AN ABSTRACT OF THE THESIS OF

Gordon Alwyn McFeters _____ for the ___PH. D._____
          (Name)   (Degree)

in ___Microbiology_____ presented on ___April 25, 1967___
          (Major)   (Date)

Title: PURIFICATION AND PROPERTIES OF STREPTOCOCCUS

LACTIS β-GALACTOSIDASE

Abstract approved:

                              W. E. Sandine

Experiments were carried out to purify and characterize the

β-galactosidase of the Streptococcus lactis 7962. Purification was

accomplished using standard procedures; however the lability of

the enzyme to numerous treatments limited the techniques that could

be used and the amount of active enzyme recovered. Gel filtration

revealed that the enzyme existed in two forms differing in size by

a factor of two (molecular weights of 5×10^5 and 10^6); the smaller

of the two forms was purified and characterized in detail. Chemical

characteristics of the two forms were the same: pH optimum, 7. 0;

temperature optimum, 37 C; and Michaelis constant, 10^{-3} M ONPG.

Ammonium sulfate exerted a stabilizing effect on the enzyme

and caused an association of the smaller unit to form a larger molec-

ular weight aggregate. Because of the size and the chemical prop-

erties of the two forms a subunit (monomer) - native enzyme (dimer)

relationship was established. The observations that para-

chloromecuribenzoate and ethylenediamine tetraacetic acid inactivat-

ed the enzyme and that ammonium sulfate prevented this inactivation

suggested that essential sulfhydryl and metal ion groups became

masked in the enzyme structure during salt stabilization. Compar-

ison of the amount of change seen in the U. V. spectra of salt-stabi-

lized and nonstabilized enzyme indicated that ammonium sulfate

stabilized the structure of the enzyme. The salt also protected the

enzyme from the inactivating effect of urea, but to a much lower

extent than was seen with the sulfhydryl antagonist or metal chelator.

Chemical analysis of the purified enzyme, when compared

with β-galactosidase from bacteria of the enteric group, showed

similarities and differences in the amino acid composition. For

example, alanine, aspartic acid, glutamic acid, glycine, isoleucine,

phenylalanine, serine, threonine and valine were present in the

same amounts.

Incubation of the purified enzyme at 27 C caused a similar

association (quaternization) as was observed in the presence of am-

monium sulfate. This indicated that hydrophobic bonding is involved

in holding the dimer form together as has been suggested for other

polymeric proteins. The same relative amount of the two enzyme

forms (monomer and dimer) observed following quaternization

was also found in enzyme extracted from log phase culture; this

suggested that an equilibrium between the two forms existed. When

enzyme was obtained from bacteria in the late logarithimic phase of growth (12 hours) a disproportionately large amount of monomer was observed. This indicated that lactose was essential for association to occur in vivo since this carbohydrate was probably depleated at that time. No evidence for dissociation of the dimer form of enzyme was seen, however, prolonged incubation at either 27 C or 5 C caused an increase in the amount of monomer form of enzyme present. This effect apparently was caused by the activation of an inactive form of enzyme, possibly monomer, as has been suggested in other proteins.

PURIFICATION AND PROPERTIES OF STREPTOCOCCUS
LACTIS β-GALACTOSIDASE

by

Gordon Alwyn McFeters

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1967

APPROVED:

Redacted for Privacy

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Date thesis is presented ____April 25, 1967____

Typed by Ruth Baines for Gordon Alwyn McFeters

ACKNOWLEDGMENTS

The author feels deeply grateful to those who helped make this thesis possible. A special word of thanks is extended to:

Dr. W. E. Sandine for his advice, understanding and many helpful suggestions.

Dr. L. W. Parks and Dr. R. R. Becker for their stimulating consultation

Dr. P. R. Elliker for his helpfulness

Mr. Bob Howard for his assistance with the amino acid analysis

Lois, Kelvin and Mike for their patience and understanding

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INTRODUCTION

β-galactosidase (E. C. 3.2.1.23 β-D-galactoside galactohydrolase) is an enzyme of prime importance in the metabolism of lactic streptococci since growth of these bacteria in milk, their natural habitat, is dependent on its function. Under these conditions lactose serves as the sole source of energy, consequently the catabolism of this carbohydrate and especially the first enzyme involved in this process (β-galactosidase) is worthy of study.

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

Extensive investigations have been done on β-galactosidase (isolated mainly from Escherichia coli) relative to its physical and kinetic properties, biosynthetic control, and comparative enzymology. The popularity of research on this enzyme is no doubt due in part to the simple and sensitive colorimetric assay procedure for β-galactosidase developed in 1950 by Joshua Lederberg.

Earlier research done in these laboratories involving a survey of over 50 strains of streptococci from the lactic group for this enzyme revealed that only one strain (S. lactis 7962) produced enzyme of sufficient quantity or stability in cell-free extracts to allow purification. This study was an extension of that research undertaken to purify and characterize the β-galactosidase of S. lactis 7962. Characteristics such as mechanism of inactivation and stabilization of the enzyme were of prime interest as well as some of its enzymatic properties.

HISTORICAL REVIEW

Some of the earliest research carried out on the characterization of β-galactosidase in microorganisms was done by Deere, Dulaney and Michelson (1938). They found the enzyme to be adaptive (inducible) with a pH optimum of about 7.4. In 1950 great impetus was given to this research area when Lederberg (1950) reported the development of a rapid, simple and sensitive assay for β-galactosidase involving the use of a chromogenic substrate. Several examples of important aspects of this research follow.

A milestone in this research was the work of Jacob and Monod (1961) who used the enzyme in a classical approach to the problem of cellular regulation of enzyme synthesis. Their data suggested that the synthesis of the enzyme was regulated by the presence or absence of the substrate (in this case) or a closely related compound. The lactose operon, of which β-galactosidase is a part also has been used in research on the process of enzyme induction and repression (Kepes, 1963; McFall and Mandelstam, 1963; Nakata and Magasanik, 1964; Müler-Hill, Rickenberg and Wallenfels, 1965; Moses and Prevost, 1966), in studies on messenger RNA (Lerve, 1965; Ben-Hamida and Schlessinger, 1965) and in work on the order of induction and deinduction of enzymes within the operon (Alpers and Tomkins, 1965). Recently Gilbert and Müler-Hill (1966)

demonstrated a protein that acts like the repressor for the lactose

operon confirming some of the suggestions made earlier by Jacob

and Monod. Another application was made of the enzyme when Rohlfing

and Crawford (1966) used the characteristics of the β-galactosidase

of enteric bacteria to determine degree of relatedness between these

organisms. This represented a novel yet quantitative approach to

the taxonomy of that group of bacteria.

