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JAMES EDWARD CITTI for the Ph. D. in Microbiology
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Title CONTROL OF ACID PRODUCTION AND β -GALACTOSIDASE
SYNTHESIS IN LACTIC STREPTOCOCCI

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The public health and economic significance of rapid acid production by lactic streptococci in controlled dairy fermentations is well known. However, the fast acid-producing characteristic of these organisms is not stable, and cultures of fast organisms have been shown to contain slow cells. These studies were carried out to characterize slow and fast acid-producing cells isolated from Streptococcus lactis. Since growth and acid production by lactic streptococci are dependent on their ability to metabolize lactose, the enzymes β -galactosidase, which hydrolyzes lactose, and galactoside-permease, which is responsible for the entry and intracellular accumulation of lactose, were also studied.

From Streptococcus lactis C2, a fast strain which produced sufficient acid to coagulate sterile nonfat milk in 18 hours at 21 C, a slow mutant was isolated which required incubation for at least 48 hours to effect coagulation. Nonfat milk cultures of these two

organisms were compared to find causes for the reduced acid production by the slow strain. These two bacteria had the same generation time (1.4 hours) in the exponential growth phase, and produced the same amount of acid per cell. The average viable population of the fast culture after 18 hours at 21 C, however, was about four times as great as the slow. Also, the fast culture was four times more proteolytic in nonfat milk than the slow. The slow organism appeared to have lost its capacity to synthesize proteolytic enzymes. Tryptic and pancreatic hydrolysates of casein stimulated acid production in milk by the slow and fast strains; acid hydrolyzed casein was not an effective stimulant. There was, therefore, a direct relationship between available nitrogen, in the form of peptides, and total growth and acid production in milk. Acid development in milk by the fast strain was also enhanced by adenine, hypoxanthine, adenosine, or inosine. Therefore, there appeared also to be a relationship between the synthesis of proteolytic enzymes in the fast strain and the availability of purine bases or nucleosides. The slow strain, however, having lost its ability to synthesize proteolytic enzymes, derived no benefit from these nucleic acid derivatives.

Synthesis of β -galactosidase by several strains of lactic streptococci was induced by lactose. The rate of hydrolysis of ortho-nitrophenyl- β -D-galactopyranoside was used to measure enzyme activity. The enzyme of all but one strain was unstable when whole cells

were sonicated or treated with toluene; the enzyme of one strain, Streptococcus lactis 7962, was stable to these treatments, which resulted in at least a five-fold increase in activity over that found in whole cells. The optimal assay conditions for toluene-treated cells of this strain were 37 C and pH 7.0 in sodium phosphate buffer. Lactose was the most effective inducer of enzyme synthesis. Methyl- β -D-thiogalactopyranoside, isopropyl- β -D-thiogalactopyranoside, and galactose were also inducers of the enzyme, but were not as effective as lactose. Melibiose, maltose, calcium lactobionate, and glucose were poor inducers of enzyme synthesis. Exogenously supplied glucose repressed induced β -galactosidase synthesis; galactose only slightly inhibited induced enzyme synthesis.

Lactose- C^{14} uptake by S. lactis 7962 was mediated by a β -galactoside-permease. Synthesis of this enzyme was induced by lactose. Maltose induced synthesis of this enzyme, but less effectively than lactose. Glucose was not an inducer. Glucose also repressed synthesis of induced β -galactoside-permease. The means of control of induced β -galactosidase and β -galactoside-permease synthesis in S. lactis was similar to that in Escherichia coli.

CONTROL OF ACID PRODUCTION AND β -GALACTOSIDASE
SYNTHESIS IN LACTIC STREPTOCOCCI

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JAMES EDWARD CITTI

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APPROVED:



Associate Professor of Microbiology

In Charge of Major



Chairman of Department of Microbiology



Dean of Graduate School

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Typed by Lucinda M. Nyberg

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CONTROL OF ACID PRODUCTION AND β -GALACTOSIDASE SYNTHESIS IN LACTIC STREPTOCOCCI

INTRODUCTION

Mixed strain lactic starter cultures are used in the dairy industry to produce hard cheeses, cottage cheese, buttermilk, sour cream, and other fermented milk products. An important constituent of these starter cultures is Streptococcus lactis, a homofermentative lactic acid bacterium which converts milk sugar, or lactose, to lactic acid. The developed acid adds flavor, consistency, and improved keeping quality to the finished product.

Rapid formation of lactic acid by starter bacteria is essential to prevent growth of pathogenic and spoilage organisms, and to maintain plant production schedules. Certain environmental factors such as antibiotics in milk or bacteriophage infection may arrest culture growth and prevent acid development. Another, and less understood, phenomenon is the irreversible loss of the fast-acid-producing ability of a lactic Streptococcus. Slow acid-forming mutants arising from fast strains of S. lactis may dominate the culture. Fast cultures develop sufficient acid to coagulate milk in 18 hours at 21 C; however, slow cultures lack this ability.

This study was undertaken to quantitatively define cultural and physiological differences between slow and fast cells isolated from

S. lactis. Growth, acid production, and proteolytic characteristics of these two types of cells were compared. Since acid production depends on the ability of S. lactis to ferment lactose, it seemed likely that differences in the capacity of these organisms to produce lactic acid might be related to differences in their ability to synthesize β -galactosidase, the lactose-hydrolyzing enzyme, and galactoside-permease, the enzyme responsible for the entry and intracellular accumulation of lactose. Therefore, systems were developed to study β -galactosidase and galactoside-permease in S. lactis. It is hoped that the results of this investigation will lead to a better understanding of the factors regulating acid production by lactic streptococci.

HISTORICAL REVIEW

Slow Acid Production by Lactic StreptococciNature of Slow Lactic Streptococci

Harriman and Hammer (1931) first reported slow variants of S. lactis. About two percent of the colonial isolates from several fast cultures were slow acid-producing bacteria when they were grown in milk at 21 C. The slow variants apparently arose spontaneously from the fast strains. The reverse type of mutation, i. e. , fast cells from slow cells, has never been observed under natural conditions (Harriman and Hammer, 1931; Garvie, 1959). However, the reversion to fast acid production by slow mutants induced by the irradiation of a fast culture has been observed recently (Wagner, 1964). Harriman and Hammer (1931) also reported that fast cultures liberated more soluble nitrogen in milk than the slow strains, and concluded from this observation that the fast strains were more proteolytic than the slow types.

Garvie and Mabbitt (1956), working with a slow variant isolated from a fast culture of Streptococcus cremoris, found the generation times and total populations of the two organisms were the same. The rates of lactose utilization and acid formation by the slow culture, however, were less than the fast. In a later paper, Garvie (1959)

reported a slowly increasing proportion of slow strains over a period of three years in cultures originally isolated as fast acid-producing. However, a moderately stable balance was observed between the two types on regular, well-controlled, propagation in milk.

Proteolytic Enzymes in Lactic Streptococci

The comparatively low proteolytic nature of slow cells of lactic acid bacteria suggested that the loss of fast acid-producing ability was due to the irreversible loss of proteolytic enzymes (Garvie and Mabbitt, 1956). The amino acid and peptide content of fresh milk is low (Deutsch and Samuelsson, 1959), and, therefore, lactic streptococci must obtain their amino nitrogen from milk proteins. Clearly, an organism lacking proteolytic enzymes to degrade milk proteins will be unable to satisfy its need for amino nitrogen. Inducible proteolytic enzymes have been demonstrated in S. lactis. Van der Zant and Nelson (1953) found that casein stimulated the synthesis of an endocellular proteinase, and a tryptic digest of casein repressed synthesis of this enzyme. Williamson, Tove, and Speck (1962) isolated an extracellular proteinase which was maximally synthesized only in the presence of casein.

Effect of Protein Fractions on Acid Production

Since acid production by slow lactic acid bacteria was limited by

their weak ability to hydrolyze milk proteins, it seems likely that protein fractions might stimulate growth and acid production. This has been verified in many laboratories. Substances such as liver fraction L (Anderson and Elliker, 1953), yeast extract (Storrs and Anderson, 1949), pancrease extract (Kizer et al., 1955), and peptone (Pollack and Lindner, 1943), when added to milk, have been shown to enhance acid development by slow cultures. Anderson and Elliker (1953) reported that peptonized milk and trypsinized milk were more stimulatory than acid hydrolyzed casein. Peptides in these adjuncts are thought to be the main growth stimulating compounds (Anderson and Elliker, 1953; Sandine, Speck, and Aurand, 1956; McAnelly and Speck, 1957).

Williamson and Speck (1962) noted that when milk was supplemented with pancrease extract, accelerated growth and acid production was accompanied by reduced proteolysis. This observation corroborated the aforementioned finding of Van der Zant and Nelson (1953) that hydrolyzed proteins repressed synthesis of proteolytic enzymes in lactic streptococci.

Effect of Free Amino Acids on Acid Production

In contrast to the effect of peptides, Anderson and Elliker (1953) found that free amino acids added to milk did not markedly increase acid production by slow lactic streptococci. Snell and his collaborators

explored this phenomenon in organisms other than S. lactis, and described several mechanisms which may account for this observation. In one case (Kihara and Snell, 1952), a high concentration of D-alanine inhibited the uptake by Lactobacillus casei of a structurally related amino acid, L-alanine, which was required for growth. However, the assimilation of a peptide containing L-alanine was not antagonized by D-alanine. In another instance (Kihara, Klatt, and Snell, 1952), cells of Streptococcus faecalis destroyed a required amino acid, tyrosine, but not its peptides, before it was used for growth. In a third study (Peter, Prescott, and Snell, 1953), Lactobacillus delbrueckii assimilated peptides of histidine with greater ease than free histidine. Mechanisms similar to these may explain why peptides, but not free amino acids, stimulate acid production by lactic streptococci.

Effect of Nucleic Acid Derivatives on Acid Production

Substances other than protein fractions have been shown to stimulate acid production in milk by lactic streptococci. Anderson and Elliker (1953) found that purine and pyrimidine bases such as adenine, guanine, and uracil accelerated growth and acid production by several strains of S. lactis and S. cremoris. This observation has been confirmed by other investigators. Dahiya and Speck (1963) reported that the rate of acid development by certain single and mixed strain lactic Streptococcus starter cultures was enhanced by the addition of

several nucleic acid derivatives; inosine was particularly effective. Koburger, Speck, and Aurand (1963) identified inosine, hypoxanthine, and adenine as stimulatory factors for acid production by a particular strain of S. lactis. A relationship between the stimulatory effects of protein digests and nucleic acid derivatives has not been established.

β -Galactosidase Synthesis in Microorganisms

Cohn (1957) and Jacob and Monod (1961) have discussed many aspects of β -galactosidase synthesis in microorganisms. The following review will be limited to topics which are directly related to the present study.

Occurrence in Microorganisms

β -galactosidase has been studied extensively in many microorganisms including Escherichia coli (Cohn and Monod, 1951), Paracolobactrum aerogenoides (Anderson and Rickenberg, 1960), Shigella sonnei (Rickenberg, 1960; Clausen and Nakamura, 1963), Bacillus subtilis (Landman, 1957), and Staphylococcus aureus (McClatchy and Rosenblum, 1963). Its activity has been demonstrated in one strain of S. lactis (Vakil and Shahani, 1962), but no biochemical or physiological properties have been reported.

Assay of β -Galactosidase

A great impetus for β -galactosidase research was the simple assay technique for the enzyme developed by Lederberg (1950). He used a chromogenic substrate, ortho-nitrophenyl- β -galactopyranoside (ONPG), which is hydrolyzed to galactose and ortho-nitrophenol. The ortho-nitrophenol liberated from ONPG forms a yellow solution, the intensity of which is proportional to enzyme activity. It is desirable to carry out this assay under conditions where the permeability of the cell membrane is not the rate limiting factor (Koppel, Porter, and Crocker, 1953). This condition may be fulfilled by preparing cell-free extracts of the enzyme or treating whole cells with toluene (Koppel, Porter, and Crocker, 1953). This latter method, toluene treatment, is easy and rapid, and is preferred by most investigators (Koppel, Porter, and Crocker, 1953).

Regulation of β -Galactosidase Synthesis

Synthesis of β -galactosidase in E. coli, the most studied microorganism in this regard, is controlled by two mechanisms: Induction and repression (Jacob and Monod, 1961). Synthesis of this enzyme is induced by lactose, which is also its natural substrate, and is repressed by glucose, a catabolite of lactose. β -Galactosides other than lactose also induce synthesis of the enzyme in E. coli. For example,

methyl- β -D-thiogalactopyranoside (TMG) and isopropyl- β -D-thiogalactopyranoside (IPTG) induce synthesis of several times more enzyme than lactose (Cohn and Monod, 1953). This may be explained by the fact that TMG and IPTG are not metabolized by this microorganism, and, consequently, there are no catabolites, such as glucose, to interfere with the induction process (Dénes, 1960). Other microorganisms respond differently to these and other inducers. This variability will be explored more fully in the discussion of results, which follows.

Although glucose inhibits synthesis of β -galactosidase in E. coli, it is not known whether or not glucose is the molecule actually responsible for repression (Faith, Giorgio, and Mallette, 1964). Some evidence has been presented that an intermediate of glucose metabolism may be the repressor (Nakada and Magasanik, 1964; McFall and Mandelstam, 1963).

Galactoside-Permease Synthesis in Microorganisms

Galactoside-permease has not been studied as extensively as β -galactosidase. Cohen and Monod (1957) have reviewed the many aspects of galactoside-permease synthesis in bacteria. This enzyme is responsible for the entry and intracellular accumulation of lactose and other galactosides. Galactoside-permease in bacteria may be studied by measuring the rate of intracellular accumulation of various

radioactive galactosides (Cohen and Monod, 1957; McClatchy and Rosenblum, 1963).

Galactoside-permease synthesis in E. coli is regulated in a manner similar to β -galactosidase (Jacob and Monod, 1961). Certain β -galactosides, such as lactose and TMG, induce synthesis of this enzyme (Rickenberg et al., 1956). Products of lactose metabolism, which repress synthesis of β -galactosidase, likewise repress synthesis of galactoside-permease (Cohen and Monod, 1957).

MATERIALS AND METHODS

Studies on Slow and Fast Acid Production

Culture and Isolation of Slow Variant

The fast acid-producing organism selected was S. lactis C2, which was obtained from the culture collection of the Department of Microbiology at Oregon State University. Slow variants were isolated from this culture by the following procedure: A milk culture of S. lactis C2 was plated by the serial dilution procedure on a lactose agar, which is described below. After incubating these plates for 24 hours at 30 C under an atmosphere of high humidity (Turner et al., 1963), 400 colonies were picked and subcultured in 10.0 ml of sterile non-fat milk, which is described below. Three of these isolates were slow, the remaining were fast. One each of a typical slow and fast acid-producing culture was selected from among these, and used throughout this study. The acid-producing abilities of these cultures were stable: The fast culture maintained its ability to coagulate milk within 18 hours at 21 C, and the slow culture retained its inability to coagulate milk within 18 hours at 21 C. The fast culture was not homogeneous, however, since slow strains, corresponding to about two percent of the total number of cells, could be isolated from it by repeating the procedure described above. The cultures were

transferred daily in sterile nonfat milk, using a 1.0 percent inoculum and an incubation temperature of 21 C.

