Number of lac^+ transductants/ml detected on lactose agar plates with bromcresol purple indicator.

System	No. transductants (lac^+)/ml
Phage lysate	0
Lac^- mutant [a/]	0
Phage lysate + lac^- mutant (1:1)	
30 min	210
45 min	130
60 min	100

[a/] Suspension contained 6.8×10^8 cells/ml.

was increased.

Comparison of *S. lactis* C2 Wild Type, Lac^- Mutants, and Lac^+ Transduction

In examining the lac^+ cells obtained from the transduction experiments previously described, it was found that the transductants had regained the ability to ferment lactose and also contained approximately the same levels of β-Pgal as the wild type. These results are summarized in Table 8. The transductants were indistinguishable from the wild type in all characteristics examined except one. The transductants were unable to produce lactic acid at the same rate as the wild type when growing in 11% milk.

Table 8. Comparison of S. lactis C2 wild type with S. lactis C2 lac^+ transductants.

Character	Wild type	Transductants		
		T2	T8	T9
Ferments				
lactose	+	+	+	+
galactose	+	+	+	+
glucose	+	+	+	+
mannose	+	+	+	+
β-Pgal (Sp. Act.) x 10^3	186	188	186	184
Lysogenic	yes	yes	yes	yes

Figure 13 shows lactic acid production of the wild type, a lac^- mutant and transductants (designated T8 and T9) when grown in milk. All initial inoculations were 1% and contained approximately 5.0 x 10^6 organisms/ml. After 6 hr at 31 C, the mutant had lowered the pH only about 0.15 units and had a cell concentration of 7.5 x 10^7. The wild type lowered the pH about 1.1 units and had a final cell concentration of 3.5 x 10^8. T9 lowered the pH about 0.9 of a unit and had a final cell concentration of approximately 1.0 x 10^8. T8 lowered the pH 0.7 units. This difference in rate of acid production between the wild type and the transductants could not be demonstrated when the cells were growing in lactose only lactic broth as shown in Figure 14. The lac^- mutant growing in lactose broth first lowered the pH slightly and then raised it again.