β-galactosidase from various strains of E. coli have been

studied and characterized very carefully. The earlier papers have

been discussed by Cohn (1957). Lederberg (1950) first described

the Michaelis constant for that enzyme (10^{-4} M ONPG). Cohn and

Monod (1951) and Kuby and Lardy (1953) further characterized the

enzyme from E. coli and found that the enzyme was activated by

sodium and potassium ions and that several compounds related to

the substrate exerted a compatative inhibition, and further that the

pH optimum was about 7.1. Hu, Wolfe and Reithel (1959) described

a purification procedure for β-galactosidase from E. coli and gave

criteria for its purity. Wallenfels et al. (1959) and Wallenfels,

Sund and Weber (1963) also described a purification technique and

showed the efficiency of various chemicals in causing dequaterni-

zation of the enzyme. However, these partially purified enzyme

preparations were contaminated with small amounts of nonenzyme

protein. This contamination was reportedly eliminated in the work

done by Craven, Steers and Anfinsen (1965) on the enzyme from

E. coli strain K-12. From the above investigations it was found

that the various characteristics of the enzyme in these preparations

were quite similar.

β-galactosidase has been isolated from many different sources

and results of charaterization studies have shown the enzymes to

be radically different. For example Got et al. (1964) identified the

enzyme in snail digestive juice with a pH optimum of 5.4 while

Barnett (1965) found it in albumin gland with a pH optimum of 2.2.

Lester and Byers (1965) found two forms of the enzyme in Neurospora

crassa with pH optima of 4.0 and 7.0. Of 181 strains of various

Mycobacteria species studied, 36 proved to possess the enzyme

(Tacquet, et al., 1966, and Szabó and Rózsa, (1965) found that

Saccharomyces fragilis also was β-galactosidase positive. The

enzyme has also been identified in a large number of bacteria be-

sides E. coli: Shigella sonnei (Rickenberg, 1960; Clausen and Nak-

amura, 1963), Paracolobacterium aerogenoides (Anderson and

Rickenberg, 1960), Staphlococcus aureus (McClatchy and Roseblum,

1963), Bacillus subtilis (Auema, 1964), and Aeromonas formicans

(Rohlfing and Crawford, 1966). Vakil and Shahani (1962) demonstrat-

ed the presence of β-galactosidase in one strain of S. lactis; however

the enzyme was not characterized at that time. Of over 50 strains

of lactic streptococci examined by Citti, Sandine and Elliker, 1965,

only S. lactis 7962 was found to have enzyme of sufficient quantity

or stability to allow purification. Preliminary information regarding

the enzyme in this organism also was contained in this article. The

organism used by these authors was first described by Hagarty

(1939); it produced acid slowly in milk at 22 C, requiring at least

24 hours of incubation to cause coagulation; accepted taxonomic tests

(Sandine, Elliker and Hays, 1962), however, have confirmed that

the bacterium is a strain of S. lactis.

Since the later stages of the research reported in this

thesis concerned the molecular structure of the S. lactis β-galacto-

sidase, this subject will be reviewed briefly. The general subject

of protein quaternary structure has been reviewed by Reithel (1963);

however no mention was made of β-galactosidase since the work

on the E. coli enzyme had not been published at that time. The first

report of this nature indicated that the β-galactosidase of E. coli

consisted of four identical subunits (Wallenfels, Sund and Weber,

1963; Zipser, 1969) which were each made of nonidentical poly-

peptide chains (Steers, Craven and Anfinsen, 1965).

Other multimeric enzymes also have been described, how-

ever two of the best understood cases are glutamic dehydrogenase

(Frieden, 1963) and aldolase (Deal, Rutter and Van Holde, 1963;

Westhead, Butler and Boyer, 1963). The former is a tetramer

(M. W. $= 10^6$) that dissociates readily; aldolase (M. W. $= 1.5 \times 10^5$)

is a trimer but dissociates into identical subunits only when exposed
to a solution of pH less than 3.0

Cowman and Swaisgood (1966) reported that a proteinase from
S. lactis underwent an in vitro temperature dependant reversible
dissociation into lower molecular weigh components; the relative
concentrations of the two forms was held in equilibrium which con-
firmed the findings of Reithel (1963) for other multimeric enzymes.
Furthermore, it has been proposed by Appel, Alpers and Tomkins
(1965) that such an equilibration process actually occurs in vivo with
the β-galactosidase of E. coli.

MATERIALS AND METHODS

Routine Procedures

Microorganisms and Media

S. lactis 7962 obtained from the American Type Collection,
Washington, D. C. was used throughout these experiments. The cul-
ture was maintained in 11 percent reconstituted, sterile, nonfat dry
milk. Sterile lactose broth was used to grow the cells required for
each experiment. These cells were grown from a 1.0 percent inoc-
ulum of a 24 hour broth culture at 32 C. The lactose broth consisted
of the following: lactose, 10 g; tryptone, 20 g; yeast extract, 5 g;
gelatin, 2.5 g; NaCl, 4.0 g; L ascorbic acid, 0.5 g; and water to 1.0
liter. This medium was autoclaved for 15 minutes at 121 C.

Buffer Solutions

Sodium phosphate buffer (0.1 M at pH 7.0) was used to wash
the bacterial cells during enzyme extraction and purification and in
the enzyme assay solution. All solutions were used at 5 C unless
otherwise stated. Ammonium sulfate (0.85 M) was added to the buf-
fer solution during some experiments as indicated.

Assay of β-galactosidase. Hydrolysis of the chromogenic

substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) was used

as a measure of enzyme activity (Lederberg, 1950). Solutions of

5 mM were prepared in the phosphate buffer and used as substrate

solution in the enzyme assay reaction. The enzyme assay was car-

ried out by adding 2.0 ml of the ONPG solution to 0.5 ml of the en-

zyme solution; the mixture was then allowed to react at 37 C, usual-

ly from 2 to 5 minutes. The reaction was terminated by the addition

of 2.5 ml of 0.5 M sodium carbonate. The absorbance at 420 mμ

was measured on a Gilford model 2000 multiple channel recording

spectrophotometer. The molar concentration of ortho-nitrophenol

(ONP) liberated was determined by multiplying the absorbancy by

the molar extinction ($1/E_{cm}^{M} = 0.238$ $mole^{-1}$ cm^{-1}) and dividing by

the reaction time in minutes. This figure then was multiplied by

10 to account for a 10-fold dilution of the enzyme in the reaction mix-

ture. The extinction coefficient was determined from a graph show-

ing the relationship between absorbancy at 420 mμ and ONP concen-

tration. One unit of enzyme activity was defined as the number of

 moles of ONP produced per minute/per ml of the original enzyme

solution. Specific activity was expressed as the units of enzyme per

mg of protein. The extinction coeficient was used to calculate the

enzyme activity as described above.

In the determination of the optimum pH for enzyme activity,

ONPG solutions were buffered at different pH values using 0.2 M sodium phosphate. Enzyme solution (0.5 ml) in 0.1 M buffer, was added to 4.5 ml of the ONPG solution at the desired pH. The pH of each of the various samples was checked with the Beckman Zeromatic pH meter upon mixing. The resulting pH was recorded as the pH for each sample.

In determining the effect of temperature on the activity of the enzyme the usual concentrations and volumes of substrate and buffer were used. Aliquots of the ONPG solution were equilibrated at various temperatures prior to addition of the enzyme solution. The assay reaction was carried out for 30 minutes because of the low activity of the enzyme solutions tested. Solutions containing 0.5 ml buffer and 2.0 ml ONPG solution were held at each temperature for the 30 minutes and then 2.5 ml sodium carbonate was added. These were used as blanks for each temperature in the spectrophotometric determination of the ONP concentration.