Media

The cultures generally were propagated in 11 percent reconstituted nonfat dry milk. This milk, as indicated, was either sterilized at 121 C for 12 minutes or pasteurized at 62 C for 30 minutes. Lactose agar consisted of the following: Lactose, 10.0 g; Difco Tryptone (Difco Laboratories, Inc., Detroit, Michigan), 10.0 g; Difco yeast extract, 10.0 g; K_2HPO_4 , 6.7 g; agar, 15.0 g; and distilled water to 1.0 liter. Some experiments were carried out in a liquid medium which was composed of the following: Lactose, 10.0 g; Difco Tryptone, 10.0 g; Difco yeast extract, 10.0 g; and distilled water to 1.0 liter.

Growth and Acid Production Studies in Milk

Comparative studies of cell growth, lactic acid formation, and total acid development were conducted in milk. Flasks containing 2.0 liters of sterile nonfat milk were inoculated with ten-hour milk cultures of slow or fast cells. Inocula were 1.0 percent unless indicated otherwise. These flasks were incubated at 21 C.

At the time of inoculation and at selected intervals, 1.0 ml portions of milk were aseptically withdrawn and diluted 10^4 , 10^6 , or 10^8 in physiological saline solution. One-milliliter of the desired dilution

was placed in a Petri plate and mixed with about 25 ml of melted lactose agar. These plates, prepared in duplicate, were incubated for 48 hours at 30 C. Colony counts were expressed as viable cells per ml of medium. Microscopic examination of both slow and fast cultures revealed that the cells were predominantly in pairs, and plate count errors due to variations in cell chain length were insignificant.

When a sample was obtained for a cell count, a second sample (10.0 ml) was withdrawn, stored in a screw-capped tube, and frozen for subsequent lactic acid and total acid determination. Just before an acid determination, the milk sample was thawed and diluted tenfold with distilled water. Coagulated samples were thoroughly mixed in a cool Waring blender at high speed for two minutes. A 10.0 ml portion of this diluted milk was assayed for lactic acid by the colorimetric technique of Ling (1951). Total acid was determined by titrating 25 ml portions of the diluted milk with 0.0094 N sodium hydroxide to a pH 8.2 end point, using a Fisher titrimeter. Lactic acid and total titratable acid were expressed in μ moles per ml of medium.

Growth and Acid Production Studies in Broth

Comparative growth and total acid formation studies were also conducted in a sterile broth medium, which was described above. Flasks containing 2.0 liters of this medium were inoculated with 1.0 percent of a ten-hour slow or fast broth culture. These flasks were

incubated at 21 C. Viable cells and total acid were determined at selected intervals throughout the fermentation by the methods described above.

Proteolysis Studies in Milk

The proteolytic abilities of the slow and fast cells in milk were determined quantitatively. Since the degree of protein hydrolysis by S. lactis may be related to the amount of free amino acids produced in the medium (Van der Zant and Nelson, 1954), proteolysis was measured by the colorimetric method of Hull (1947), using the precaution which was recently reported (Citti, Sandine, and Elliker, 1963). Low levels of proteolysis could not be detected in milk sterilized by autoclaving (Citti, Sandine, and Elliker, 1963); therefore, the milk was pasteurized at 62 C for 30 minutes. Viable cell counts were conducted by the above described procedure. Flasks containing 250 ml of milk were inoculated with 1.0 percent of a ten-hour milk culture of slow or fast cells. The flasks were incubated at 21 C. At three-hour intervals, 5.0 ml samples for proteolysis determinations and 1.0 ml samples for cell counts were withdrawn aseptically from the flasks. Proteolytic activity was expressed as μ grams of tyrosine released per ml of medium.

Addition of Acid-Promoting Stimulants to Milk

The effect of various substances on acid development in milk by slow and fast strains of S. lactis C2 was also investigated. Pasteurized nonfat milk was supplemented to a concentration of 0.5 μ moles/ml with a filter-sterilized solution of adenine, hypoxanthine, adenosine, or inosine. An acid hydrolysate of casein (Casamino Acids, Difco Laboratories, Inc., Detroit, Michigan), a pancreatic digest of casein (N-Z-Amine, type A, Sheffield Chemical, Norwich, New York), or a tryptic digest of casein (N-Z-Case, Sheffield Chemical) was added to milk, before its pasteurization, to a concentration of 1.0 percent. Since acid hydrolysis destroys or partially destroys some of the amino acids found in casein (Fox and Foster, 1957), the following amino acids were added to a concentration of 0.02 percent in milk supplemented with the acid hydrolysed casein: DL-tryptophan, DL-serine, DL-tyrosine, and DL-threonine. Sterile milk was supplemented to a concentration of 0.25 percent with catalase (technical grade, Nutritional Biochemicals Corp., Cleveland, Ohio) or catalase which had been inactivated by heating at 100 C for ten minutes. These fortified milks were inoculated with slow or fast cultures of S. lactis C2, incubated at 21 C, and checked for total acid development as described above.

Studies on β -Galactosidase

Microorganisms

Several strains of S. lactis were used during the initial stage of β -galactosidase studies. S. lactis C2, C10, and E were obtained from the culture collection of the Department of Microbiology at Oregon State University, and S. lactis 7962, 7963, and 11454 were obtained from the American Type Culture Collection, Washington D. C. Cultural and biochemical characteristics of these bacteria have been reported by Sandine, Elliker, and Hayes (1962). As discussed below, only S. lactis 7962 possessed desirable β -galactosidase properties, and this organism was used throughout most of this study. A strain of E. coli B, obtained from the Department of Microbiology, Oregon State University, was also included during assays for β -galactosidase as a comparative control. All cultures were maintained by daily transfer in a lactose liquid medium, described below, with incubation at 32 C.

Media

Cultures were routinely propagated in a liquid medium which consisted of the following: Lactose, 10.0 g; Difco Tryptone, 20.0 g; Difco yeast extract, 5.0 g; gelatin, 2.5 g; sodium chloride, 4.0 g; sodium acetate, 1.5 g; ascorbic acid, 0.5 g; and distilled water to

1.0 liter. The pH of this medium was adjusted to 7.0 before sterilization at 121 C for 20 minutes. For enzyme induction experiments, the above medium was prepared without lactose, sterilized, and cooled. A filter-sterilized solution of lactose, maltose, or glucose was then added to this sugar-free broth to a concentration of 0.01 g/ml. In some experiments, maltose was added to a concentration of 0.002 g/ml.

Buffer Solutions

Cells were washed, suspended, and assayed in one of the following buffer solutions: 0.05 M sodium phosphate (pH 6.0 to 8.0), 0.05 M potassium phosphate (pH 7.0), 0.05 M tris(hydroxymethyl)amino-methane (tris) (pH 7.0), 0.05 M tris with 0.05 M sodium chloride (pH 7.0), or 0.05 M tris with 0.05 M sodium phosphate (pH 7.0). When indicated, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ or reduced glutathione was added to a concentration of 0.0004 M.

Harvesting of Cells

Culture samples were immediately chilled and spun down at 3,000 times gravity in a centrifuge refrigerated at 1 C. The harvested cells were washed twice with cold buffer.

Preparation of Whole Cell Suspensions

Washed cells were suspended in buffer solutions. Suspensions of E. coli B contained approximately 3×10^8 cells/ml and all S. lactis strains, 3×10^9 cells/ml.

Preparation of Toluene-Treated Cell Suspensions

Washed cells were suspended in buffer solution. Suspensions of E. coli B contained approximately 3×10^7 cells/ml; S. lactis 7962, 3×10^8 cells/ml; and all other S. lactis strains, 3×10^9 cells/ml. Four-ml volumes of these suspensions were treated with 0.2 ml of toluene-acetone solution (one part toluene to nine parts acetone), and incubated for five minutes at 25 C with vigorous agitation. The treated suspensions were immediately assayed for enzyme as described below.

Preparation of Cell-Free Extracts

Washed cells of S. lactis 7962 or S. lactis C2 were suspended in sodium phosphate buffer to a concentration of about 3×10^9 cells/ml, and ruptured in a Raytheon sonic oscillator for 20 minutes. The cell debris was removed by centrifugation at 9,000 time gravity for 12 minutes at 1 C. The supernatant liquid was diluted ten-fold with buffer, and assayed for enzyme as outlined below.

Assay of β -Galactosidase

The chromogenic substrate ortho-nitrophenyl- β -D-galactopyranoside (ONPG) was used to measure enzyme activity (Lederberg, 1950). Solutions of 0.005 M ONPG were prepared in the above buffers. A 1.0 ml volume of whole-cell suspension, toluene-treated cell suspension, or cell-free extract was incubated with 4.0 ml of ONPG solution for 15 minutes at, unless otherwise indicated, 37 C. Color development was stopped by adding 5.0 ml of cold 0.5 M sodium carbonate to the reaction mixture. Cells were removed from the assay mixture by centrifugation at 9,000 times gravity for 12 minutes at 1 C. The absorbance of the supernatant liquid was measured at 420 m μ . The μ moles of ortho-nitrophenol liberated from ONPG were determined from a standard curve, which was prepared by measuring the change in absorbance produced by varying concentrations of ortho-nitrophenol. The amount of ortho-nitrophenol liberated per minute was directly proportional to the quantity of cells in the assay mixture. The dry weight of a cell suspension was obtained from a standard curve relating cell suspension optical density at 420 m μ to cell dry weight (Society of American Bacteriologists, 1957). The specific activity of β -galactosidase is expressed as μ moles of ortho-nitrophenol liberated from ONPG per minute. The differential rate of β -galactosidase induction, P, is defined as the differential rate of synthesis, and was

obtained from the slope of a plot of enzyme units per ml against cell dry weight per ml.

Induction of Enzyme Synthesis

Lactose was used to induce β -galactosidase synthesis by the several strains of S. lactis and the one strain of E. coli. Using a 1.0 percent inoculum, a 12-hour culture was inoculated into broth which contained 0.01 g/ml lactose. The inoculated broth was incubated at 32 C for ten hours. The cells were then immediately harvested and assayed for enzyme as described above. Also, seven- to ten-hour cultures of S. lactis 7962 were prepared in this manner and used to find the optimal assay pH and temperature.

The inducing ability of galactose, glucose, methyl- β -D-thiogalactopyranoside (TMG), isopropyl- β -D-thiogalactopyranoside (IPTG), ortho-nitrophenyl- β -D-galactopyranoside (ONPG), para-nitrophenyl- β -D-galactopyranoside (PNPG), melibiose, calcium lactobionate, and borate was compared to that of lactose, with use of a modified procedure of Dénes (1960). A 12-hour culture of S. lactis 7962, which was grown at 32 C in maltose broth, served as the inoculum. Three-ml of this culture were inoculated into 297 ml of broth containing 0.002 g/ml maltose as the only carbohydrate. This culture was incubated for three hours at 32 C, and then divided into several equal portions. A filter-sterilized solution of the compound or compounds

to be tested was added to one of these portions to a concentration of 0.01 M, or, in the case of borate (as boric acid, pH 7.0), to 0.05 M. The cultures were incubated for an additional 4.5 to 10.5 hours. During this period, cell samples were obtained at 1.5 hour intervals, and immediately chilled, harvested, treated with toluene, and assayed for enzyme activity as described above.

Procedure for Catabolite Repression Studies

The effect of glucose and galactose on enzyme synthesis was examined by a modified method of Dénes (1960). A 12-hour culture of S. lactis 7962 grown in maltose broth at 32 C served as the inoculum. Two-ml of this culture were inoculated into 198 ml of broth which contained 0.002 g/ml maltose as the sole carbohydrate. After incubating for three hours at 32 C, the culture was divided into five portions of 40 ml each. A filter-sterilized lactose solution was added to four of these cultures to a concentration of 0.01 M. After an additional incubation period of 1.5 hours, a solution of glucose, galactose, or glucose and galactose was added to one of three lactose-induced cultures. Each of these sugars was present at a concentration of 0.005 M. One of the five cultures contained no lactose, glucose, or galactose, and another contained lactose, but no glucose or galactose. Samples were removed from each of these five cultures at 1.5 hour intervals throughout this experiment, and immediately chilled. The cells were

harvested, treated with toluene, and assayed for β -galactosidase as described above.

Studies on Galactoside-Permease

Assay of Galactoside-Permease

The presence and activity of a galactoside-permease in S. lactis 7962 was measured by the intracellular accumulation of radioactive lactose, maltose, or TMG. Solutions of lactose-1-C¹⁴ (California Corporation for Biochemical Research, Los Angeles, California), maltose-1-C¹⁴ (California Corporation for Biochemical Research), or TMG-C¹⁴ (New England Nuclear Corp., Boston, Massachusetts) were prepared in sodium phosphate-chloramphenicol buffer (0.05 M sodium phosphate, pH 7.0, and 50 μ g/ml chloramphenicol). The specific radioactivity of each solution was 20 μ curies/mmole, and each compound was present at a concentration of 0.1 M.

Media

The growth media used for this study were of the same composition as those used for β -galactosidase studies, and were described above.

Preparation of Cell Suspensions

Resting cell suspensions of S. lactis 7962 were prepared in the

following manner: The microorganism was grown at 32 C in 200 ml of broth which contained 0.01 g/ml lactose, 0.01 g/ml maltose, 0.01 g/ml glucose, or 0.01 g/ml lactose and 0.01 M glucose. The cells were harvested during the exponential growth phase when they attained a population corresponding to 2.5 mg dry weight per ml. These cells were washed twice with 0.05 M sodium phosphate buffer, pH 7.0, in a centrifuge refrigerated at 1 C, and suspended to a concentration of 35 mg dry weight per ml in cold sodium phosphate-chloramphenicol buffer.

Sampling Procedure

A 10.0 ml quantity of a cell suspension was mixed with a 10.0 ml quantity of one of the above radioactive solutions, and this mixture was incubated at 32 C for 20 to 40 minutes. A 2.0 ml portion was withdrawn from this mixture at various intervals, and immediately chilled to 0 C in a glass centrifuge tube, which had been placed in an ice bath. Each sample was washed three times with ice-cold sodium phosphate buffer in a refrigerated (1 C) centrifuge, and then suspended in 2.0 ml of distilled water.