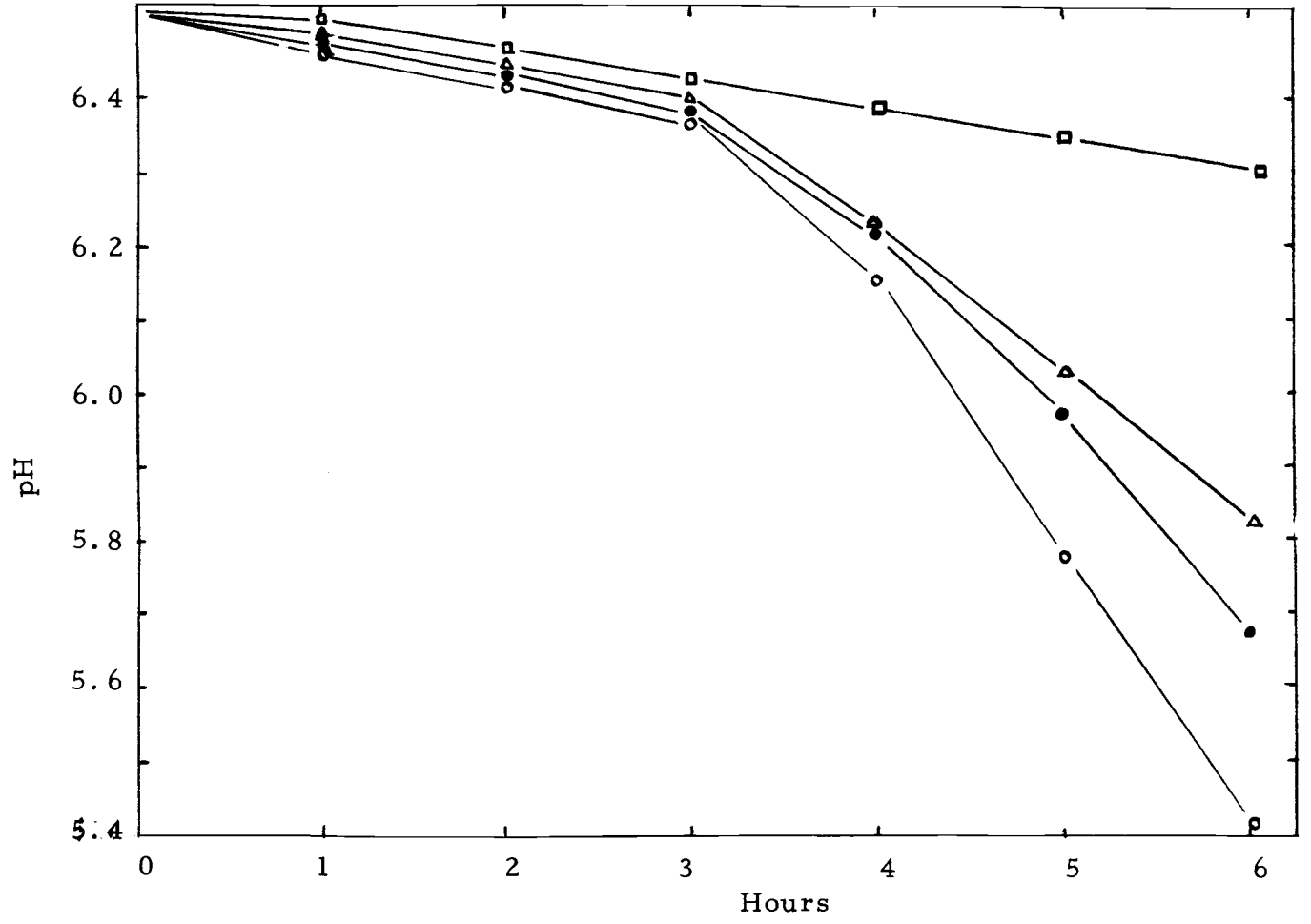


Figure 13. Lactic acid production of S. lactis C2 growing in milk (31C, 1% inoculum). Wild type (o), transformants T_8 (▲) and T_9 (●) and lac$^-$ mutant (□).