Protein determination. The method of Lowry et al. (1951) was used to determine the protein concentration of enzyme preparations. The protein concentration was determined by multiplying the absorbancy of the protein assay mixture at 500 mμ by a milligram extinction ($1/E_{cm}^{mg}$ = 0.599 mg^{-1} cm^{-1}). This extinction coefficient was calculated from a standard curve of various concentrations of bovine serum albumin assayed by the Lowry method. The protein

concentration was expressed as mg per ml. In most of the column

separations the relative protein concentration was determined meas-

uring the absorbancy of each sample at 280 mμ . These results were

expressed as absorbancy at 280 mμ (A_{280}).

Growth curve determination. Side-arm flasks (50 ml) were

autoclaved and 10 ml of lactic broth were added to each. The flasks

were inoculated as described above and incubated at 32 C. At var-

ious time intervals, the absorbancy at 650 mμ of each was measured

using a Coleman spectrophotometer. The results were expressed

graphically as A_{650} versus time.

Purification Procedures

Harvesting and Washing of Cells

Cells grown on lactose broth for 10 hours, inoculated and

incubated as described above, were harvested at 1 C by centrifuga-

tion at 4000 x g. These growth conditions allowed maximal enzyme

production as indicated by Citti, Sandine and Elliker (1965). These

cells were washed three times with the cold (O C) 0.1 M sodium

phosphate buffer.

Extraction

The cells grown in 1.0 L of medium, when washed in the

above manner and resuspended in 50 ml of cold buffer, were then ready for extraction. This suspension was treated for one hour in a Raytheon 10 KC sonic oscillator at 100 percent properly tuned voltage. The resulting cell-free extract (crude extract) was stored at 5 C if not used or treated further immediately. Various other extraction procedures such as grinding with different abrasives and use of a pressure extrusion cell were tested but were unsuitable as they resulted in the destruction of enzymatic activity.

Nucleic Acid Precipitation

Protamine sulfate was used to precipitate the nucleic acids from the crude extract. Solid reagent was added such that the ratio of protamine sulfate to protein (mg/ml) minimized the 260 to 280 absorbancy ratio after the solution was held at 20 C for 15 minutes and was centrifuged at 12000 x g for 10 minutes. As part of the purification procedure routinely used, the crude extract was allowed to stand for 45 minutes at 20 C prior to centrifugation. The nucleic acid fraction, the large cellular debris and the unbroken cells were removed in this process.

Ammonium Sulfate Fractionation

Ammonium sulfate fractionation was used to accomplish further purification and concentration of the enzyme. Varying amounts

of solid salt was added to aliquots of the above enzyme extract and dissolved. These were allowed to stand at O C for 12 hours then centrifuged at 0 C for 10 minutes at 12000 x g. The active enzyme was found to be precipitated under these conditions by an ammonium sulfate concentration of from 1.3 to 2.5 M. In the routine purification procedure that was used the extract was brought to 1.3 M, held at O C for 4 hours, centrifuged as above and the supernatant brought to 2.5 M. This was held at O C for 4 hours, centrifuged as before and the precipitate retained. Upon careful resuspension of this precipitate in buffer at O C, only 50 percent of the original enzyme activity in the crude extract was recovered. Enzyme activity as well as protein concentration was followed through the above stages. The resulting enzyme solution (partially purified) was used in further purification and other procedures.

Ion Exclusion Chromatography (Gel Filtration)

Sephadex G-200 was used to achieve further purification of the enzyme and as an analytical procedure. Sephadex columns (1 by 20 cm, 2.5 by 38 cm and 2.5 by 90 cm), resin bed dimensions were used. The resin was prepared in the usual manner and added to the columns at 20 C. Most of the preparative and analytical separations were done with a 2.5 by 38 cm column since the 2.5 by 90 cm column resulted in excessive enzyme inactivation and the smaller

column did not hold enough sample.

Following the addition of the resuspended resin to the column, it was placed in a Gilson refrigerated fraction-collector and percolated with buffer for at least 48 hours prior to use. The flow rate was not regulated so even with minimal head pressure the flow rate decreased steadily, giving better resolution but slower separations. Blue dextran (MW = 2 x 10^6) was used to determine the void volume (V_o) and to check the column packing for irregularities. Fraction collection was done with a Gilford linear base, refrigerated fraction-collector at 5 C, using the drop counter set at 100 drops per fraction. The drop counter mal-functioned during later phases of the research so timed collection was necessary for some of the separations. When this was done, the drop rate was determined and the timer set to allow for the collection of 100 drops per fraction. Sample was added to the sample applicator disc of the column immediately after the level of eluant dropped below the applicator. Fraction collection was started at this time. When the sample had entered the resin, 2.0 ml of cold buffer was used to rinse the sides of the sample applicator and to "chase" the sample. Following this, 5 ml of buffer was added to the applicator disc and the column cap screwed firmly into place; the eluant bottle then was attached. The eluant bottle, (a 2 l battery jar), was only slightly higher than the level of the resin. The fractions were analyzed for A_{280} and enzyme activity

starting with the first fraction before the void volume. The elution

data were recorded as fraction (tube) number, each of which repre-

sented a certain tube number after void volume minus one, as des -

cribed above. Enzyme activity data were determined directly follow-

ing the collection of the last fraction to minimize post-separation in-

activation.

Disc Electrophoresis

Disc electrophoresis was carried out using a discontinuous

pore-sized column (Ornstein and Davis, n. d.). The lower 2 inches

was a small pore gel of 7.5 percent acrylamide, pH 8.9. Above the

small pore gel was a 0.25 inch spacer of large pore gel which was

3.0 percent acrylamide at pH 6.7. A sample (50 to 100 μl) of

enzyme solution (about 200 μg protein/ml) was added to 150 μl of

large pore gel and this filled the last 0.25 inch of the 2.5-inch column.

The electrophoresis was carried out at 5 C at a constant current of

3 milliamps per sample. The gels were removed from the columns

and stained with Amido Schwarz.

Specialized Procedures

Sulfhydryl Titration

A 10^{-3} M solution of p-chloromercuribenzoate (PCMB) was

prepared in 1 M NaOH and subsequently purified using the method of

Neilands (1965). This procedure was repeated three times and the

resulting solution was used in the sulfhydryl titration (Boyer, 1954).

The procedure was carried out using the Gilford Model 2000 multiple

channel recording spectrophotometer at room temperature (24 C).

The change in absorbancy at 250 mμ, after the addition of 0.1 ml

of 10^{-3} M PCMB to a mixture of 2.5 ml of buffer (20 C) and 0.1 ml

of enzyme solution, was recorded. Moles of PCMB reacting with

the protein sulfhydryl groups were calculated by use of the molar

extinction coefficient (E_{cm}^{M} = 7.6 x 10^{3} mole^{-1} cm^{-1}). These data

obtained with the stabilized and nonstabilized partially purified en-

zyme preparations and with the pooled fractions of the two peaks

following gel filtration were compared.