Counting of Samples

The radioactivity of each washed cell suspension was determined with a liquid scintillation counter (Tri-Carb, model 3000, Packard

Instrument Co., Inc., Downers Grove, Illinois). The counting fluor solution consisted of the following: 2,5-diphenyloxazole (Packard Instrument Co., Inc.), 4.0 g; 1,4-bis-2-(5-phenyloxazolyl)benzene (Packard Instrument Co., Inc.), 0.2 g; naphthalene, 60 g; absolute methyl alcohol, 100 ml; ethylene glycol, 20 ml; and dioxane to 1.0 liter.

One-half ml of a cell suspension, which had been incubated with a radioactive substrate and washed as described above, was placed into a glass counting vial (Packard Instrument Co., Inc.), and mixed thoroughly with 19.5 ml of the above fluor solution. Cells which had been incubated at 0C with the radioactive compound being tested, and which had been washed as described above, served as a control and blank. Each counting sample was prepared in triplicate, and each was counted twice for ten minutes at a voltage setting of 19 percent. The results are expressed in counts per minute (CPM) per mg cell dry weight.

RESULTS

Studies on Slow and Fast Acid Production

Growth Characteristics in Milk

Initial experiments demonstrated the growth characteristics of the slow and fast cells of S. lactis C2 in milk. Typical growth curves of parallel slow and fast cultures plotted semilogarithmically are shown in Figure 1. Although a 1.0 percent inoculum of each culture was used, approximately four times as many fast cells existed throughout the various phases of growth. However, it may be seen that the exponential phase generation times were the same (1.4 hours) for each type of culture.

These results suggested that if there were initially present as many slow cells as fast, there would be present throughout the fermentation the same number of these two types of cells. Figure 2, however, shows that this was not the case. When the amount of inoculum was adjusted so as to provide nearly equal amounts of fast and slow cells in each culture at the beginning of the incubation period, the fast strain overtook the slow in total population during the late phases of growth. Although there were only 1.3 times as many fast cells as slow cells present initially, the difference in population increased to nearly three after 18 hours of incubation. Despite this

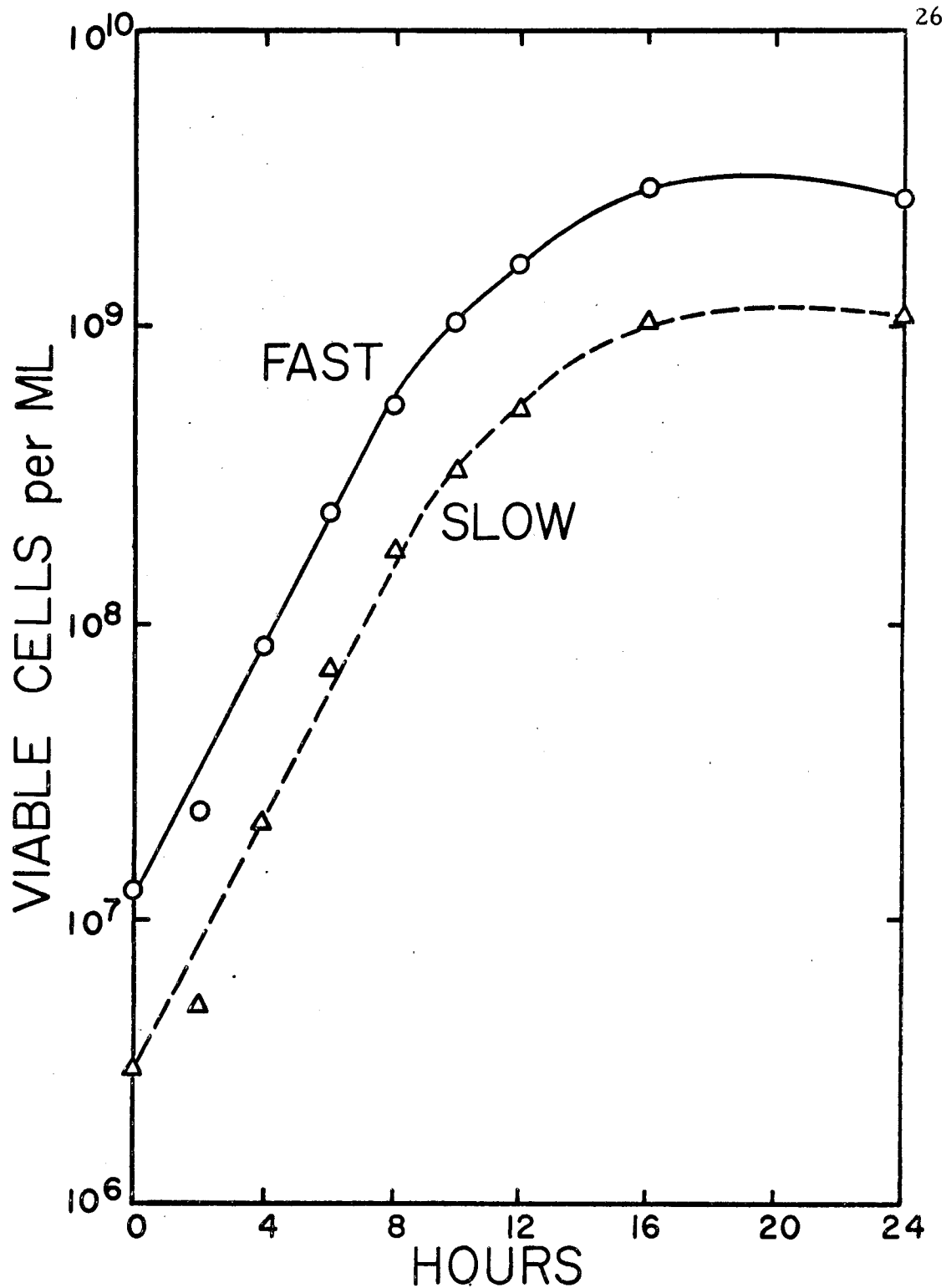


Figure 1. Comparison of growth of slow and fast cultures of *S. lactis* C2 incubated at 21 C in sterile nonfat milk. Data plotted semilogarithmically.

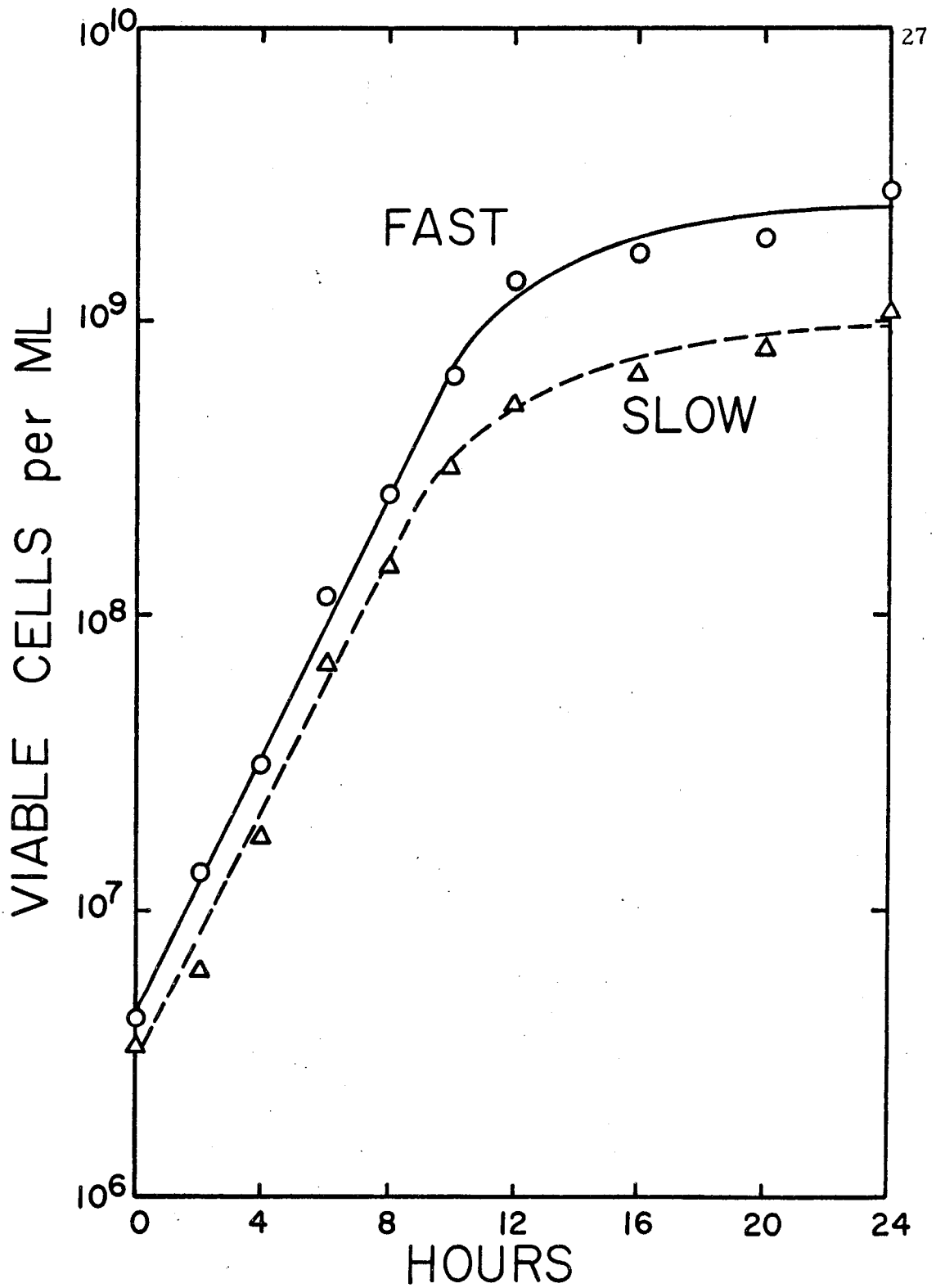


Figure 2. Comparison of growth curves of slow and fast cultures of *S. lactis* C2, starting with approximately equal numbers of each, upon incubation at 21 C in sterile nonfat milk. Data plotted semilogarithmically.

adjustment to equalized the number of the two types of cells, the slow culture was still unable to coagulate milk in 18 hours at 21 C; the fast culture was consistent in its ability to do this.

Inspection of Figure 2 also reveals that the fast culture was in logarithmic growth a few hours longer than the slow culture. Since every 1.4 hours of exponential growth at 21 C resulted in a doubling of the population, the fast culture attained a higher cell population. These differences between the fast and slow strains were constant and repeated experimentally several times.

Acid Development in Milk

Comparative studies of lactic acid and total titratable acid formed by the cultures in milk were conducted. As illustrated by a typical arithmetic plot shown in Figure 3, growth and acid production were directly related. Although the total amount of acid produced by the slow culture was less than that of the fast, the amounts of acid produced per cell were the same.

Proteolysis in Milk

Figure 4 shows the relative proteolytic abilities of the slow and fast cultures in milk. Throughout its growth, the fast culture was more proteolytic than the slow. From these data, the proteolytic ability per cell was calculated, and, from 9 to 15 hours, the fast cells

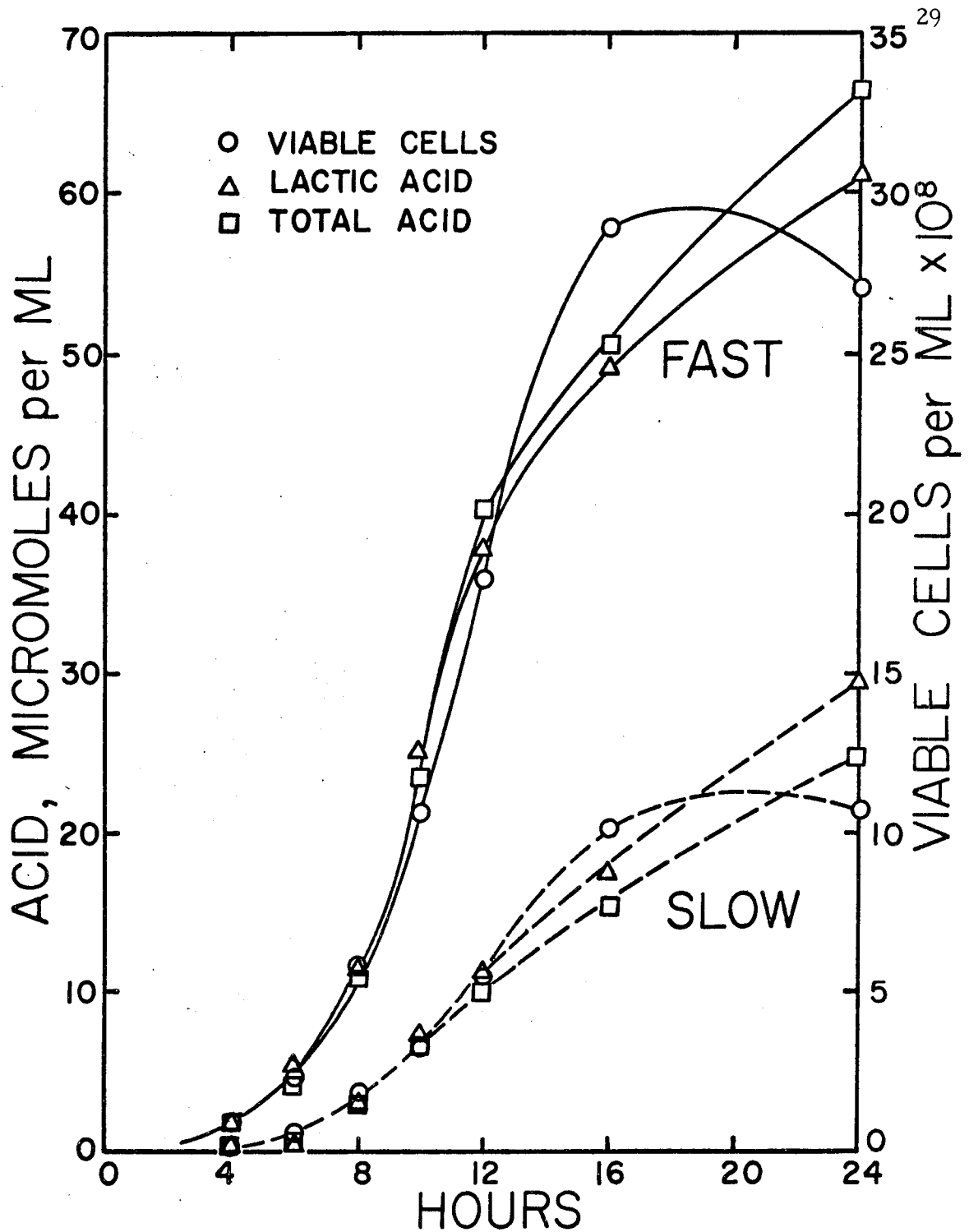


Figure 3. Comparison of total acid, lactic acid, and viable cells of *S. lactis* C2 in sterile nonfat milk at 21 C. Data plotted arithmetically.