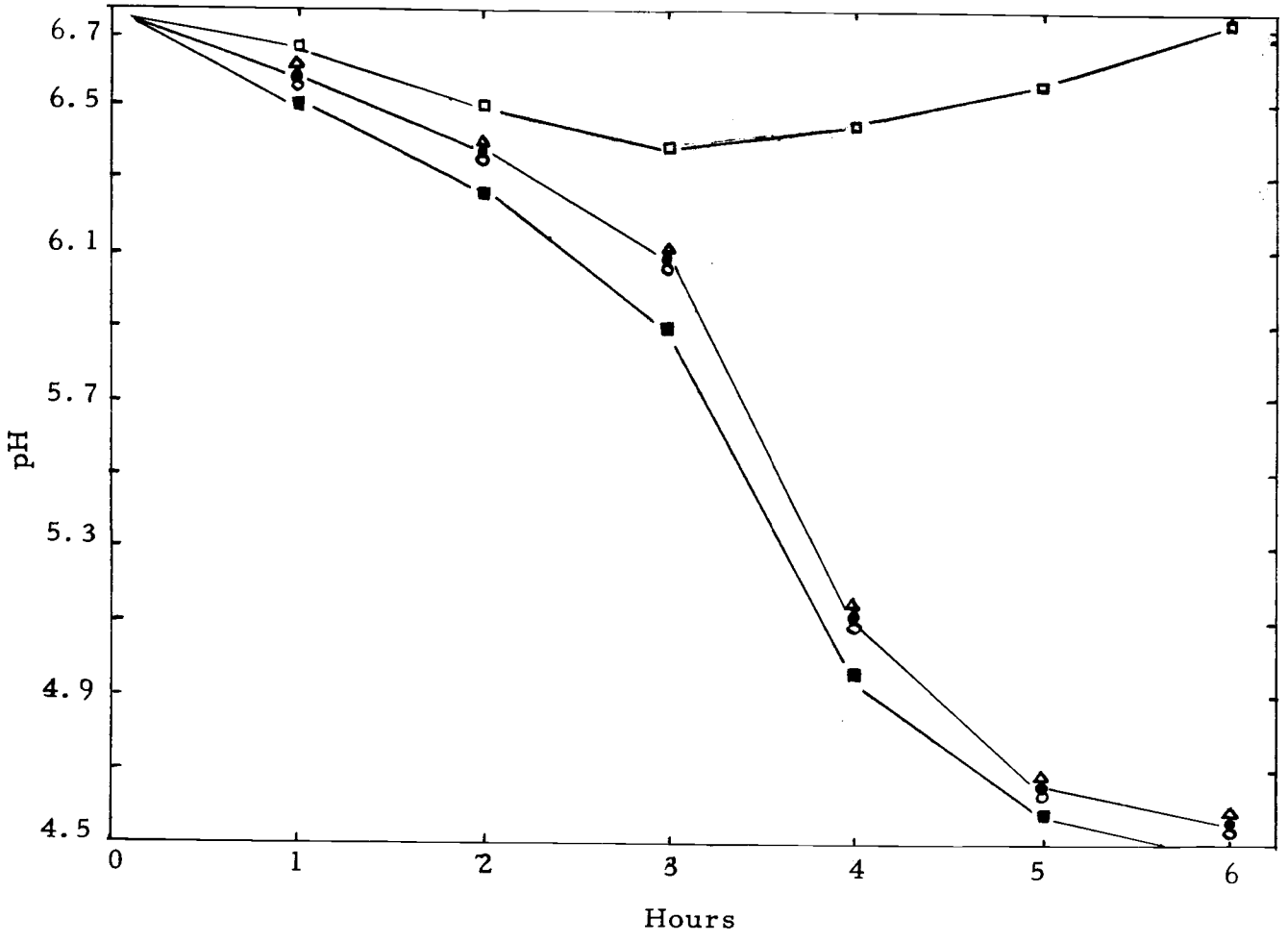


Figure 14. Lactic acid production of S. lactis C2 growing in lactose only lactic broth. Wild type (o), transformants T_8 (△) and T_9 (●), and lac$^-$ mutant (□). Lac$^-$ mutant growing in glucose only lactic broth (■), wild type gave similar curve.

Final cell concentration was 9.0×10^7. When the lac$^-$ mutant was grown in glucose only broth the pH was lowered over 2.0 units and the final cell concentration was 6.9×10^8. The rate of acid production of the wild type and transductant T9 and T8 were essentially the same, pH was lowered 2.0 units and the final cell concentration was approximately 4.0×10^8. Because it appeared that the lac$^-$ mutants could ferment glucose and produce lactic acid at a high rate when grown in broth, it was thought that the addition of glucose to 11% NFM might restore the lactic acid production of the lac$^-$ mutant in milk. Results are given in Figure 15. Addition of 1% glucose to 11% NFM only restored the ability of the lac$^-$ mutant to produce acid at a rate similar to the slow lactic acid transductant T8. The presence of 1% glucose in the milk did not appreciably affect the rate of acid production of the wild type or lac$^+$ transductants.

The slow nature of acid production by the lac$^-$ mutants and also the transductants when grown in milk, indicated that possibly the proteolytic ability of these organisms had been impaired. Proteolytic activity of the lac$^-$ mutant, lac$^+$ transductant T8 and the wild type was examined during growth in milk. Samples were assayed at 5 hr during the lactic acid rate determination experiments. Table 9 summarizes the results.

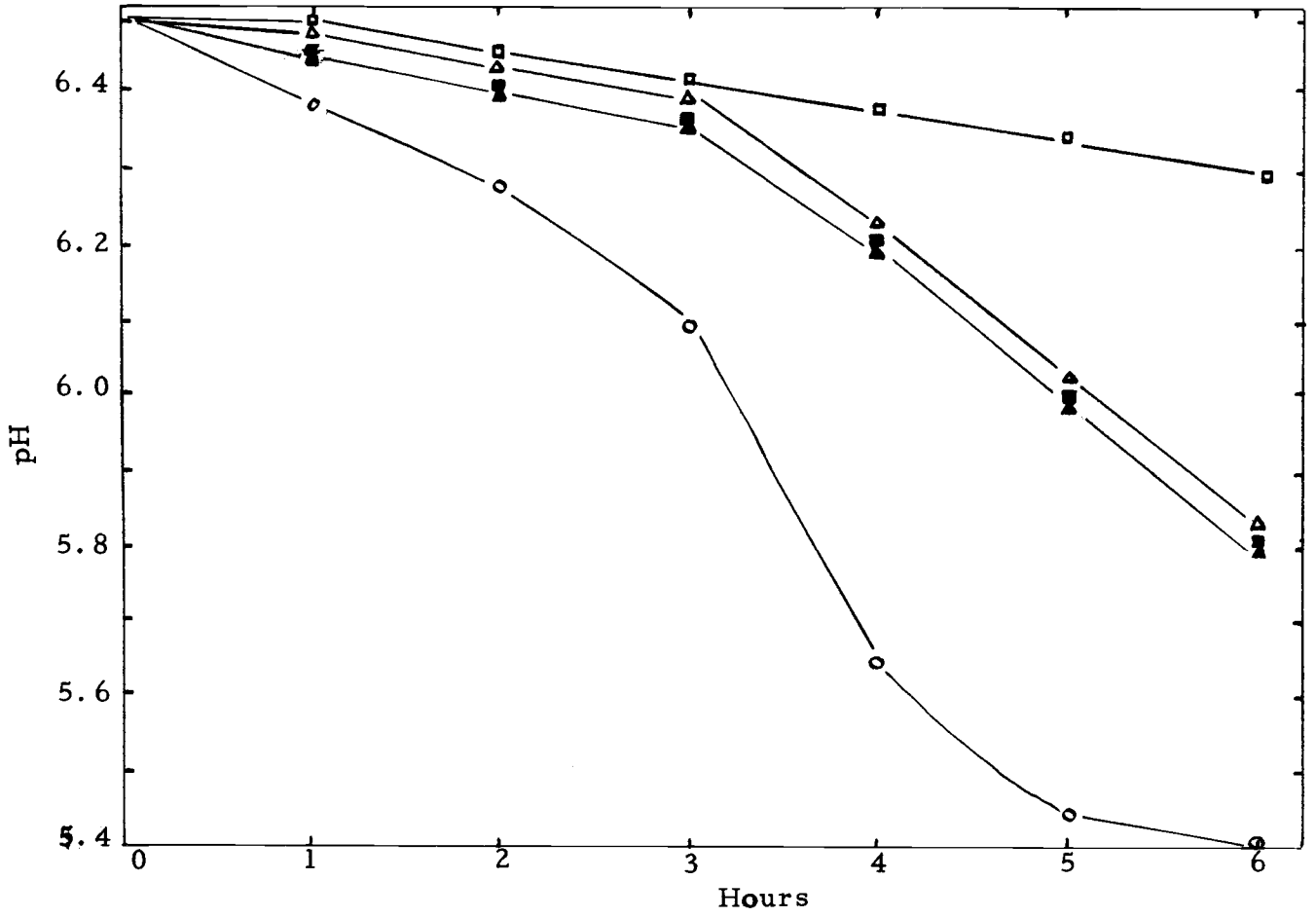
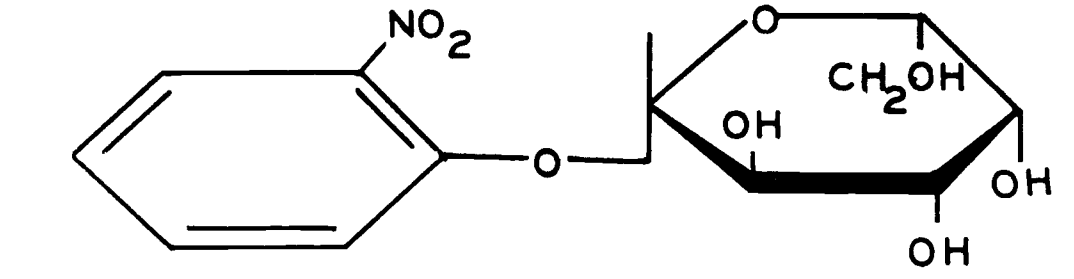