Molecular Weight Determination

The elution volume (V_{e}) exhibited when enzyme fractions

(peaks) were eluted from the G-200 Sephadex column was replotted

semi-logarithmically using the standard procedure of Dasgupta,

Boroff and Rothstein (1966). This gave a good estimation of the

molecular weight of enzyme represented by the two peaks since the

conditions for separation of the S. lactis enzyme were essentially

the same as described in the "standard" procedure cited above.

The theoretical justification for such a correlation was discussed

by Laurent and Killander (1964).

Amino Acid Composition

Peak II fractions from one of the G-200 preparative separations were pooled and dialyzed exhaustively (at least 12 2-liter volumes of double distilled water) and then lyophylized. Samples of this protein were hydrolyzed for 24 or 48 hours at 110 C in concentrated hydrochloric acid. Samples were examined on the Beckman Spinco model 120 B amino acid analyzer. Following this analysis, another sample of protein was oxidized with performic acid using the procedure of Moore (1963). This oxidized the half-cystine residues to cysteic acid which could be detected on the analyzer at lower concentrations than cystine. Protein so treated was then hydrolyzed as described above and analyzed for cysteic acid residues.

Blue-Shift Detection Procedure

Nonstabilized, partially purified enzyme (0.1 ml) maintained at 0 C was added to 2.5 ml of cold (0 C) buffer and the A_{250} compared to the salt-stabilized enzyme under the same conditions. The spectra of the two samples from 220 to 360 mμ was determined using the Cary model 11 recording spectrophotometer.

Kinetic Observations

The Gilford model 2000 multiple channel recording spectro-
photometer was used for following the kinetics of hydrolysis of ONPG.
A Hakke temperature control unit was attached to the jacket of the
curvette chamber of the spectrophotometer and the temperature was
maintained at 37 C. A wavelength setting of 420 mμ was used. Dif-
ferent concentrations of enzyme were placed in each of the curvet-
tes along with substrate (5 mM ONPG) which was also prewarmed to
37 C.

Michaelis Constant (Km) Determination

The method of Woolf as described by Haldane (1957) in which
the Michaelis -Menton equation is re-written as $v = V - K_{m(S)} \frac{v}{}$
was used. In this equation, v = units of enzyme, V = maximum
units of enzyme, (S) = ONPG concentration and Km = Michaelis
constant. By plotting v versus $v/(s)$, a straight line with a negative
slope results where the v intercept is V and the K_m is V divided by
the $v/(S)$ intercept. The reagents were prepared as described above
except that varying concentrations of ONPG in buffer were used.
The reactions were carried out at 37 C. The enzyme activity of each
fraction was measured and calculated as before.

Sucrose Density Gradient Centrifugation

Gradients of sucrose were prepared from 20 to 5 percent (bottom to top) in water at room temperature by means of gradient block using constant mixing in the 20 percent side. A wire inoculating loop bent in a series of regular S curves and driven by an electric mixer at maximum speed was used for the mixing. Solutions (2.4 ml) were placed in each side, the mixer started and the valve opened, allowing the mixture to drain into the gradient centrifuge tubes. Each gradient took about 6 minutes to complete. The sample (0.2 ml) was carefully layered onto the top of the gradient and the tubes placed in the SW 39 rotor. The samples were then centrifuged in the Beckman Spinco model L-2 ultracentrifuge for 8.5 hours at a speed of 30,000 rpm. Following the centrifugation the tubes were carefully removed and 22 3-drop fractions were collected from bottom to top. To each fraction 1.0 ml of ONGP solution was added followed by incubation at 37 C for 15 minutes; 1.0 ml of 0.5 M sodium carbonate then was added to stop the reaction. The enzyme activity of each fraction was measured and calculated as stated above.

Proteinase Assay

The level of proteinase activity in cell-free enzyme extracts

of S. lactis 7962 was measured using Azocoll (Calbiochem, n. d.) as

the substrate. Various dilutions of enzyme in different stages of

purification were made in 5 ml of buffer and 25 mg of Azocoll then

was added. This mixture was incubated at 37 C with constant swirl-

ing for 15 minutes and the reaction was terminated by filtering

through Whatman No. 1 filter paper using gravity flow; this removed

the substrate from the reaction mixture. The A_{580} was determined

on the filtrate using appropriate blanks and the values then used as

a measure of proteinase activity.

Test for Ribosome-bound Enzyme

The method of Zipser (1963b) was followed in an effort to

detect ribosonaly bound enzyme.

RESULTS

Purification of the Enzyme

Extraction Procedures

Several different extraction procedures were attempted with most methods causing complete or nearly complete inactivation of the enzyme. These findings suggested that the enzyme was quite unstable and only the process finally developed to yield reasonably active enzyme will be described here.

Sonic treatment of washed cell suspensions of S. lactis 7962 provided cell extracts with measurable enzyme activity. Figure 1 shows the effect of sonic treatment time on enzyme and protein release from a washed cell suspension. Protein was liberated to a maximum level within 30 minutes and then remained constant; the enzyme activity in solution, however, reached a maximal level in 80 minutes and then decreased rapidly. The difference in the time required to achieve maximum protein concentration and enzyme activity suggested that the enzyme was associated with particulate material. However, no evidence to indicate that the enzyme was bound to cellular particles was found even though several attempts were made.
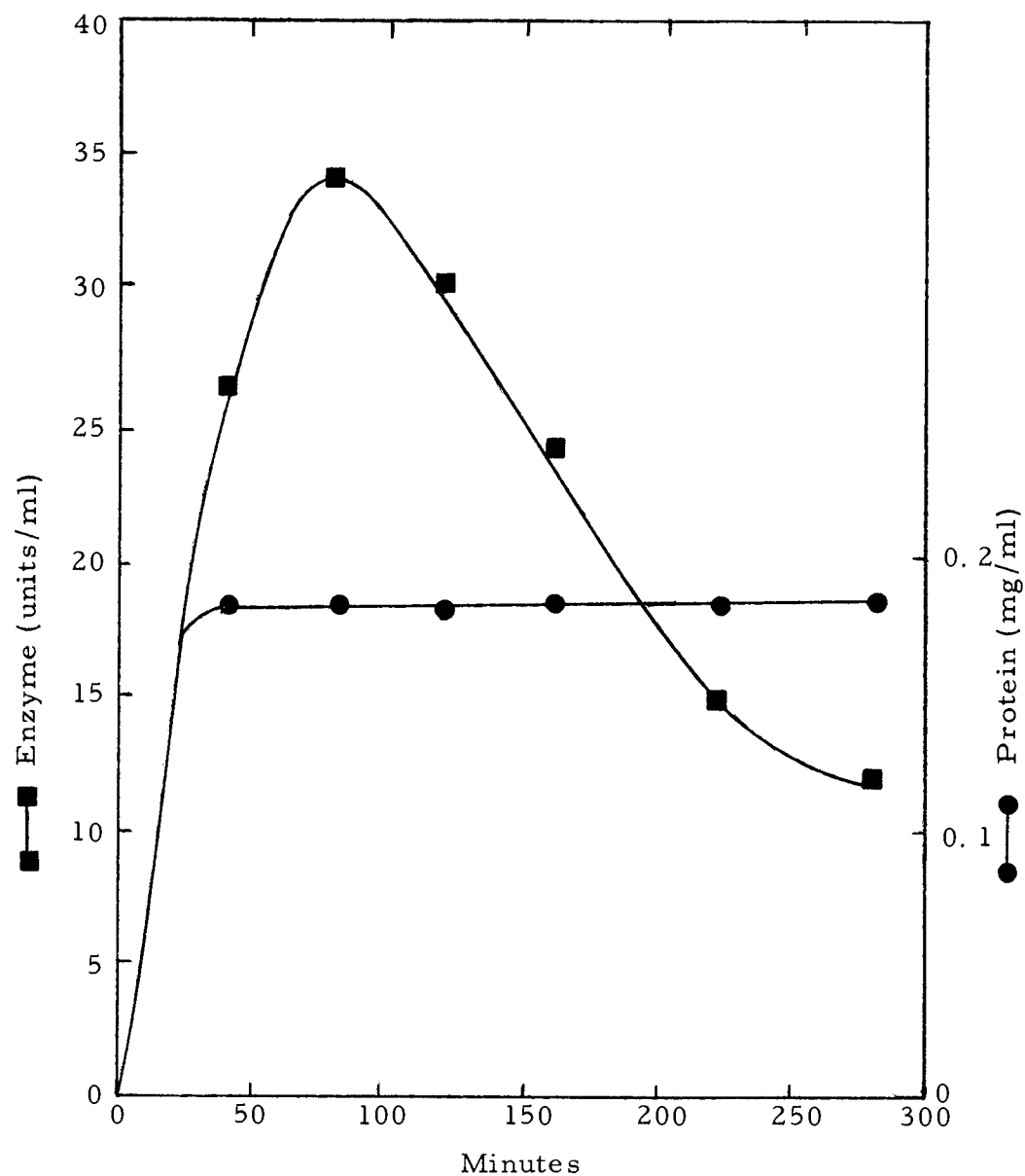
Figure 1. Effect of sonication time on protein and β-galactosidase release from Streptococcus lactis 7962. A 1:10 dilution of the cell suspension prior to sonication provided an absorbancy of 0.45 at 420 mμ.