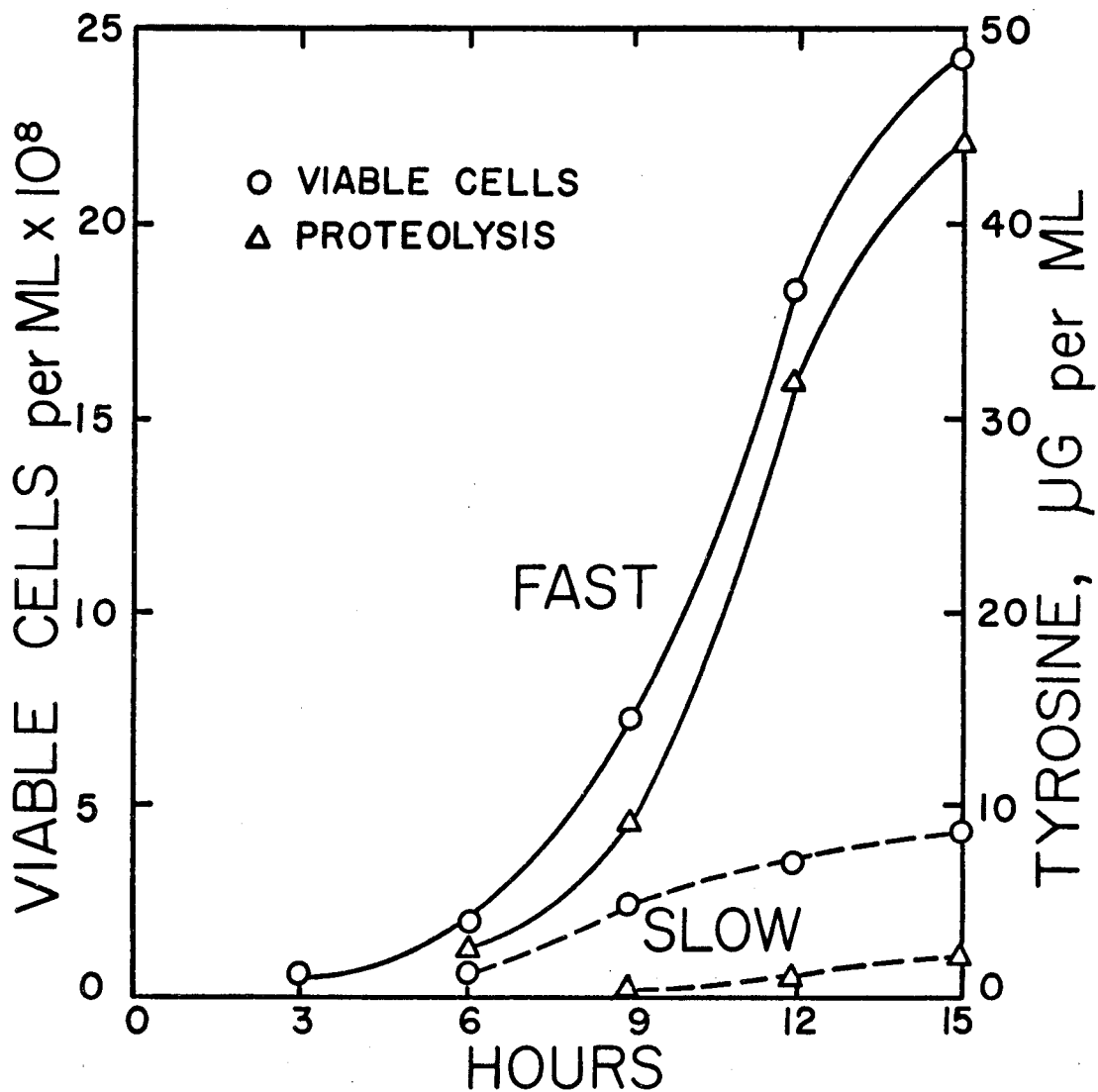


Figure 4. Comparison of cell populations and proteolysis of slow and fast cultures of *S. lactis* C2 incubated in pasteurized nonfat milk at 21 C. Data plotted arithmetically.

were four times more proteolytic than the slow.

Growth and Acid Production in Broth

It was desired to perform enzyme analyses on slow and fast cultures. However, cells could not be collected from milk cultures by centrifugation or filtration, and attempts to develop a clear medium in which the slow and fast characters of these organisms were expressed have not been successful. Furthermore, the two types of cultures revealed identical growth and acid-forming characteristics in a lactose-Tryptone-yeast extract medium as shown in Figure 5.

Effect of Various Supplements on Acid Development

Acid production by slow and fast strains of S. lactis C2 in milk which was fortified with various substances was also examined.

Casein hydrolysates. The effect of casein hydrolysates on acid production is shown in Table I. Tryptic and pancreatic digests stimulated acid development by both the slow and fast bacteria. Acid hydrolyzed casein, however, had no appreciable effect on acid development by either organism. The acid digest of casein was supplemented with amino acids that are known to be destroyed or partially destroyed by acid hydrolysis (Fox and Foster, 1957); however, acid production by the slow or fast strain still was not significantly enhanced.

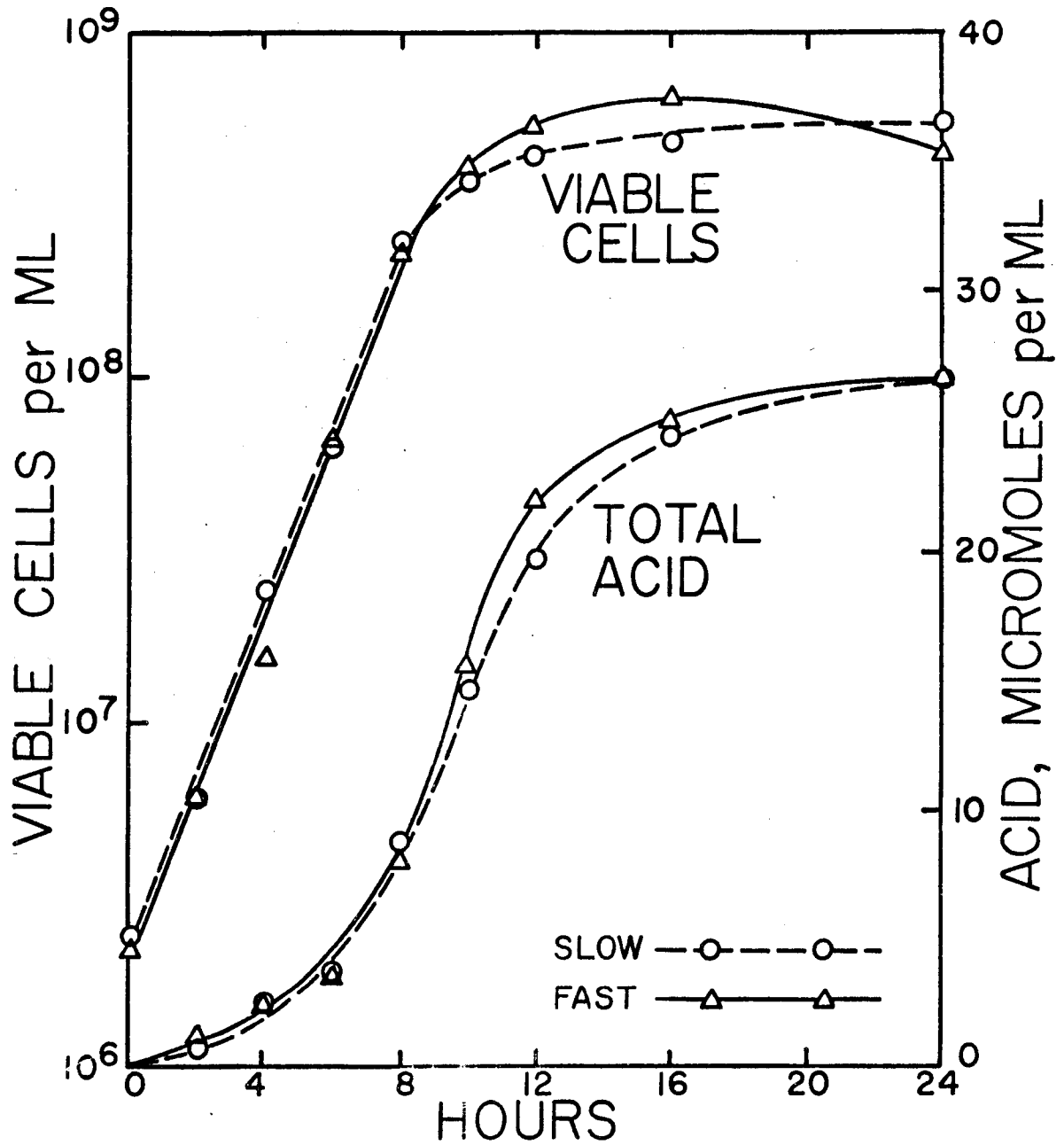


Figure 5. Comparison of total acid and viable cells produced by slow and fast cultures of *S. lactis* C2 incubated in sterile broth at 21 C. Viable cell data plotted semi-logarithmically; acid data plotted arithmetically.

TABLE I. EFFECT OF CASEIN HYDROLYSATES ON ACID PRODUCTION IN PASTEURIZED NON-FAT MILK BY SLOW AND FAST STRAINS OF S. LACTIS C2 INCUBATED FOR 10 HOURS AT 21 C.

Strain	Casein digest	Type of digest	Percent	Total acid, μmoles/ml	Percent increase
fast	none (control)	-	-	30	-
fast	N-Z-Case	tryptic	1.0	70	134
fast	N-Z-Amine	pancreatic	1.0	64	113
fast	Casamino	acid	1.0	32	nil
fast	Casamino ¹	acid	1.0	35	nil
slow	none (control)	-	-	4	-
slow	N-Z-Case	tryptic	1.0	24	500
slow	N-Z-Amine	pancreatic	1.0	16	300
slow	Casamino	acid	1.0	4	nil
slow	Casamino ¹	acid	1.0	5	nil

¹Supplemented with 0.02 percent tryptophan, serine, tyrosine, and threonine.

Purine bases and nucleosides. The effect of purine bases and nucleosides on acid production in milk by the slow and fast strains is shown in Table II. The rate of acid production by the fast culture was increased by adenine, adenosine, inosine, and hypoxanthine. However, none of these compounds affected acid development by the slow bacterium. As shown in Figure 6, acid production by the fast strain in milk fortified with adenosine was accompanied by nearly proportional increases in viable population and proteolytic activity.

Catalase. Figure 7 illustrates the effect of catalase on acid development by the slow and fast strains. Milk cultures which were un-supplemented or supplemented with inactivated catalase served as comparative controls. Catalase stimulated only slightly the rate of acid formation by the fast strain; the slow strain was stimulated more markedly. However, as also shown in Figure 7, both the incubation time and total acid development required to effect coagulation were sharply reduced in the catalase-fortified slow and fast cultures.

Studies on β -Galactosidase

Enzyme Activity of Lactose-Induced Cells

The β -galactosidase specific activities of whole cell suspensions of various strains of S. lactis and one strain of E. coli are presented in Table III. The specific activity of enzyme of untreated cells of the

TABLE II. EFFECT OF NUCLEIC ACID DERIVATIVES ON ACID PRODUCTION IN PASTEURIZED NONFAT MILK BY SLOW AND FAST STRAINS OF S. LACTIS C2 INCUBATED AT 21 C.

Strain	Nucleic acid derivative	Conc., μmoles/ml	Culture age, hr.	Total acid, μmoles/ml	Percent increase
fast	none (control)	-	12	42	-
fast	adenosine	0.5	12	57	36
fast	adenine	0.5	12	54	28
fast	inosine	0.5	12	56	33
fast	hypoxanthine	0.5	12	54	28
slow	none (control)	-	16	8	-
slow	adenosine	0.5	16	8	nil
slow	adenine	0.5	16	8	nil
slow	inosine	0.5	16	8	nil
slow	hypoxanthine	0.5	16	8	nil

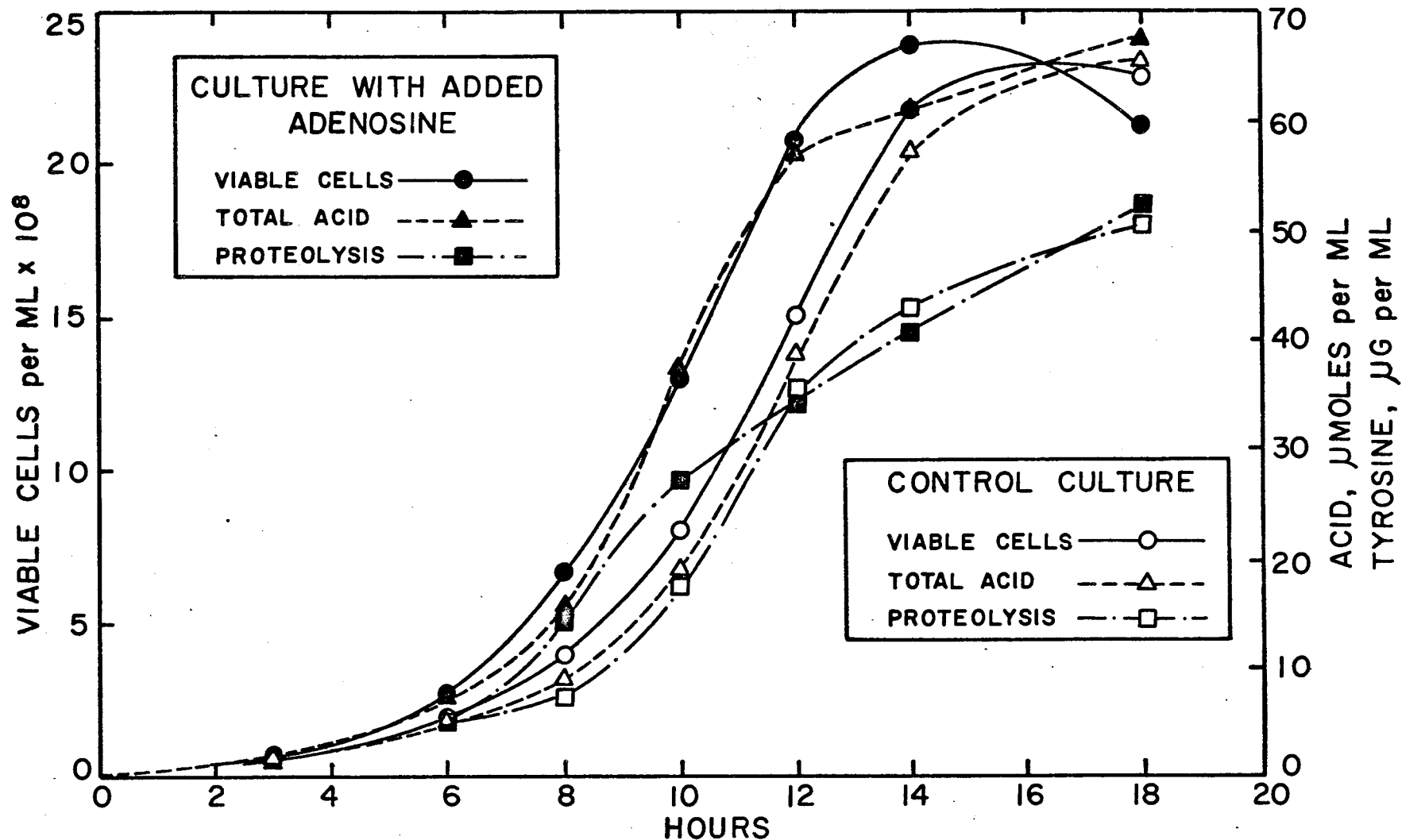


Figure 6. Effect of adenosine on proteolysis, cell growth, and acid production by a fast strain of *S. lactis* C2 grown in pasteurized nonfat milk at 21 C.