Figure 15. Lactic acid production of S. lactis C2 growing in milk with 1% glucose added. T_8 (▲), lac⁻ mutant (■). Controls without glucose are also shown, T_8 (▲) lac⁻ mutant (□) and wild type (○).

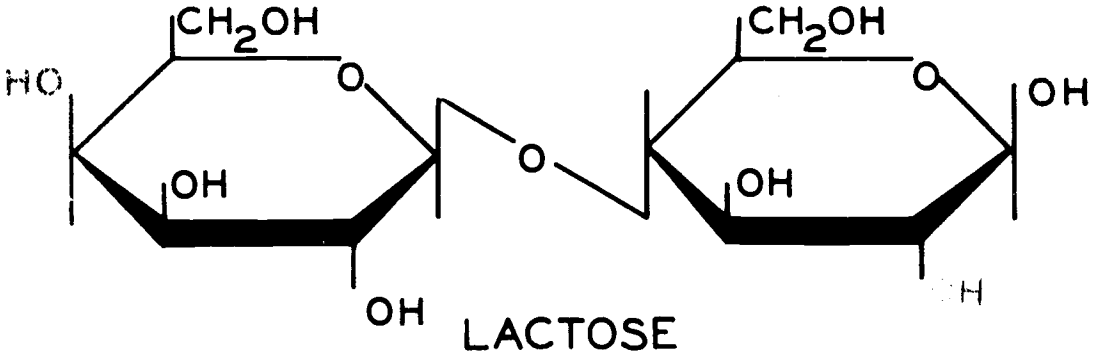
Table 9. Proteolytic activity of S. lactis C2 wild type, lac$^-$ mutant and transductant.

Organism	μg/ml Tyrosine	
	11% NFM	11% NFM + 1% glucose
S. lactis C2		
wild type	56.0	16.0
lac$^-$ mutant	6.0	8.0
lac$^+$ transductant	6.0	8.0
uninoculated control	0.0	0.0

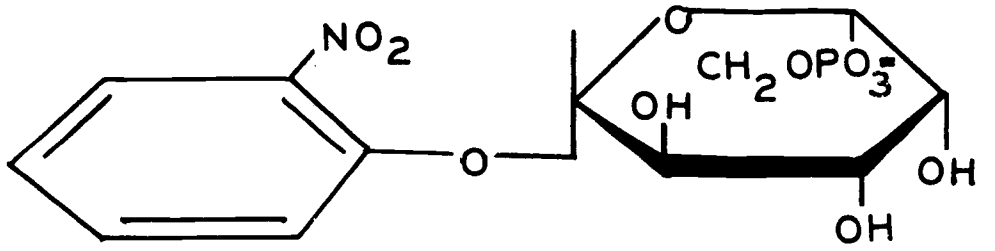
The wild type growing in 11% NFM exhibited considerable proteolytic activity as expressed as liberated tyrosine. Both the lac$^-$ mutant and lac$^+$ transductant, however, exhibited very little proteolytic activity. Even when 1% glucose was added to the milk, the proteolytic ability of the lac$^-$ mutant and the lac$^+$ transductant was not significantly increased; in fact, glucose appeared to inhibit proteolysis by the wild type.