Nucleic Acid Precipitation

Protamine sulfate was used to precipitate the nucleic acid fraction of the crude extract. This treatment was carried out prior to the ammonium sulfate fractionation because the protamine was precipitated when the first salt addition was made. Figure 2 shows the decrease in A_{260}/A_{280} ratio in the crude extract as increasing amounts of protamine sulfate were added. A one to one ratio of protamine to protein (mg/ml) gave maximal decrease in the A_{260}/A_{280} ratio. This procedure, followed by centrifugation, resulted in the removal of the unbroken cells, cellular debris and the nucleic acid fraction.

Ammonium Sulfate Fractionation

Fractional precipitation with ammonium sulfate allowed further purification and concentration of the enzyme. Table 1 shows typical results obtained following the partial purification process. Usually 90 percent of the protein was eliminated during this salting procedure, leaving the enzyme in the remaining 10 percent. The total enzyme activity was decreased by 50 percent while the specific activity increased to about 400 percent over that percent in the crude extract (Table 1). These data also emphasized the lability of the enzyme; furthermore, the enzyme was very sensitive to freezing,
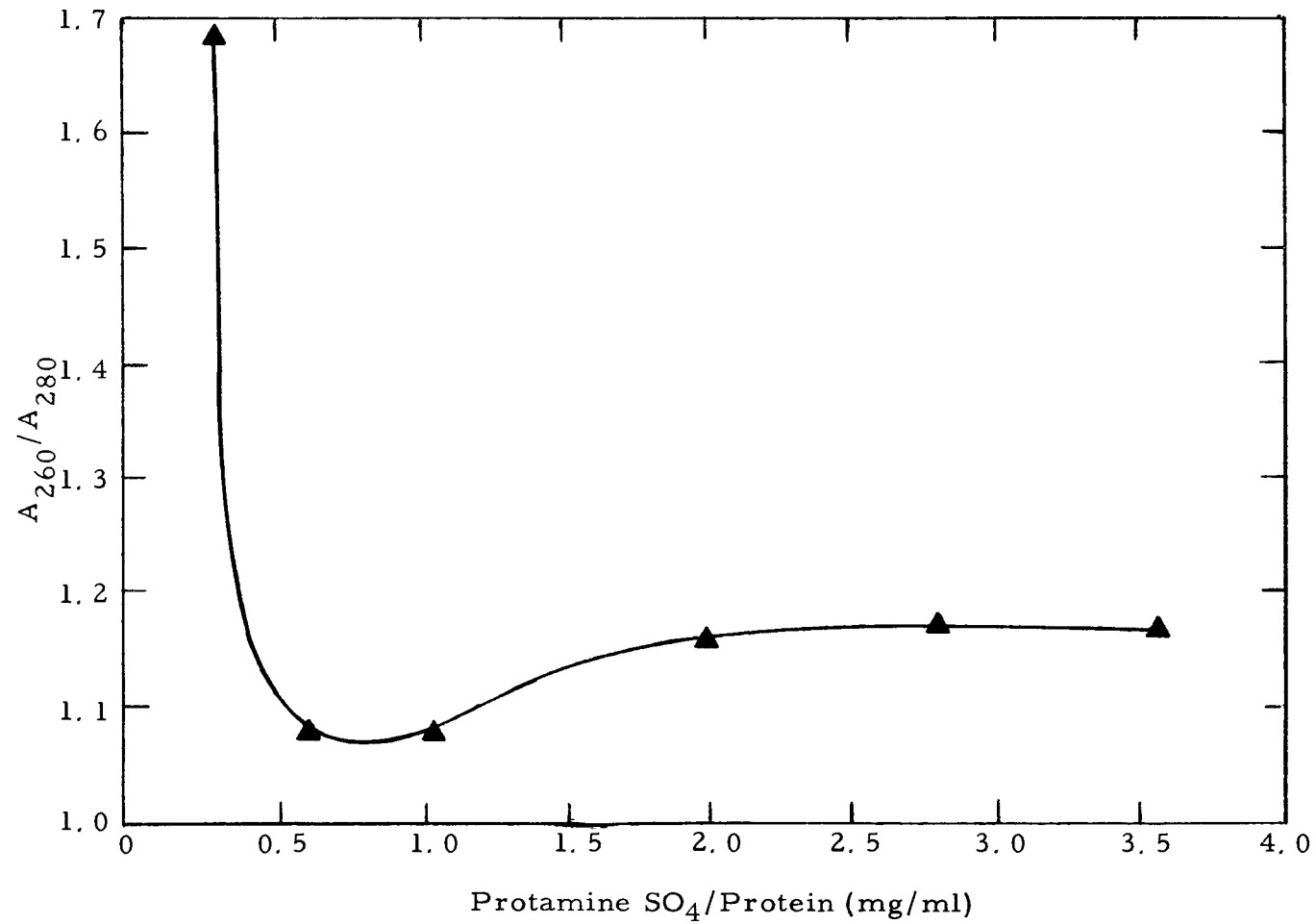
Figure 2. Effect of protamine sulfate on 260/280 absorbancy ratio of crude extract of Streptococcus lactis 7962.