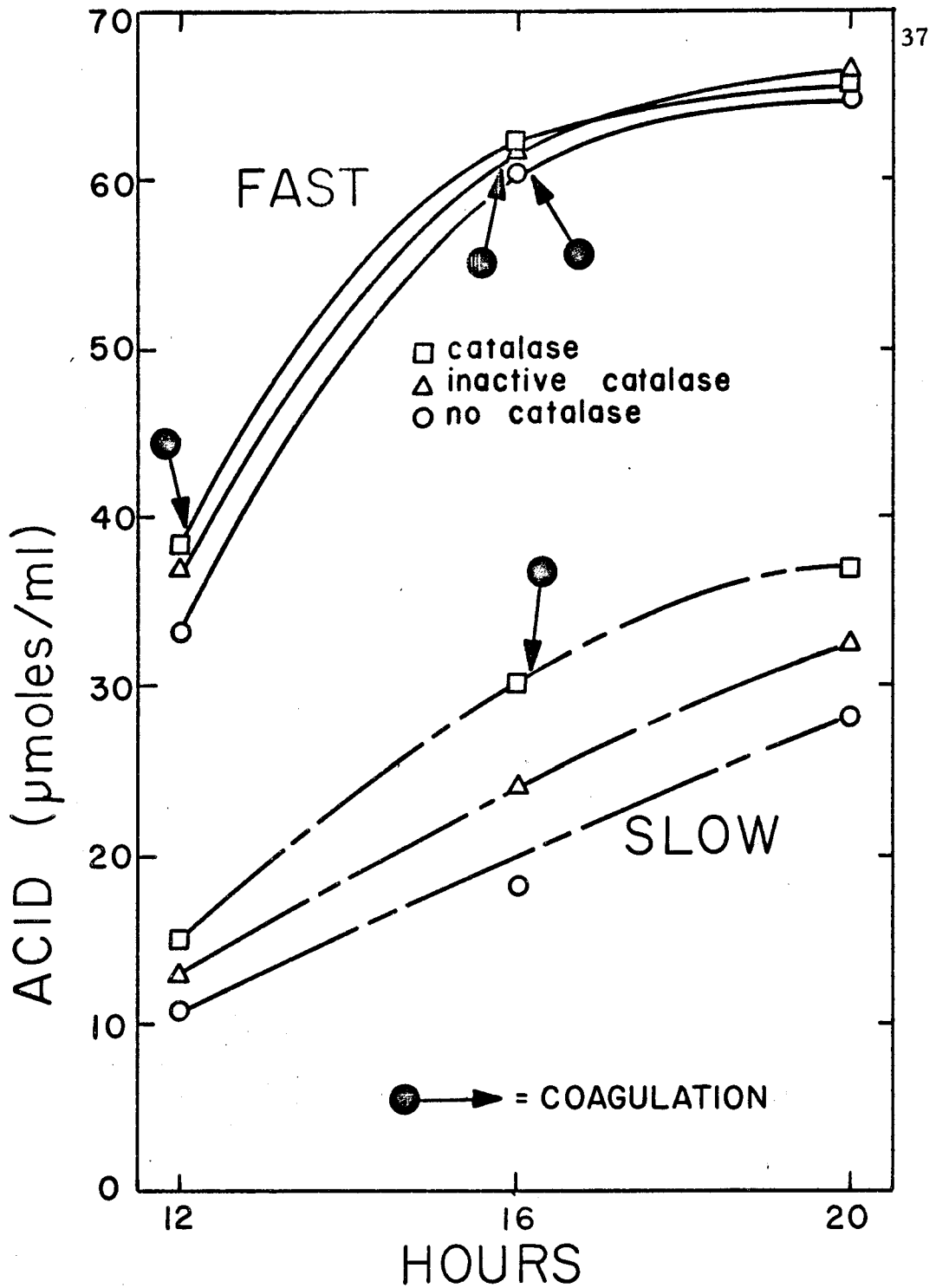


Figure 7. Effect of catalase on production of acid and coagulation of milk by slow and fast strains of *S. lactis* C2 grown in sterile nonfat milk at 21 C.

TABLE III. SPECIFIC ACTIVITY OF β -GALACTOSIDASE IN UNTREATED WHOLE CELLS AND TOLUENE-TREATED CELLS OF VARIOUS ORGANISMS. ¹

Culture	Untreated cells	Toluene-treated cells
<u>Escherichia coli</u> B	0.40	6.6
<u>Streptococcus lactis</u> C2	0.015	<0.001
<u>S. lactis</u> C10	0.033	<0.001
<u>S. lactis</u> E	0.014	<0.001
<u>S. lactis</u> 7963	0.020	<0.001
<u>S. lactis</u> 11454	0.031	<0.001
<u>S. lactis</u> 7962	0.050	0.77

¹ Assays were in sodium phosphate buffer with 0.0004 M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ at pH 7.0 and 37 C.

S. lactis strains ranged from 0.014 to 0.050. The enzyme in cells of E. coli B, assayed in the same manner, had a specific activity of 0.40; however, this activity increased more than ten-fold after toluene treatment. The β -galactosidase activity of all but one strain of S. lactis was destroyed upon toluene treatment. Manganese ion (as MnCl_2), which is known to stabilize the β -galactosidase in Streptococcus faecium (Buecher and Brock, 1962) did not stabilize the enzyme in the other strains. Neither decreasing the time of toluene treatment from five minutes to one minute, nor reducing the assay incubation temperature from 37 to 30 or 25 C affected the apparently unstable nature of the enzyme in these S. lactis strains. In a follow-up study, cell

suspensions of 40 Streptococcus cremoris strains were assayed for β -galactosidase in this manner. None of these strains produced an enzyme stable to toluene treatment.

The enzyme in S. lactis 7962, however, was stable and about ten times more active after toluene treatment. Increased toluene treatment times of up to 20 minutes did not change the activity of the enzyme in this bacterium. Although the cells were no longer viable after toluene treatment, microscopic examination revealed no cell lysis. The enzyme in this strain was also stable in cell-free extracts prepared by sonic treatment. However, its specific activity (0.43) was nearly one-half that of toluene-treated cells (0.77). In contrast, stable enzyme in cell-free extracts of S. lactis C2 could not be prepared by sonic treatment, disruption in a French press, or grinding with alumina or extra-fine glass beads. Neither manganese ion or reduced glutathione stabilized the enzyme in these preparations. S. lactis C2 was also sonic treated in the presence of lactose, the natural substrate of the enzyme. The cell-free extract was passed through a Sephadex G-50 column to remove the lactose prior to enzyme assay. Enzyme stability could not be preserved by this procedure.

Optimal Enzyme Assay Conditions

Lactose-induced cells of S. lactis 7962 were used to determine the optimal β -galactosidase assay conditions. The effect of assay

incubation temperature on enzyme activity is shown in Figure 8. The activity of toluene-treated cells increased with temperature up to 40 C, and then dropped sharply. A temperature of 37 C was used for the routine assay of β -galactosidase. In contrast to toluene-treated cells, untreated whole cells exhibited increasing activity with temperature up to 50 C. The drop in activity beyond this temperature was less rapid than with toluene-treated cells. The effect on toluene-treated cells of the assay solution pH is shown in Figure 9. The optimal pH was about 7.0. Table IV shows the effect on enzyme activity of various buffers used to prepare the assay solution. Assays performed in sodium phosphate buffer gave the highest enzyme activity, and tris buffer gave the lowest activity. Some activity was restored if tris buffer was supplemented with sodium chloride, or, especially, sodium phosphate.

Differential Induction Rates

Figure 10 shows the induction rate of β -galactosidase of cells grown in a lactose broth. The rate of induction approached a constant value of 0.82 units/mg, which was maintained for the remaining period of growth. Figures 11 and 12 compare the effect of various compounds on the rate of induction. (The data shown in Figures 11 and 12 were obtained six months after the data presented elsewhere on β -galactosidase synthesis, and show slightly lower differential

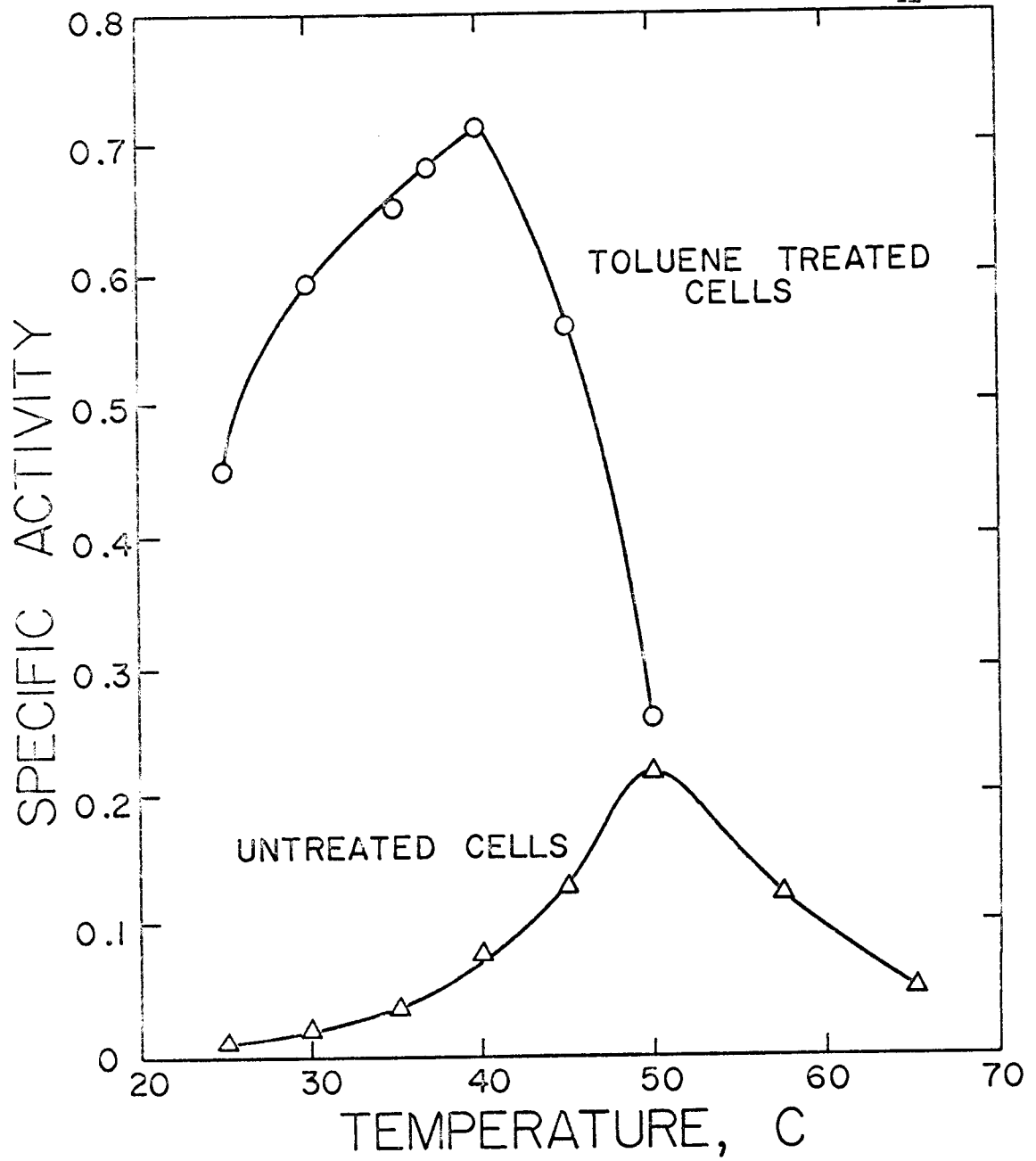


Figure 8. Effect of temperature on specific activity of β -galactosidase in untreated and toluene-treated cells of S. lactis 7962.