ONPG



LACTOSE



ONPG-6-PO_4

Figure 16. Structures of ONPG, lactose, and ONPG-6 phosphate.

DISCUSSION

Survey of Lactose Hydrolyzing Enzymes in Lactic Streptococci

The enzyme β-gal could be demonstrated in only one organism, S. lactis 7962 (Table 1); the other lactic streptococci examined contained varying amounts of β-Pgal activity and essentially no β-gal could be detected. These results are consistent with a similar survey undertaken by McKay et al., 1970. In the course of the present study it was also found that whole cells of S. cremoris HP, suspended in phosphate buffer could not hydrolyze ONPG-6-P, but could slowly hydrolyze ONPG if incubated for one hour or more. Whole cells treated with toluene:acetone were able to hydrolyze ONPG-6-P (10 min assay) but could no longer hydrolyze ONPG even when the assay time was considerably extended. S. lactis UN exhibited a similar response (McKay et al., 1970). These results indicate that an untreated cell still has a PTS capable of slowly phosphorylating ONPG, thus making it available for hydrolysis by β-Pgal. Intact whole cell membranes, however, are impermeable to ONPG-6-P. Treatment with toluene:acetone apparently results in a loss of a functional PTS and thus loss of the ability to hydrolyze ONPG, but at the same time yields a membrane that is no longer impermeable to ONPG-6-P and therefore hydrolysis of this

compound is possible. The differences that exist between S. lactis 7962 and the rest of the lactic streptococci have led investigators to question whether or not 7962 is actually a lactic streptococci.

Optimal Enzyme Assay Condition

β-Pgal activity in toluene:acetone-treated cells of S. cremoris HP exhibited maximal activity over a narrow range of both pH (7.0) and temperature (37 C). β-Pgal which had been purified approximately 12-fold from the same organism, exhibited maximal activity over a wider range of pH (5.5 - 7.5) and temperature(48-55 C) (Johnson and McDonald, 1974). In toluene:acetone-treated cells, sodium ion was required for maximal activity and could not be replaced by potassium ion. In studies of the partially purified enzyme (Johnson and McDonald, 1974) Mg^{2+} and Li^{+} inhibited enzyme activity slightly.

Induction of β-Pgal

Lactose was the best inducer of β-Pgal over the range of concentrations tested. Galactose-grown cells, although eventually reaching the same maximal level of β-Pgal as lactose-grown cells, exhibited a slower rate of induction, especially during the initial hours after shifting. This result is just the opposite for induction of β-gal in S. lactis 7962 where galactose was a better inducer of

enzyme activity (Citti et al., 1965). Also β-Pgal activity of cells grown in carbohydrates normally considered non-inducers (glucose and mannose), showed some degree of fluctuation. The characteristic low specific activity of 12-hr old cells grown in mannose or glucose showed an increase during the first few hours after shifting into fresh medium containing the same sugar (Table 4). This fluctuation was also observed when cells were shifted to broth with no carbohydrate, or containing either TMG, ONPG, IPTG or ONPG-6-PO_4. Although cells carried on lactose or galactose showed considerable fluctuation in enzyme levels also, the level of β-Pgal found in these cells was always higher than found in glucose or mannose-grown cells. When the effect of glucose on enzyme induction was examined more closely it was found that cells shifted at 12 hr from mannose broth to broth containing both galactose (0.005 gm/ml) and glucose (0.001 gm/ml) gave only a basal level of enzyme typical of glucose or mannose-grown cells (Fig. 7). The normal inducing action of galactose was prevented by trace amounts of glucose. Lactose induction was only partially inhibited by the presence of glucose under similar conditions (Fig. 7 and 8). These results indicate that lactose is an ideal combination of a rapidly metabolizable sugar (glucose) and also some form of the actual inducer (galactose). A mixture of the free sugars did not show any inducing ability. When galactose served as the sole

carbohydrate, S. cremoris HP grew very slowly, indicating that the rate of β-Pgal induction, which is also slow in broth containing only galactose, may be a direct reflection of the organism's ability to metabolize the inducer.