Table 1. Typical data obtained before and after the partial purification of the β-galactosidase of S. lactis 7962

	Crude Extract	After $(NH_4)_2SO_4$ Fractionation
Protein (μg/ml)	2.15	1.40
Enzyme (u/ml)	0.290	0.766
Specific Activity	0.135	0.547
Volume (ml)	100	20
Total Protein	215	28
% Original Protein	100	13
Total Enzyme Units (u/ml x ml)	29	14.3
% Original Enzyme (u/ml)	100	264
% Original Specific Activity	100	406

liophilization, dialysis and to various inorganic ions and organic solvents. Therefore, maintenance of enzyme activity during further purification and storage was difficult to achieve.

Gel Filtration

When partially purified enzyme samples were run through a G-200 Sephadex column, sufficient activity remained in the eluant to allow detection. Figure 3 shows a plot of typical separation data where about 10 percent of the enzyme activity added to the column was recovered. Virtually all of the protein that was added to the column was recovered by fraction number 50. However, since no more active enzyme was eluted from the column after fraction 25, subsequent experiments were shortened to that point. The low recovery of activity following gel filtration was expected in view of the extreme lability of the enzyme to dialysis.

The enzyme elution data in Figure 3 suggested that there were two forms of the enzyme, differing in molecular weight or size. The possibility of sonication being responsible for liberation of a smaller enzyme form (Peak II) from the larger molecular weight unit (Peak I) was investigated. Cells were sonicated for 45 minutes and half of the sonicate removed and chilled; the remaining half was sonicated for an additional 15 minutes. The two samples then were treated the same through partial purification and then separated
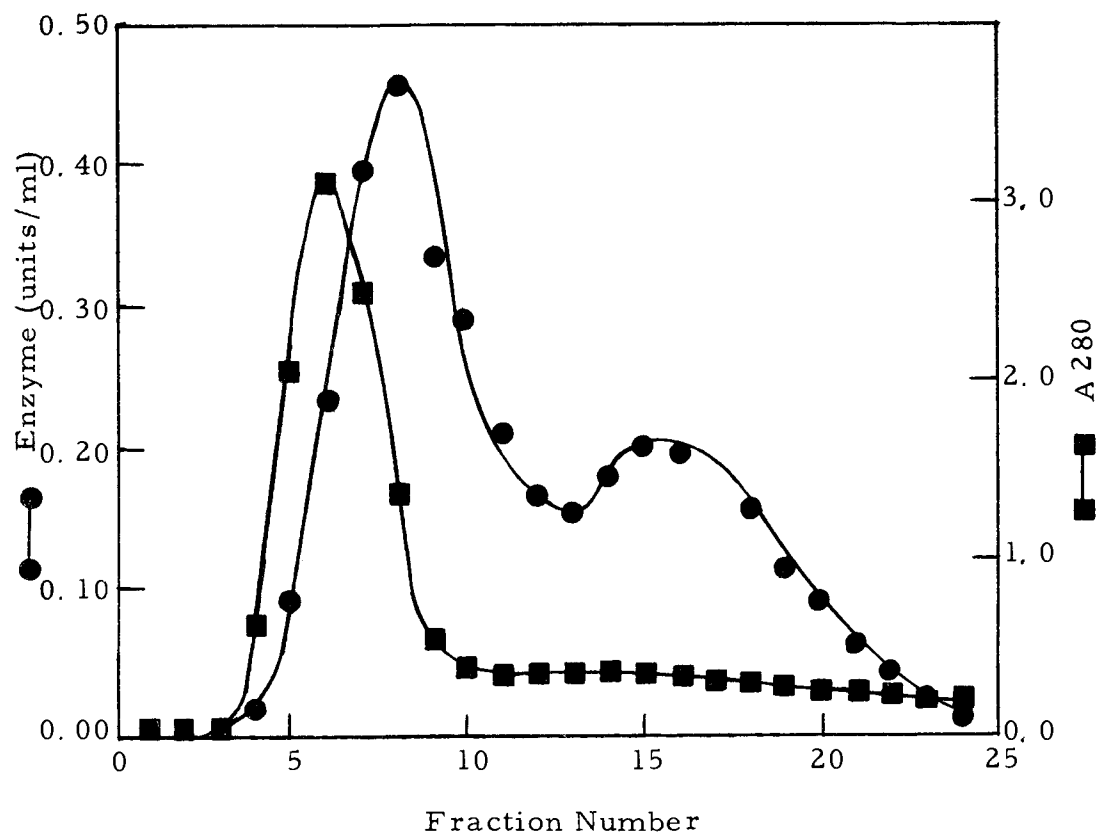
Figure 3.   Sephadex G-200 gel filtration of partially purified
β-galactosidase from Streptococcus lactis 7962 using
a 2. 5 by 38 cm column at 5 C eluted with 0. 1 M sodium
phosphate buffer, pH 7. 0.   Each fraction contained 100
drops.

at 5 C by use of two identical Sephadex G-200 columns, each mounted over a fraction collector. The additional sonication resulted in only quantitative changes in both enzyme fractions, indicating that the two forms were not produced by the sonication but probably existed as such within the cell.

Figure 3 shows a typical enzyme elution pattern of cell extracts taken from the log phase of growth; peak I is greater than twice as large as peak II. The protein elution pattern indicated that the majority of the protein in the partically purified preparation emerged from the column immediately after the V_0. Peak 1 enzyme activity was retained slightly and it emerged two fractions after the peak of protein. However, peak II enzyme activity was retained longer and was eluted later when the protein concentration was lower. Thus, the column yielded at this point a more highly purified preparation and data presented later will show that peak II enzyme is quite free of nonenzyme protein. Peak I enzyme however was purified only two to three-fold over the partically purified preparation, while peak II was purified an additional ten-fold, giving a specific activity of 4000 percent over that of the crude extract.

Properties of the Enzyme

Disc Electrophoresis

Gel cylinders containing enzyme were removed from the

tubes and immersed in ONPG solution at 37 C. When this test for

enzyme was attempted on samples that were exposed to as little as

0. 5 milliamp of current per sample for 5 minutes, complete enzyme

inactivation resulted. Identical samples, however, that were not ex-

posed to any current did show high activity when analyzed in the same

way.

Ribosome-bound Enzyme

The results of several experiments carried out according to

Zipser (1936b) to detect ribosome-bound enzyme were negative.

Centrifugation studies on the enzyme also failed to give evidence that

the enzyme was particulate in nature.