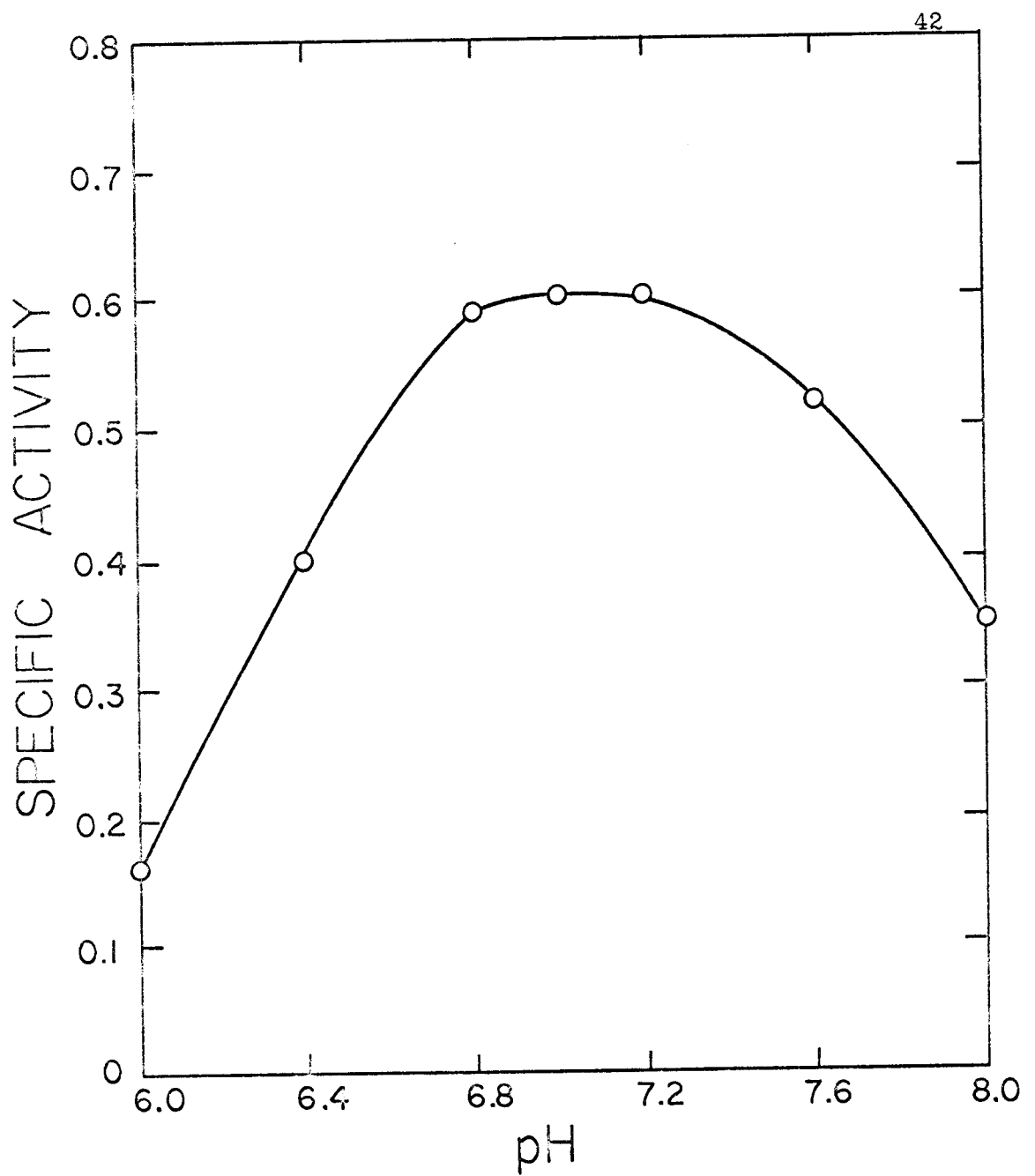


Figure 9. Effect of pH on specific activity of β -galactosidase in toluene-treated cells of S. lactis 7962.

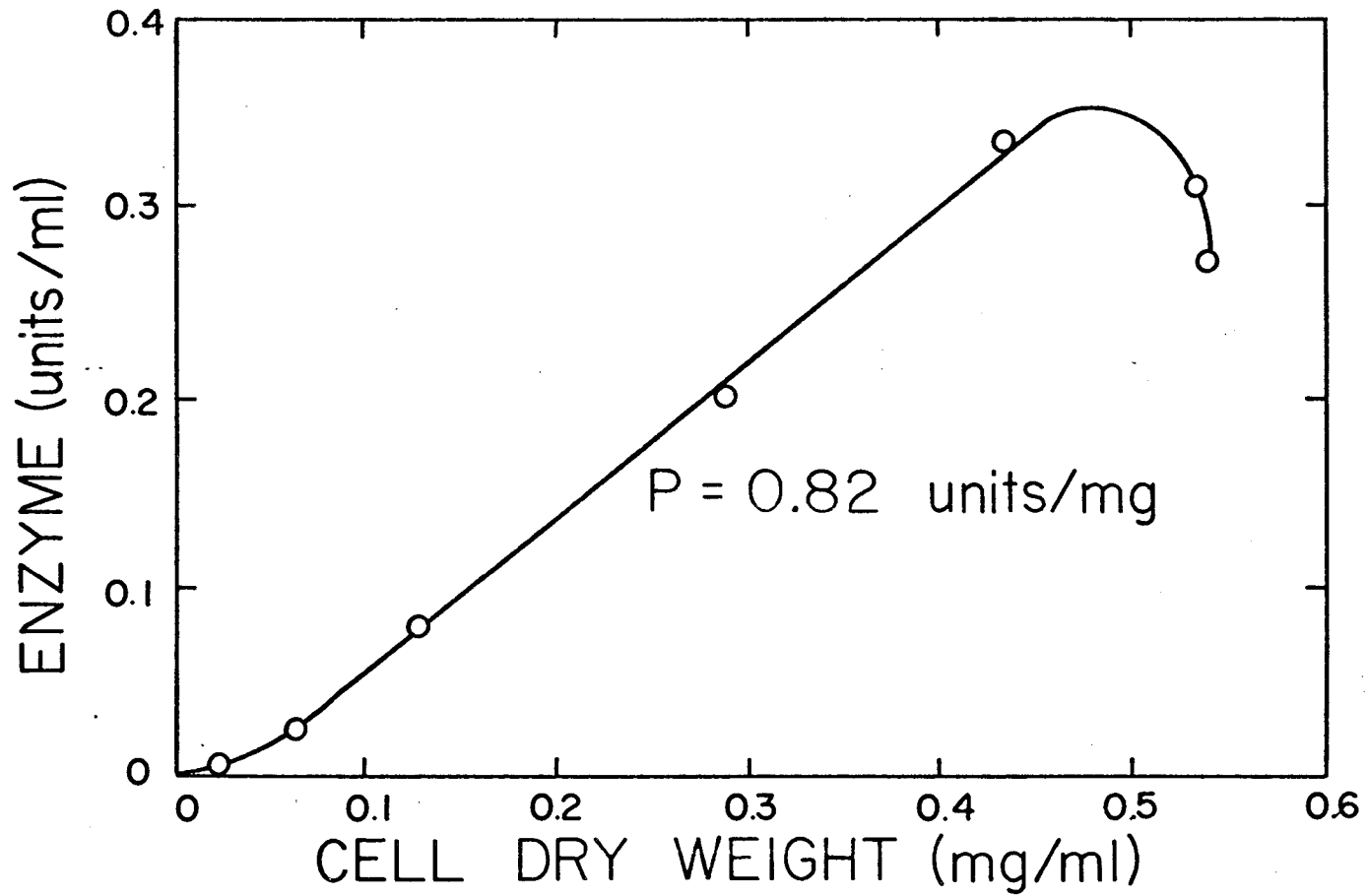


Figure 10. Differential rate of β -galactosidase induction (P) of *S. lactis* 7962 grown in lactose broth.

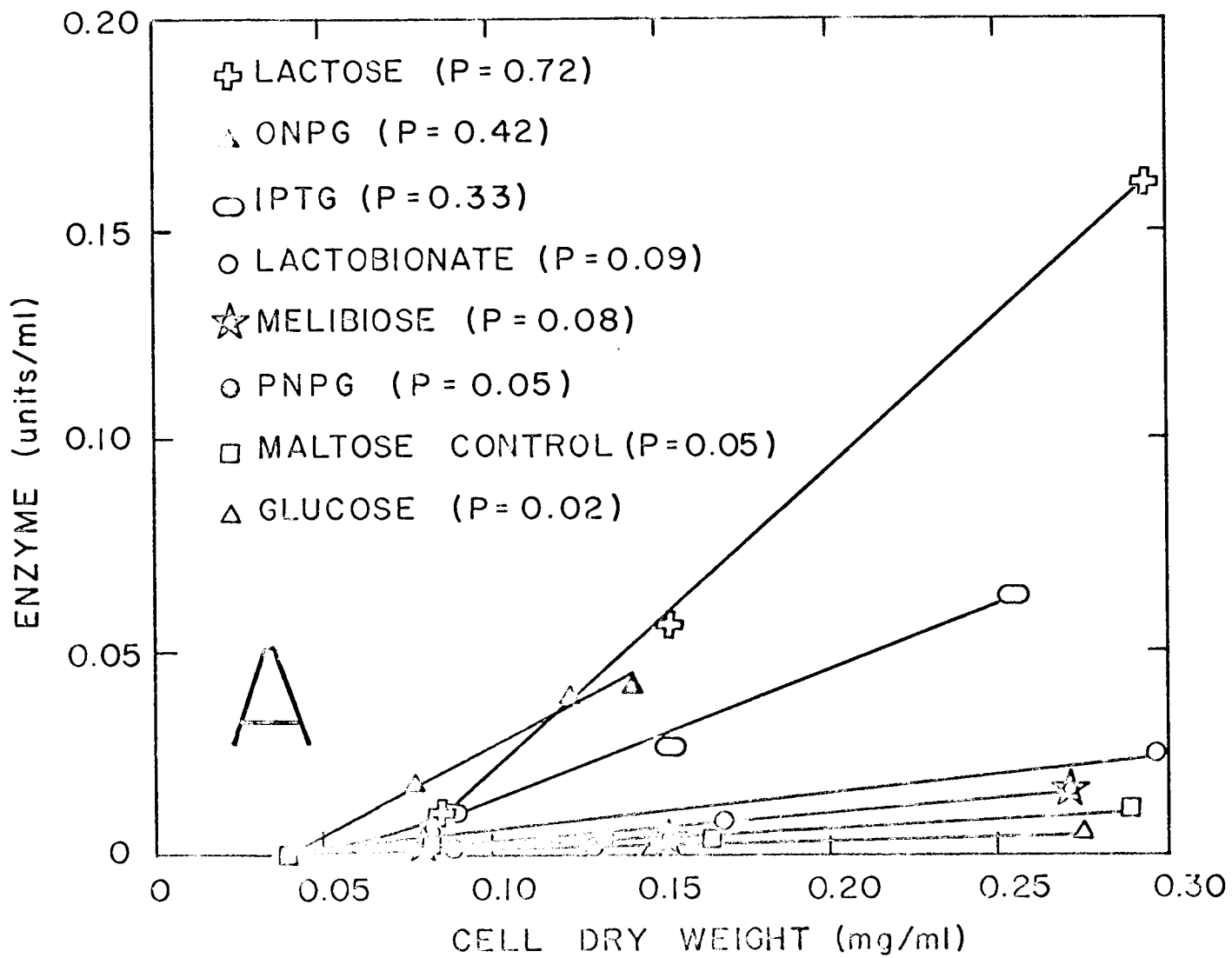


Figure 11. Effect of various compounds on the differential induction rate (P) of p-galactosidase of *S. lactis* 7962.

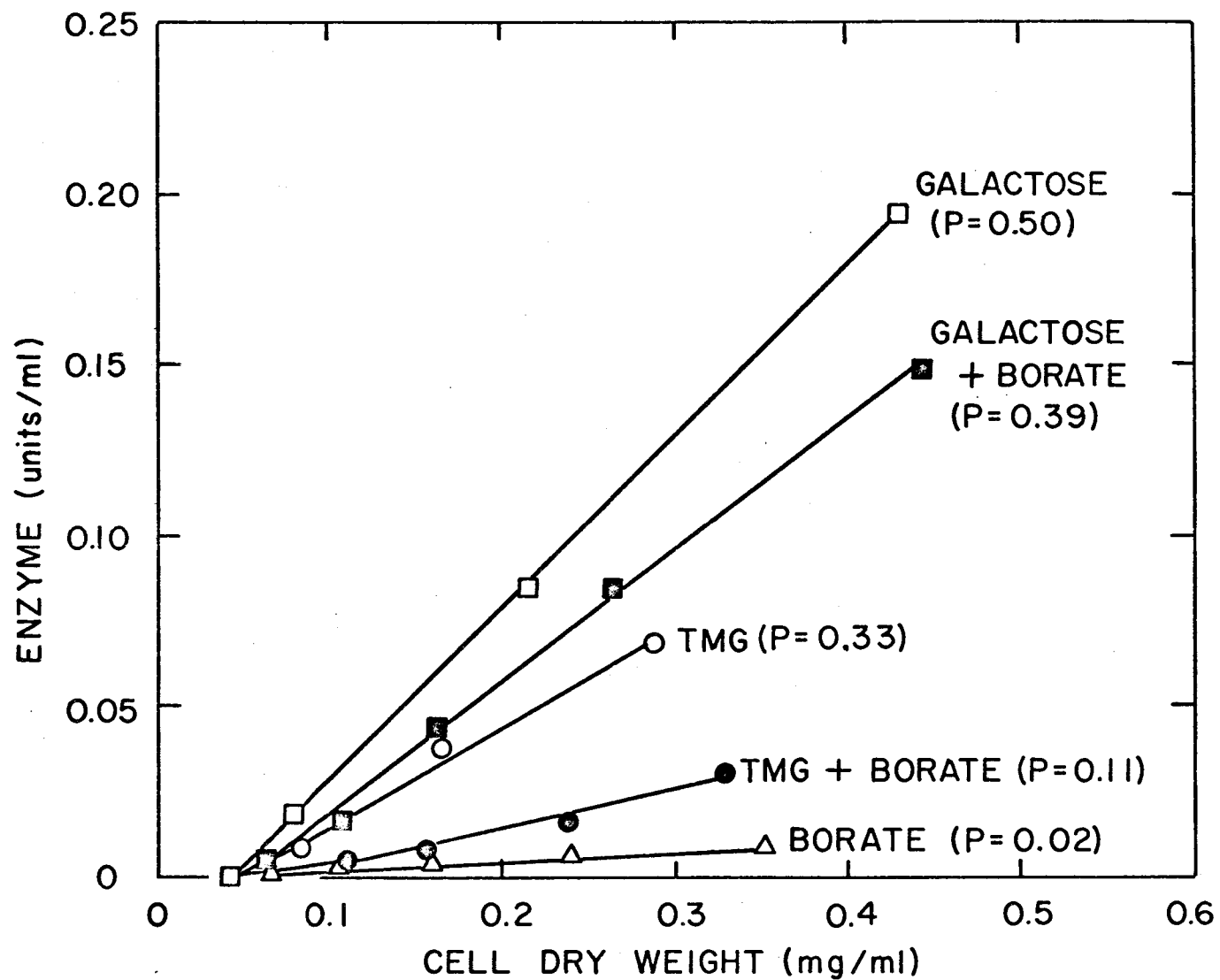


Figure 12. Effect of various compounds on the differential induction rate (P) of β -galactosidase of *S. lactis* 7962.

TABLE IV. EFFECT OF VARIOUS BUFFERS ON β -GALACTOSIDASE ACTIVITY.¹

Assay buffer solution	β -Galactosidase specific activity
0.05 M sodium phosphate	0.75
0.05 M potassium phosphate	0.48
0.05 M tris(hydroxymethyl)aminomethane (tris)	0.02
0.05 M tris with 0.05 M sodium chloride	0.27
0.05 M tris with 0.05 M sodium phosphate	0.51

¹All assays were performed at 37 C and pH 7.0 with toluene treated cells of a 10 hour lactose-induced culture of S. lactis 7962.

rates of induction. For example, the rate induced by lactose is 0.72 in Figure 11 and 0.82 in Figure 10. This difference in response to inducers appears not to be due to a variation in the microorganism. A culture preserved by freezing at the time of the earlier experiments was later compared to a culture maintained by daily transfers throughout this study. The growth and induction characteristics of both these cultures were identical.)