The basal level of enzyme in the presence of non-inducing sugars and the nature of the fluctuations found seemed to indicate a kind of repression-depression of basal β-Pgal always present in the cell and not an actual synthesis of new enzyme. This was indicated by the fact that this basal increase occurred even when 12-hr mannose-grown cells were shifted to starvation conditions and actual cell growth did not occur. Also trace amounts of glucose in the presence of galactose, stopped production of β-Pgal above the characteristic basal level.

This repression-depression hypothesis was examined using cell-free extracts of S. cremoris HP grown on glucose and lactose. When the extracts were mixed, no decrease in β-Pgal activity in the lactose-grown cells could be demonstrated. This would indicate that glucose grown cells do not produce an inhibitor type substance that had the ability to repress previously formed β-Pgal.

The other explanation of the fluctuation of the basal level of enzyme was that an actual synthesis of new enzyme occurs for a short period after the cells are introduced into fresh media. This possibility was examined by studying the effect that chloramphenicol

had on β-Pgal induction. Addition of chloramphenicol which inhibits protein synthesis at the translational level, completely stopped induction of β-Pgal (Fig. 9 and 10). This was the case for both cells growing in lactose or galactose and also for the basal fluctuation exhibited by cells grown in glucose or mannose. These results indicated that even when cells were grown in non-inducing carbohydrates, a slight stimulation of synthesis of new β-Pgal occurred after transfer to the fresh medium representing a mechanism whereby the cell maintains a low but detectable level of hydrolyzing enzyme.

No gratuitous inducers of β-Pgal were found, although cells inoculated into media containing TMG, IPTG, ONPG or ONPG-6-PO_4 did exhibit the basal level of enzyme already described. This is in contrast to findings in S. lactis 7962 where β-gal was slightly induced by both TMG and also ONPG (Citti et al., 1865). Also in E. coli, TMG and IPTG are better inducers of β-gal than either lactose or galactose (Cohn and Monod, 1953).

Relationship of β-Pgal Activity and Proteolytic Activity

A rapid rate of lactic acid production from lactic streptococci is important in many industrial fermentations. It has been shown that slow acid production can be the result of a loss of proteolytic

ability of the cells (Garvie and Mabbitt, 1956). Since the peptide and amino-acid content of fresh milk is low (Deutsch and Samuelson, 1959) an organism with impaired proteolytic ability would be at a disadvantage. The addition of certain protein hydrolysates to milk greatly increases the ability of slow lactic acid producers to produce acid despite their poor proteolytic ability (Koburger et al., 1963). Studies in this laboratory were directed toward determining if slow lactic acid-producing cells with reduced proteolytic ability also had decreased levels of β-Pgal and whether or not the addition of protein hydrolysates would effect enzyme level.

Results indicated that the rate of lactic acid production was independent of the level of β-Pgal present in the cell (Table 3). Although S. cremoris 175 when grown in plain 11% NFM produced very little lactic acid over a 13-hr period, addition of protein hydrolysates greatly increased the amount of acid produced. This increase was accomplished without significant change in the level of β-Pgal. It appears therefore that impaired protein synthesis that may result from an inability of the cell to efficiently hydrolyze the milk protein does not affect β-Pgal synthesis.

Effect of Acriflavin on S. lactis C2 and 7962

The results given in Table 4 indicate that while S. lactis 7962 is much more sensitive to acriflavin treatment with respect to

survivors than C2, no lac$^-$ mutants could be isolated from 7962. Lac$^-$ mutants were readily obtained from C2, however, as a result of acriflavin treatment. The lactose system of S. lactis 7962 is quite similar to that found in E. coli (Citti et al., 1965). Acriflavin causes mutations by eliminating extra chromosomal DNA that may be present in the growing cell. Results presented here indicate that the lactose-hydrolyzing enzyme of S. lactis 7962 (β-gal) may be carried on the host chromosome as it is in E. coli and not affected by acriflavin. In S. lactis C2, however, it would appear that the enzyme of this organism is carried extrachromasomally, on a plasmid-like particle and thus subject to acriflavin treatment. Plasmids have been identified in some S. lactis organisms (Guerry et al., 1973 Cords et al., 1974).

Two types of indicator plates were used in this study. Lactic (lactose) agar with bromcreosol purple indicator, and L. agar with neutral red indicator. It was found that the lactic agar was best used when picking lac$^+$ colonies (yellow) from a lawn of lac$^-$ colonies (white). L. agar was preferred when picking lac$^-$ colonies (white) from a lawn of lac$^+$ colonies (red).