Stability in Buffer

The partially purified enzyme was quite unstable in buffer at

5 C, especially at low protein concentrations. Figure 4 presents

some results in this regard; when resuspended in 0. 1 M phosphate

buffer at 5 C, the more concentrated solution revealed a much

higher stability than the dilute solution. Even in the more concen-

trated form, the activity decreased exponentially with an initial in-

activation rate of 5 percent per day. In the more dilute solution

the initial rate of decrease in enzyme activity was one percent per

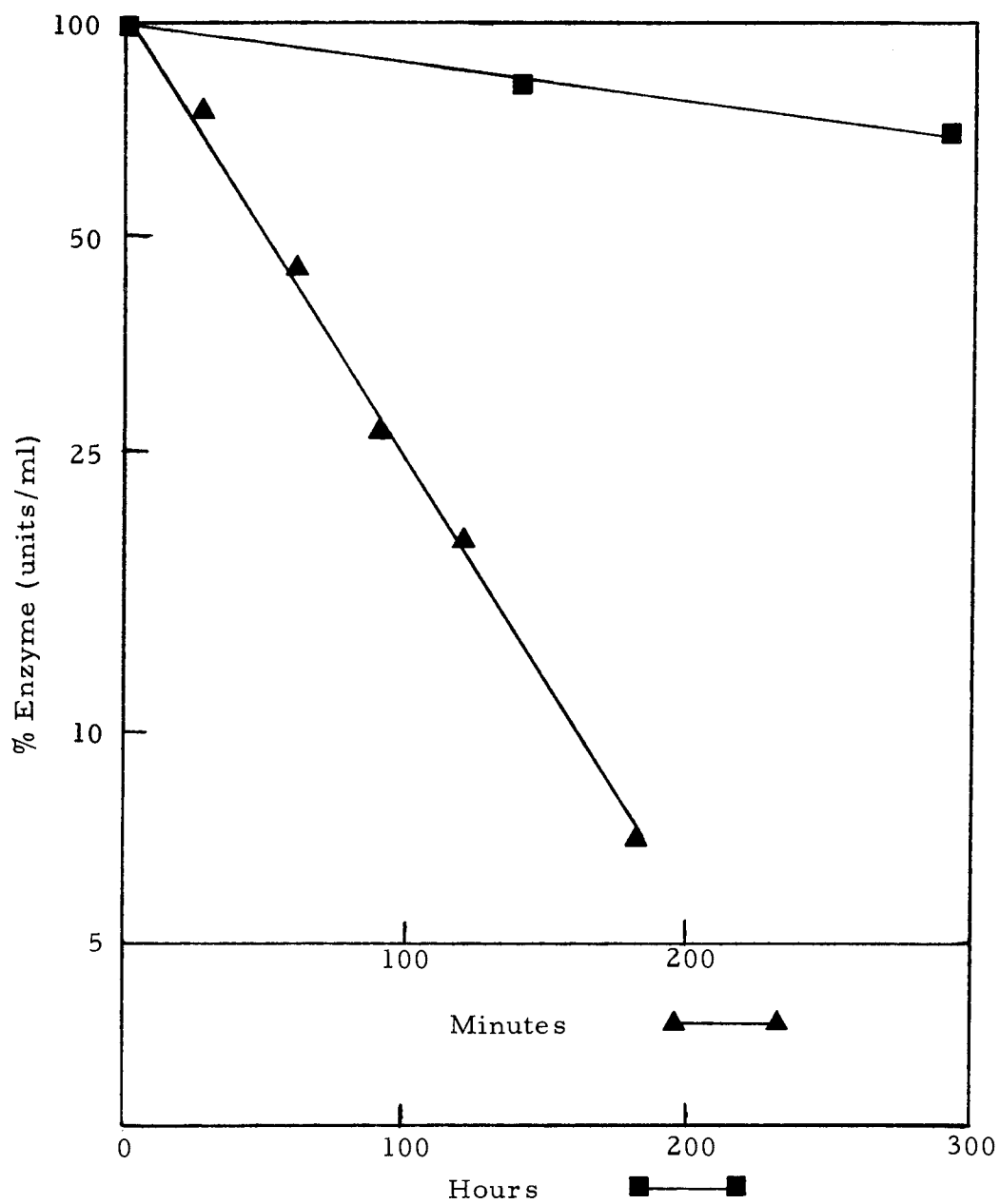hour. The lability of the enzyme to various chemicals and procedures

Figure 4. Effect of storage at 5 C on enzyme activity on partially
purified β-galactosidase from Streptococcus lactis 7962.
The protein concentrations were 0. 1 mg/ml (▲), with
time measured in minutes and 0. 5 mg/ml (■), with the
time measured in hours.

has already been mentioned. Tris (hydroxymethyl aminomethane) buffer also allowed extremely rapid and complete inactivation.

Effect of Metal Ions

The addition of metal ions (as salts) was attempted in an effort to stabilize the enzyme. Various salts of iron, magnesium and manganese were not effective in this regard. Calcium added as calcium chloride (10^{-2} M) did afford some protection.

Ammonium Sulfate Effect

During the purification procedure it was observed that ammonium sulfate protected the enzyme from activity loss. Figure 5 shows the effect of varying concentrations of ammonium sulfate on enzyme activity, both 15 minutes and 18 hours after the addition of the salt to the extract. It can be seen that in the case of the 15-minute exposure time that maximal enzyme activity occurred at a salt concentration of about 1.4 M. In the case of the 18-hour exposure time at 5 C, marked stimulation of enzyme activity occurred, with 1.25 M ammonium sulfate exhibiting the maximal effect; in this case the activity of the control aliquot (O M) dropped from 13 to 2 units but at 1.0 M salt concentration an eight-fold stimulation over the control was noted. The decrease in the activity above 1.3 M salt concentrations was caused by precipitation of the enzyme. This same
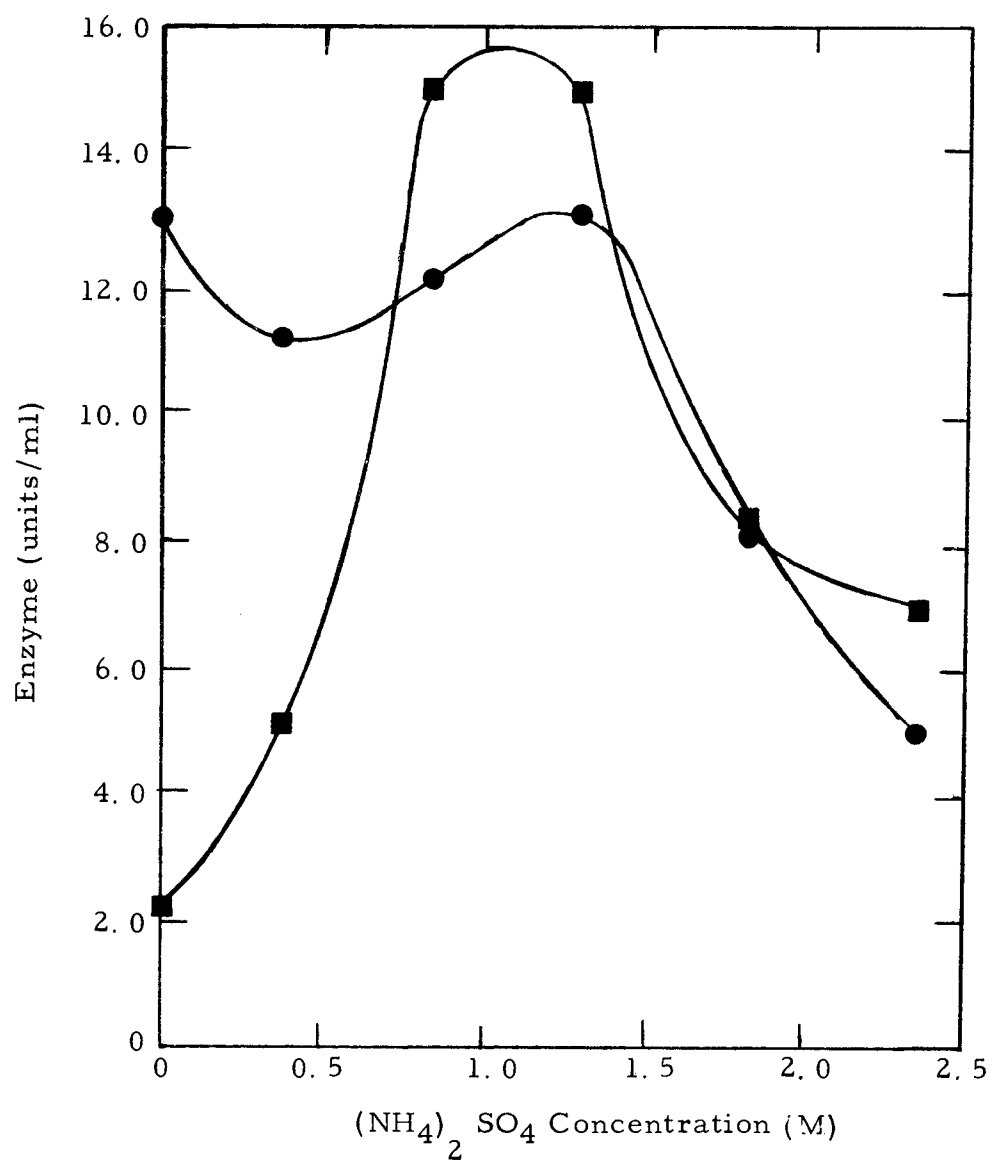
Figure 5. Effect of various concentrations of ammonium sulfate on β-galactosidase activity of Streptococcus lactis 7962 upon storage for 15 minutes (●) and 18 hours (■) at 5 C.