Certain nonmetabolizable compounds, such as IPTG and TMG, induce higher rates of β -galactosidase synthesis than lactose in E. coli (Cohn and Monod, 1953). However, these compounds induced synthesis of β -galactosidase in S. lactis 7962 less effectively than lactose. Rotman (1964) recently showed that borate plus galactose

induced a greater rate of β -galactosidase synthesis in E. coli than either galactose or borate alone; however, as shown in Figure 12, this was not true of S. lactis 7962. Borate also caused a 30 to 50 percent reduction in growth. Melibiose and lactobionate were also poor inducers. The assay substrate, ONPG, induced synthesis of enzyme less effectively than lactose. PNPG was not an inducer. Both ONPG and PNPG, which were hydrolyzed by the cells, retarded growth of the culture.

Repression of Enzyme Synthesis

Figure 13 demonstrates the effect of exogenously supplied glucose and galactose on β -galactosidase synthesis in lactose-induced cells of S. lactis 7962. Glucose especially repressed enzyme synthesis; galactose only slightly inhibited synthesis.

Studies on Galactoside-Permease

Uptake of Lactose

Figure 14 shows the rate of uptake of lactose- C^{14} by cells of S. lactis 7962 which were grown in a medium containing lactose, maltose, glucose, or lactose and glucose. Lactose grown cells accumulated lactose- C^{14} at a higher rate than maltose or, especially, glucose grown cells. Cells which were grown in a medium containing both

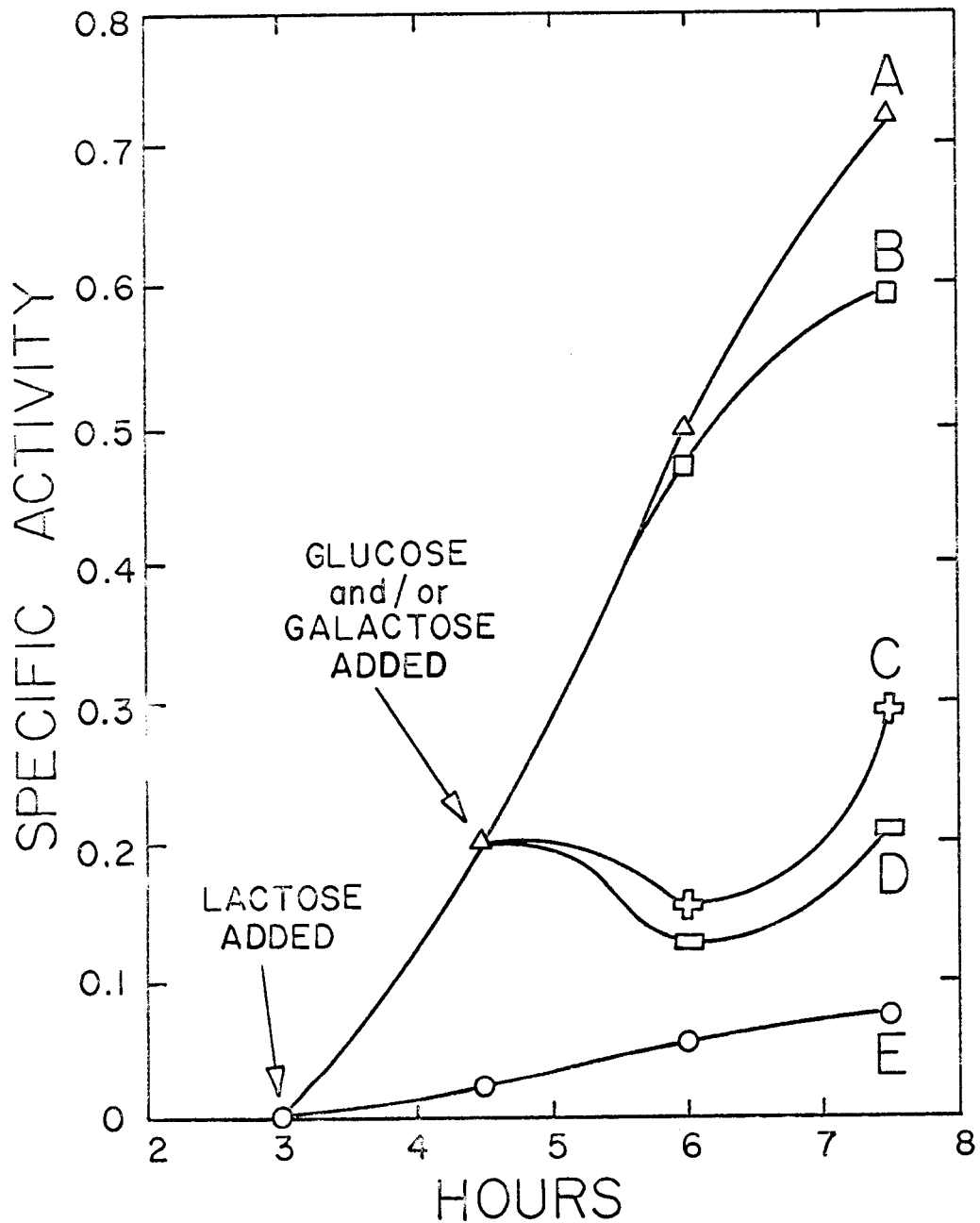


Figure 13. Effect of various sugars on synthesis of β -galactosidase in *S. lactis* 7962. Maltose-grown cells were suspended in broth and lactose added at 3 hours. No other additions, curve A; galactose added, curve B; glucose added, curve C; glucose and galactose added, curve D; non-lactose control, curve E.

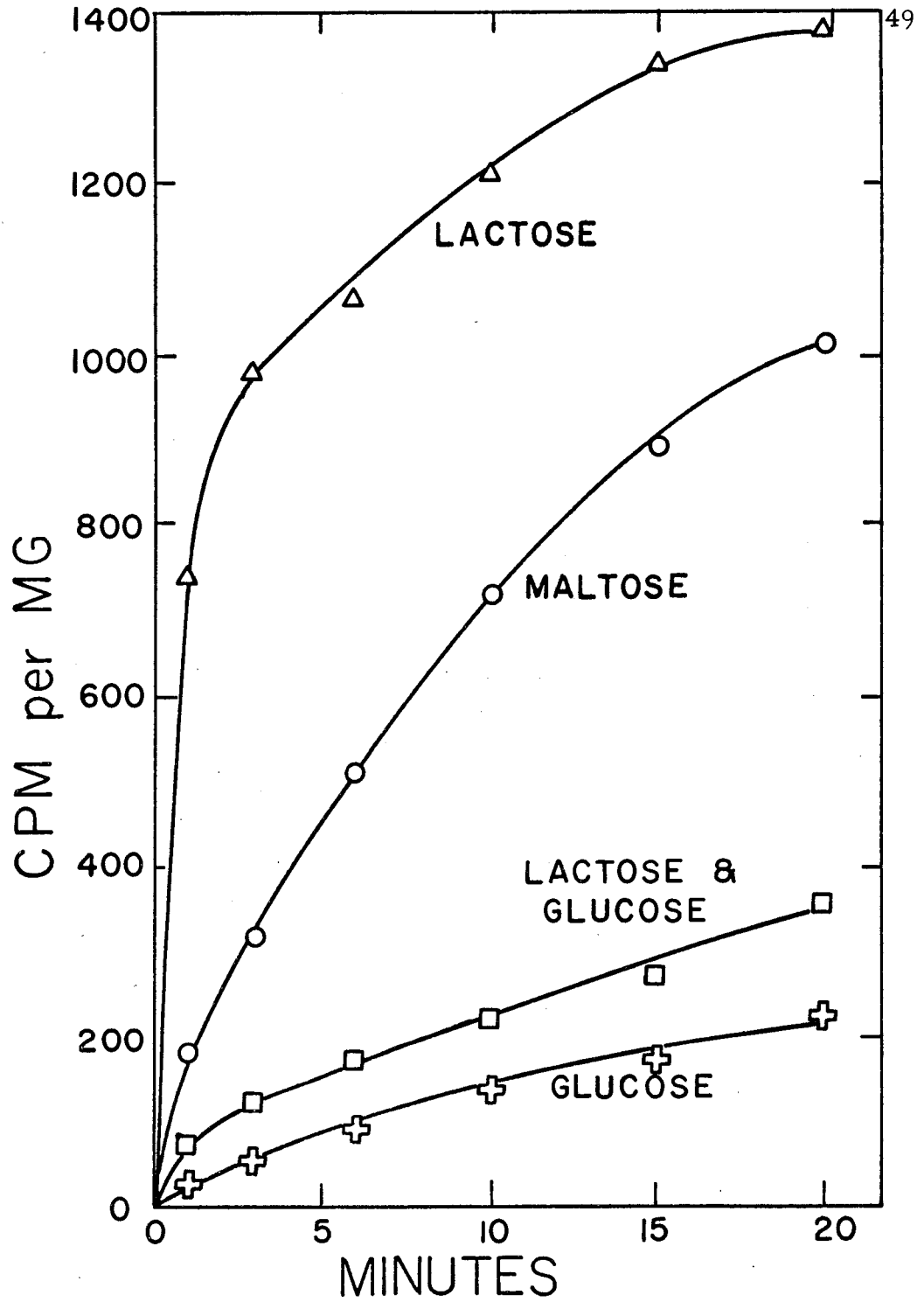


Figure 14. Intracellular accumulation of lactose-C¹⁴ by resting cell suspensions of *S. lactis* 7962 grown in broth containing lactose, maltose, glucose, or lactose and glucose.

lactose and glucose accumulated lactose-C¹⁴ at a noticeably lower rate than cells grown in the lactose medium.

Uptake of TMG and Maltose

Suspensions of lactose and maltose-grown cells of S. lactis 7962 were also checked for their ability to take up TMG-C¹⁴ and maltose-C¹⁴. As shown in Figure 15, the rate of maltose-C¹⁴ uptake by maltose grown cells greatly exceeded the rate of maltose-C¹⁴ uptake by lactose grown cells. Figure 15 also illustrates the inability of either maltose or lactose grown cells to accumulate TMG-C¹⁴ at a rapid rate. The uptake of TMG-C¹⁴ by cells grown in a lactose medium, however, was greater than cells grown in a maltose medium.

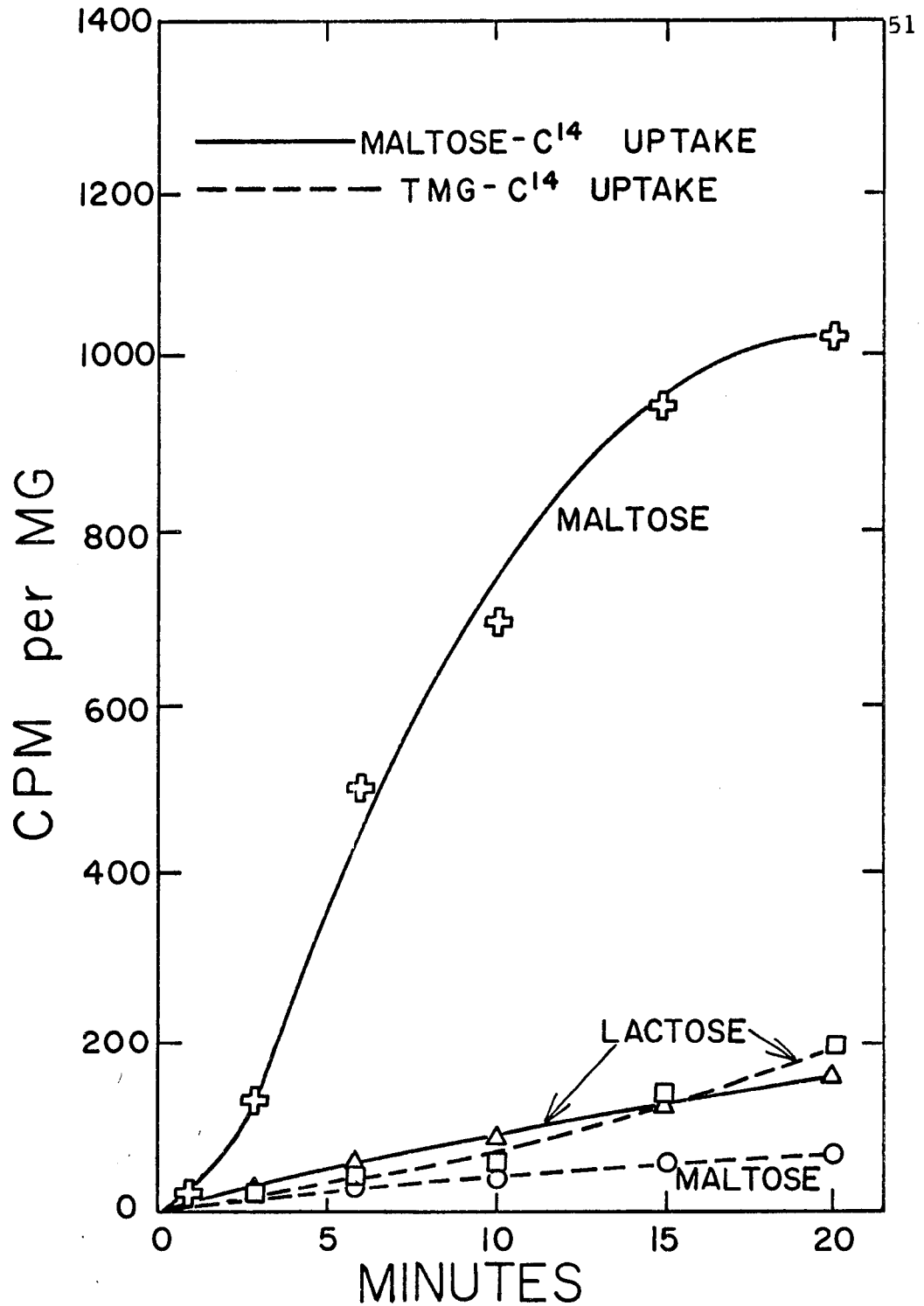


Figure 15. Intracellular accumulation of maltose- C^{14} or TMG- C^{14} by resting cell suspensions of *S. lactis* 7962 grown in maltose or lactose broth.