Comparison of S. lactis C2 Wild Type and Lac$^-$ Mutants

The lac$^-$ mutants obtained from acriflavin treatment of S. lactis C2 were examined to see if other mutant characteristics could be

identified. S. lactis C2 wild type is an extremely fastidious organism requiring a very complex medium for growth. Thus, most mutations would tend to be lethal. It was hoped that by identifying other mutants, a linked character with the lactose-fermenting region could be determined, and thus eventually aid in mapping experiments. Results given in Table 8 indicate that with the exception of the ability to ferment lactose (including lactose transport and β-Pgal activity) the mutants examined were indistinguishable from the wild type. All mutants examined were the same. The importance of proteolic activity will be discussed later.

An interesting aspect of the lac^- mutants was their ability to grow in galactose and yet not produce any β-Pgal. Growth in galactose by the mutants was slightly slower than the wild type growing in galactose. The question therefore arises as to what the actual role of galactose is during induction of β-Pgal and the nature of the actual inducer. In S. aureus, galactose-6-PO_4 (a product of hydrolysis of lactose-6-PO_4) has been shown to play a role in the induction process (Morse et al., 1968). Studies to determine the actual inducer in Streptococcus organisms have been hampered by the fact that unlike staphylococci, streptococci are impermeable to many phosphorylated compounds. Attempts to increase the permeability of the cell membrane and still leave the cell viable have been unsuccessful.

Induction of Prophage by UV Irradiation

Prophage induction by UV irradiation could be demonstrated by only one strain of lactic streptococcus examined, S. lactis C2. The lac^- mutants and also the lac^+ transductants obtained from this strain were also susceptible to induction by the method used. Although S. lactis 7962 and S. cremoris HP did not lyse by the irradiation treatment employed, these organisms could still carry prophage. The method used to lyse S. lactis C2 was highly specific with respect to length of irradiation and dosage. Possibly by varying these parameters, these other strains could be induced to lyze. Recently, Lowry (1974) demonstrated lysogeny in several lactic streptococci using UV-light and mytomycin C as inducers. Attempts to isolate an indicator strain for the phage released from C2 have not been successful (McKay and Baldwin, 1973). It was important to carry out the actual irradiation and inoculation into Henning's broth in complete darkness at least for the first hour after irradiation. It was found that any light allowed to come in contact with the cells during this period of time would stop the lysis from occurring. This would indicate that a type of photoreactivation mechanism is present in S. lactis C2.

Transduction of Lactose Fermenting Character

The lactose fermenting characteristic was transduced into lac^- cells of S. lactis C2 at a maximal rate of 210 lac^+ cell appearing from 6.8×10^8 lac^- recipient cells. This compares well with results of McKay, Cords and Baldwin (1973). In the course of these experiments, no spontaneous reversion of the lac^- mutant to lac^+ was ever observed. This fact along with the results of acriflavin treatment of S. lactis C2 previously described would argue for the existence of the lactose-fermenting character on a plasmid in these cells.

Characterization of the Lac^+ Transductants

Upon initial examination, all the lac^+ transductants isolated from bromcreosol indicator plates were indistinguishable from the parent S. lactis C2 wild type (Table 8). Transductants had regained full β-Pgal activity and once again could ferment lactose only lactic broth at a rate that was essentially the same as the wild type.

When the transductants were examined for lactic acid production in milk, however, it was found that the transductants were somewhat slower in the rate at which they lowered the pH (lactic acid production). Slow lactic acid production in milk is usually an indication of poor proteolytic ability, while the cells are still able to ferment

lactose. Thus cells of poor proteolytic ability might be designated lac^+, pr^-, while the wild type exhibits a lac^+ pr^+ phenotype. Upon examination of the lac^- mutants it was found that these cells were indeed pr^-.

It is important to note that in the lactic streptococci, pr^- cells cannot be detected when grown in lactic broth. This is probably due to the large amount of small peptides already present in this media. It is only when the cells are grown in milk and forced to hydrolyze casein to get their required amino nitrogen, that the pr^- phenotype can be demonstrated.

When the lac^- mutants of S. lactis were grown in lactose only lactic broth, it was found that the pH initially was lowered slightly, and then increased again. Lactic streptococci other than S. cremoris can generate ATP by hydrolysing arginine with subsequent formation of ammonia. Normally the production of lactic acid is so great that it masks any slight increase in pH due to ammonia formation. In the lac^- mutants, however, the pH is only affected by ammonia formation since lactic acid is not formed and the subsequent increase in pH can be observed.

In these experiments, lac^- was the only phenotype selected. Yet as a result of this selection all the lac^- mutants obtained were also pr^-. These data along with a recent report by Pearce and Skipper (1974), into the nature of the proteolytic enzymes of the

lactic streptococci, indicate that certain proteolytic enzymes of these organisms are carried on a plasmid. This plasmid is lost during acriflavin treatment just as the lactose-fermenting character is lost. Westhoff et al. (1971a, b and c) have characterized both intra and extracellular proteases in lactic streptococci. The nature of the protease responsible for the pr^- phenotype examined in this study is not known.