DISCUSSION

Slow and Fast Acid Production

These studies on slow and fast acid-producing isolates of S. lactis C2 provide additional evidence regarding the relationship between proteolysis and acid production in milk by lactic streptococci. It was noted that while fast and slow acid-producing cells exhibited the same generation time during the logarithmic growth phase and formed the same amount of acid per cell, the former were about four times as numerous because of an extended logarithmic growth phase; the fast cells also were about four times as proteolytic as the slow cells. This suggests that a direct relationship may exist between available nitrogen and amount of total acid produced in milk culture. Specifically, the following explanation seems tenable: The inability of fastidious lactic streptococci to synthesize amino acids to meet their nitrogen requirements makes them dependent on exogenous sources of supply. When this exogenous source is undegraded protein, as is the case in milk, these organisms become dependent on their proteolytic enzymes to make nitrogen available. This regulates the total acid production and exerts a limiting effect in the case of slow acid-producing cells.

Tryptic and pancreatic digests of casein were shown to stimulate acid production in milk by both slow and fast strains of S. lactis C2.

Acid development by the slow strain was especially enhanced. This finding supports the above observation that acid production in milk by lactic streptococci is limited by their ability to synthesize proteolytic enzymes, which are needed to hydrolyze the milk casein in order to form peptides readily metabolized by the cell. These preparations are rich sources of peptides (Sheffield Chemical, 1960 and 1962), which have been shown to be stimulatory for lactic acid bacteria (Anderson and Elliker, 1953; Sandine, Speck, and Aurand, 1956; McAnelly and Speck, 1957). Acid hydrolyzed casein did not significantly stimulate acid production in milk by either fast or slow cells. However, under these conditions, essentially all of the nitrogen is in the form of free amino acids (Difco Laboratories, Inc., 1953), which are known to have little effect on acid development by lactic streptococci (Anderson and Elliker, 1953).

The rate of acid production by the fast strain was also increased by adenine, hypoxanthine, adenosine, and inosine. Since both nucleic acid derivatives and enzymatic digests of casein enhanced acid production by the fast strain, there may be a relationship between the ability of this organism to synthesize purine bases or nucleosides and proteolytic enzymes. At this time, such a relationship can be only speculative. Since these purine bases and nucleosides might be incorporated into ribonucleic or deoxyribonucleic acids, they may be involved in the regulation of the synthesis of proteolytic enzymes.

By comparison, acid production by the slow strain of S. lactis C2 was not stimulated by any of the above purine bases or nucleosides. The slow strain, however, had lost its capacity to synthesize proteolytic enzymes, and therefore was immune to the beneficial effect the nucleic acid compounds may have in stimulating the synthesis of these enzymes.

There is currently some interest in treating raw milk with hydrogen peroxide and catalase to destroy objectionable bacteria in milk to be used for cheese making (Paul-Lewis Laboratories, Inc., Milwaukee, Wisconsin). Therefore the effect of catalase on acid production in milk by lactis streptococci was studied. Catalase was shown to stimulate acid production only slightly by the slow and fast strains of S. lactis C2. However, milk coagulation was effected with less total acid development. Catalase may have stimulated the production of rennet-like enzymes in these bacteria. Although this observation was especially unusual, it was not directly related to the present investigation, and, therefore, was not studied further.

At least one important question remains unanswered regarding slow lactic acid-producing streptococci: What causes the apparent rapid rate of loss of proteolytic ability resulting in the appearance of at least two percent slow acid-producing cells in a fast culture? This is more than 10,000 times as great as the spontaneous mutation rate ($1 \text{ in } 10^6$) one expects for particular types of mutants. Studies

recently completed (Wagner, 1964) indicate that different types of mutations in lactic Streptococcus organisms may result in slow acid production. This may explain the unusually high rate of change in acid producing capabilities by these bacteria.

Lack of suitable plating methods to distinguish between slow and fast acid-producing colonies of lactic streptococci have hindered studies on factors influencing the numbers of slow cells present in a fast acid-producing culture. However, results of this study, and also that of Harriman and Hammer (1931) and Garvie (1959), suggest that about two percent is a stable proportion. Possible reasons for this are obscure. Garvie (1959) also points out that altered culture conditions for mixed-strain starters may change the balance between fast and slow strains. The practice of supplementing mother culture milk with protein digests such as pancrease extract (Procheez, Wilson Company, Chicago, Illinois), however, helps minimize the effects of slow strains. The availability of nitrogen in these preparations may make synthesis of inducible proteolytic enzymes (Williamson and Speck, 1962; Williamson, Tove and Speck, 1964) unnecessary since it has been shown (Williamson and Speck, 1962) that addition of pancrease extract reduces proteolysis of starter cultures.

β -Galactosidase

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) found that toluene-treated cells of E. coli could be used for the assay of β -galactosidase. In the present study, however, work with treated cells was restricted to one strain of S. lactis. This was necessary since it was the only strain among 40 lactic streptococci tested 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, but further efforts in this regard are continuing.

The optimal conditions for the assay of the β -galactosidase in S. lactis 7962 were typical of those for other microorganisms in which the enzyme has been studied (Koppel, Porter, and Crocker, 1953; Anderson and Rickenberg, 1960). Maximal enzyme activity was observed with toluene-treated cell suspensions which were assayed at about 37 C in sodium phosphate buffer of pH 7.0. The requirement for sodium ion to maximally activate the enzyme when ONPG was the substrate was evident; similar results were obtained with E. coli by Cohn and Monod (1951) and with P. aerogenoides by Anderson and Rickenberg (1960).

The ability of certain compounds to induce β -galactosidase in S. lactis 7962 differed from that of other microorganisms. The thio-galactosides TMG and IPTG have been shown to induce enzyme synthesis in E. coli more effectively than lactose, the natural substrate (Cohn and Monod, 1953). It was expected that the same might be true with S. lactis since glucose, and immediate product of the enzyme, was found to repress enzyme synthesis greatly. However, although TMG and IPTG were inducers of β -galactosidase synthesis in S. lactis 7962, they were not as effective as lactose. In this respect, other microorganisms also have been reported to differ from E. coli. For example, IPTG was an ineffective inducer in S. aureus (McClatchy and Rosenblum, 1963), and TMG was inactive in S. faecium (Buecher and Brock, 1962). Lactose was the only galactoside with induced β -galactosidase synthesis in P. aerogenoides (Anderson and Rickenberg, 1960). However, TMG induced synthesis of about 200 times more enzyme than lactose in Shigella paradysenteriae and S. sonnei (Rickenberg, 1960). These various responses in bacteria to inducing compounds may indicate permeability differences between organisms or differences in enzyme induction site specificity.

Induction response to galactose and melibiose also revealed differences between S. lactis 7962 and other microorganisms. In the present study, galactose induced synthesis of the enzyme nearly 70 percent as effectively as lactose in S. lactis. Galactose, however,

was a poor inducer in E. coli (Koppel, Porter, and Crocker, 1953) and S. sonnei (Clausen and Nakamura, 1963), but was more active than lactose in S. aureus (McClatchy and Rosenblum, 1963). Melibiose, which is an α -galactoside, was almost as effective as lactose in E. coli (Koppel, Porter, and Crocker, 1953) and S. sonnei (Rickenberg, 1960), and induced 40 times more enzyme in S. paratyphosiae (Rickenberg, 1960). Melibiose was neither an inducer of the enzyme in S. aureus (McClatchy and Rosenblum, 1963) nor, as found in the present study, in S. lactis 7962.

Recently, Rotman (1964) reported an increased rate of synthesis of β -galactosidase in E. coli by the addition of borate to galactose. This was interpreted to indicate that borate interacted with the transcription mechanism of the lactose operon. This effect could not be demonstrated during the present studies with S. lactis.

It was found that exogenously supplied glucose severely inhibited β -galactosidase synthesis by lactose-induced cells of S. lactis 7962. The rate of enzyme synthesis was therefore regulated not only by lactose, the inducer, but by a metabolite of lactose. Nakada and Magasanik (1964), working with E. coli, presented evidence that a product of glucose metabolism, rather than glucose, was actually responsible for the repression of β -galactosidase synthesis. McFall and Mandelstam (1963), also working with E. coli, suggested that glucose acts as a repressor by virtue of its rapid intracellular

conversion to galactose. Galactose, however, only slightly inhibited β -galactosidase synthesis in S. lactis 7962. This observation and the fact that galactose was nearly as effective an inducer of enzyme as lactose indicate that galactose is not the catabolite repressor of β -galactosidase synthesis in S. lactis.

Galactoside-Permease

The uptake of a metabolite by a bacterium may be mediated by passive diffusion or a permease transport system (Cohen and Monod, 1957). Lactose uptake by S. lactis 7962 was shown to be accomplished by a β -galactoside-permease. If lactose transport was the result of only passive diffusion, the rate of lactose uptake would have been found to be independent of the particular carbohydrate used for cell growth. However, the rate of lactose uptake differed in cells which were grown in lactose, maltose, and glucose media. Of these three sugars, lactose was the most effective inducer of permease synthesis. Maltose also appeared to induce synthesis of the enzyme, but less effectively than lactose. Cells grown in a glucose medium exhibited little β -galactoside-permease activity. The response of β -galactoside-permease synthesis to lactose, maltose, and glucose was similar to that of β -galactosidase synthesis.

Glucose also inhibited synthesis of β -galactoside-permease synthesis in lactose induced cells. Glucose, or a metabolite of glucose,

therefore, served as a catabolite repressor of enzyme synthesis.

These findings show that, like β -galactosidase synthesis, β -galactoside-permease synthesis in S. lactis 7962 is controlled by two mechanisms: induction and catabolite repression. These mechanisms also regulate synthesis of β -galactoside-permease and β -galactosidase in E. coli (Jacob and Monod, 1961).

It was found that the rate of entry of TMG into cells of S. lactis 7962 was slow. This observation may explain the inability of TMG to induce synthesis of β -galactosidase more effectively than lactose. In E. coli, the β -galactose-permease mediates the rapid intracellular accumulation of TMG (Rickenberg, et al., 1956). Since this compound is not metabolized by E. coli, there are no metabolic products to interfere with β -galactosidase induction. Consequently, TMG induces synthesis of more β -galactosidase than lactose (Cohn and Monod, 1953). It was hoped during the present studies that TMG would also be found an effective inducer of β -galactosidase synthesis in S. lactis. This could have been of practical significance in preparing cells of lactic acid bacteria with increased lactose fermenting potential. The use of these cells in fermentations may have provided time saving and other advantages to the dairy industry.

An enzyme which mediates the permeation of maltose was also found in S. lactis 7962. As shown in Figure 15, maltose induced synthesis of this permease; lactose was a poor inducer of this enzyme.

Concluding Remarks

The above studies have only touched on the many aspects of the regulation of acid production and the synthesis of β -galactosidase and β -galactoside-permease in lactic streptococci. Numerous problems, some of which are discussed below, remain to be explored.

Although the loss of the fast acid-producing characteristic of lactic streptococci was related to the loss of proteolytic ability, the reason for the high rate of mutation of fast to slow is yet to be discovered. Many different types of mutations may directly or indirectly result in the loss of proteolytic ability. Methods of selecting and characterizing these various types of mutants must be developed. Another possible explanation for the loss of proteolytic ability, which may warrant study, is the mutagenic action of organic peroxides (Dickey, Cleland, and Lotz, 1949). Organic peroxides may be formed from hydrogen peroxide accumulating in these lactic streptococci, which are catalase negative.

One of the first functions carried out by lactic streptococci in milk is the hydrolysis of lactose. This function requires the biosynthesis of inducible β -galactosidase. The more proteolytic nature of the fast strain may allow greater synthesis of β -galactosidase by providing a greater quantity of amino acids to the cell. The loss of proteolytic ability may result in an impaired ability to synthesize this

enzyme. Therefore, it may be rewarding to study the relationship between proteolysis and β -galactosidase synthesis. One approach to this problem is to measure the incorporation of radioactive peptides into β -galactosidase. A study of this type would also require developing a procedure for the purification of β -galactosidase in S. lactis. Experiments of this nature in other organisms have been performed. For example, Fox (1961) measured the incorporation of C^{14} -peptides into M-protein, streptolysin O, and proteinase of a group A Streptococcus. Incorporation of these peptides was dependent on proteolytic activity.

It was noted in the present work that the β -galactosidase of only one strain of over 40 lactic streptococci was stable in toluene-treated or sonic-treated cell preparations. Since conventional methods of enzyme stabilization failed, new approaches for the study of the enzyme in these β -galactosidase-labile strains are needed.

SUMMARY

From S. lactis C2, a fast strain which produced sufficient acid to coagulate sterile nonfat milk in 18 hours at 21 C, a slow mutant was isolated which required incubation for at least 48 hours to effect coagulation. Nonfat milk cultures of these two organisms were compared to find causes for the reduced acid production by the slow strain. These two bacteria had the same generation time (1.4 hours) in the exponential growth phase, and produced the same amount of acid per cell. The average viable population of the fast culture after 18 hours at 21 C, however, was about four times as great as the slow. Also, the fast culture was four times more proteolytic in nonfat milk than the slow. The slow organism appeared to have lost its capacity to synthesize proteolytic enzymes. Tryptic and pancreatic hydrolysates of casein stimulated acid production in milk by the slow and fast strains; acid hydrolyzed casein was not an effective stimulant. There was, therefore, a direct relationship between available nitrogen, in the form of peptides, and total growth and acid production in milk. Acid development in milk by the fast strain was also enhanced by adenine, hypoxanthine, adenosine, or inosine. Therefore, there appeared also to be a relationship between the synthesis of proteolytic enzymes in the fast strain and the availability of purine bases or nucleosides. The slow strain, however, having lost its ability to

synthesize proteolytic enzymes, derived no benefit from these nucleic acid derivatives.

Synthesis of β -galactosidase by several strains of lactic streptococci was induced by lactose. The enzyme of all but one strain was unstable when whole cells were sonicated or treated with toluene; the enzyme of one strain, S. lactis 7962, was stable to these treatments, which resulted in at least a five-fold increase in activity over that found in whole cells. The optimal assay conditions for toluene-treated cells of this strain were 37 C and pH 7.0 in sodium phosphate buffer. Lactose was the most effective inducer of enzyme synthesis. TMG, IPTG, and galactose were also inducers of the enzyme, but were not as effective as lactose. Melibiose, maltose, calcium lactobionate and glucose were poor inducers of enzyme synthesis. Exogenously supplied glucose repressed induced β -galactosidase synthesis; galactose only slightly inhibited induced enzyme synthesis.

Lactose uptake by S. lactis 7962 was mediated by a β -galactoside-permease. Synthesis of this enzyme was induced by lactose. Maltose induced some synthesis of this enzyme; glucose was not an inducer. Glucose also repressed synthesis of induced β -galactoside-permease. The means of control of induced β -galactosidase and β -galactoside-permease synthesis in S. lactis was similar to that in E. coli.

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