Lactic streptococci are known to be very unstable with respect to proteolytic activity. Strains revert (or mutate) to slow lactic acid producers, as a result of losing their proteolytic ability, spontaneously at a rate of about 1%. The pr^- strains obtained by this type of spontaneous mutation, however, are still lac^+ with respect to fermentation. On the other hand, spontaneous mutation to lac^- was never observed in this laboratory. The difference in stability of the lactose and the proteolysis characters suggests that they are carried on different plasmids. Whether or not this is actually the case remains to be determined. A method whereby large numbers of cells can be examined for proteolytic ability directly is not available as yet.

A possible model of the lactose and proteolytic genes in S. lactis C2 might be constructed as follows: Genes responsible for lactose fermentation are carried on one plasmid. These genes would include β-Pgal and also one or more of the enzymes

functioning in the PTS for lactose. These genes, being closely linked, could be transduced into a lac^- cell and restore full ability to ferment lactose. The genes responsible for proteolytic activity might be carried on a second plasmid, and transduced independently of the lactose marker. Thus when transductants are selected for lac^+ phenotype alone, they have not regained their full proteolytic ability. This would mean that when acriflavin treatment is employed in obtaining lac^- mutants, pr^- mutants are formed concurrently. Indeed all the lac^- mutants examined up to this time have also been pr^-.

Possibilities of Improving the Lactic Fermentation

Results of these studies indicate at least two areas where genetic manipulation might be employed to improve lactic fermentation by lactic streptococci. One area is the use of transduction to stabilize the pr characteristic. Macrina and Balbinder (1972) have demonstrated a mutant F plasmid obtained from Salmonella typhimurium when transduced into E. coli exhibits unique stability in this organism. If stability of this nature could be developed for the pr characteristic in streptococci, the appearance of slow acid production in the fermented dairy product industry would be greatly reduced. Economically this would be of great benefit to the dairy industry, since slow acid production means loss of product.

The second area where genetic manipulation could be used involves lactose fermentation itself. Results given in Figure 9 indicate that in S. cremoris HP lactic acid production lags until β-Pgal levels reach a certain level. This lag is not as long in cells inoculated into glucose presumably because induction of β-Pgal is not critical in this case. (Fig. 14) It might be possible to increase the production of β-Pgal in an organism by creating stable merodiploids for the β-Pgal marker. A higher level of β-Pgal might tend to decrease the lag time for lactic acid production, thus decreasing the production time of many fermented dairy products. Such merodiploids have been constructed in E. coli K12 (Fowler, 1972) and the level of β-gal determined. It was found that the merodiploids contained approximately twice as much β-gal as found in the haploid cell. In order to create merodiploids in lactic streptococci, however, a method of selecting for the merodiploid cell must first be developed. Current methods of plating would not work since these methods only select for ability to ferment lactose and not the amount of β-Pgal present in the cell.

SUMMARY

The relationship between β-Pgal activity, lactic acid production and proteolytic activity of certain lactic streptococci was examined in detail.

Lactose was the fastest inducer of β-Pgal while induction of enzyme during growth on galactose lagged behind but eventually reached levels comparable to lactose grown cells. The amount of β-Pgal varied during the growth of the cell. Cells inoculated into fresh broth showed an increase in β-Pgal levels even if glucose or mannose served as the carbohydrate. This increase was much less than that found in cells grown in lactose or galactose and was taken to be the normal basal response of this enzyme.

Proteolytic ability of the cell did not directly affect the amount of β-Pgal found in the cell. Proteolytic activity did affect the rate of lactic acid production, less activity being associated with a slower rate of lactic acid production. In normal fast lactic acid-producing cells with normal proteolytic ability, it was found that lactic acid production lagged until amount of β-Pgal reached a certain level when lactose served as the sole carbon source. This lag was not as pronounced when cells were grown in glucose, presumably because β-Pgal induction was not necessary in this case.

A transduction system found in S. lactis C2 was employed to further study certain aspects of lactose metabolism found in these organisms. It was found that the lactose fermentation character could be transferred from lac^+ wild type cells to lac^- mutant cells by transduction. The lac^+ transductants thus obtained were similar in all respects to the wild type with the exception of their proteolytic ability. Further studies indicated that the lac^- mutant cells used in the transduction experiments were also proteolytic deficient (pr^-). Thus the selection for lac^+ transduction yield cells that could utilize lactose once again but had failed to pick up the genetic material needed to regain their proteolytic ability. Milk (11% NFM) was the only medium employed that demonstrated this difference. Additional studies indicate that the genes for the lactose-fermenting character and those for one or more of the proteolytic enzymes may be carried on different plasmids. These plasmids differ in their stability in the host. This would be similar to the situation found in Streptococcus faecalis where drug resistance characters are carried on three different plasmids (Clewell et al., 1974).

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