experiment was carried out using β-galactosidase of E. coli pre-

pared as a crude extract and only a slight stimulation (4%) of enzyme

activity occurred at 0.85 M ammonium sulfate. Combinations of

ammonium and sulfate ions with various other ions were tested in

an attempt to duplicate this salt effect; all such compounds failed to

stabilize or stimulate the enzyme.

Figure 6 shows the effect of 0.85 M ammonium sulfate on a

partially purified enzyme solution in phosphate buffer at 5 C. Upon

addition of the salt, there was an immediate decrease in enzyme ac-

tivity followed by a rapid and then more gradual increase. The

activity of the control solution (without salt) decreased as was seen

before.

Since the presence of ammonium sulfate allowed preparation

of a more stable enzyme, it was felt that gel filtration purification

could be more readily accomplished in the presence of this salt.

Therefore, enzyme treated for 30 hours in 0.85 M ammonium sulfate

and then centrifuged to insure the absence of any insoluble particles

was run through a Sephadex G-200 column previously equilibrated

with buffer containing 0.85 M salt. Enzyme separated in this manner

was compared with an aliquot of the same enzyme preparation

separated on a non salt equilibrated column. The enzyme activity

data from both separations can be seen in Figure 7. In the unstab-

ilized sample the usual profile of two enzyme activity peaks can be
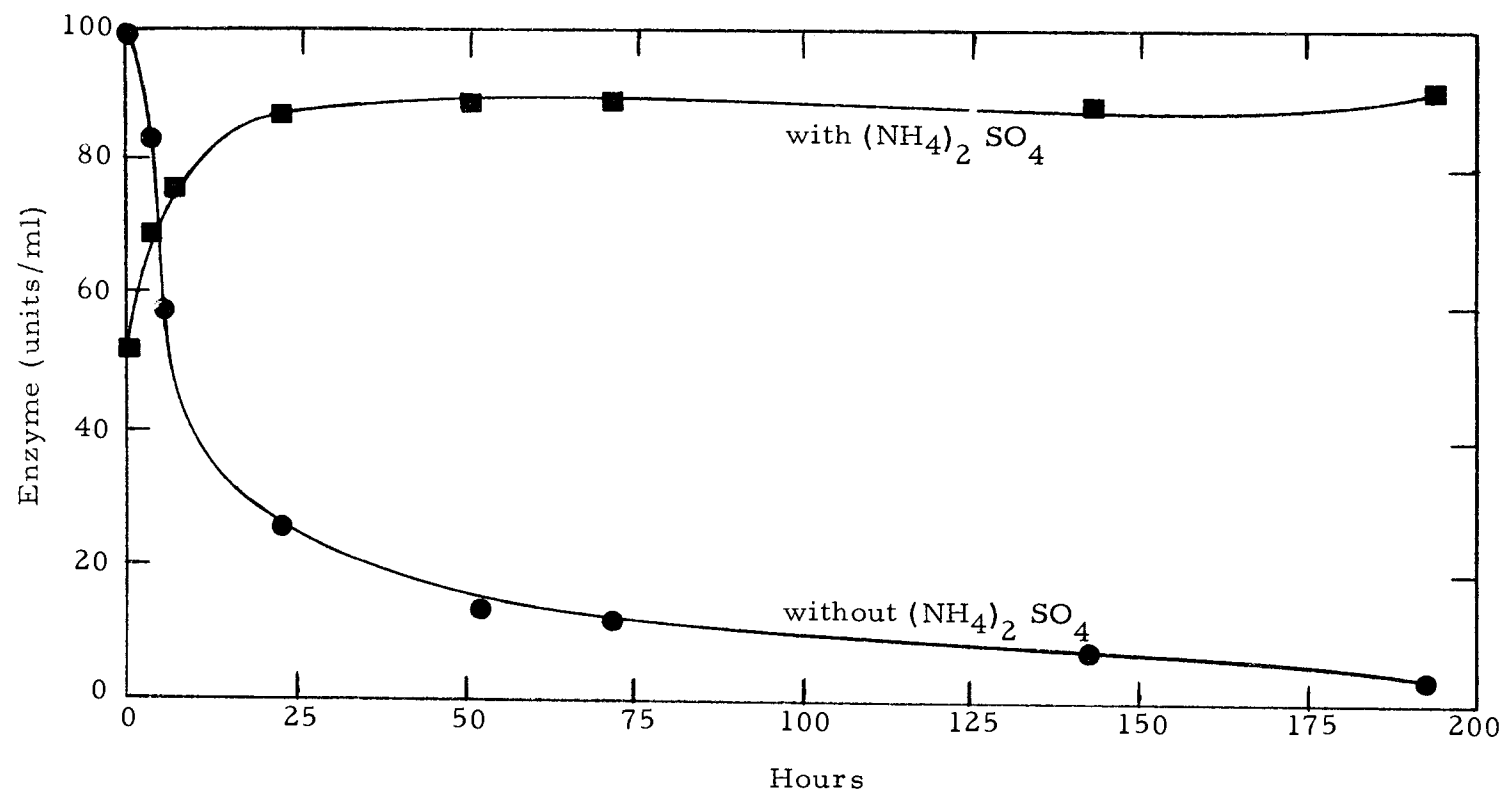
Figure 6. Effect of 0. 85 M ammonium sulfate on the stability of β-galactosidase in Strepto-
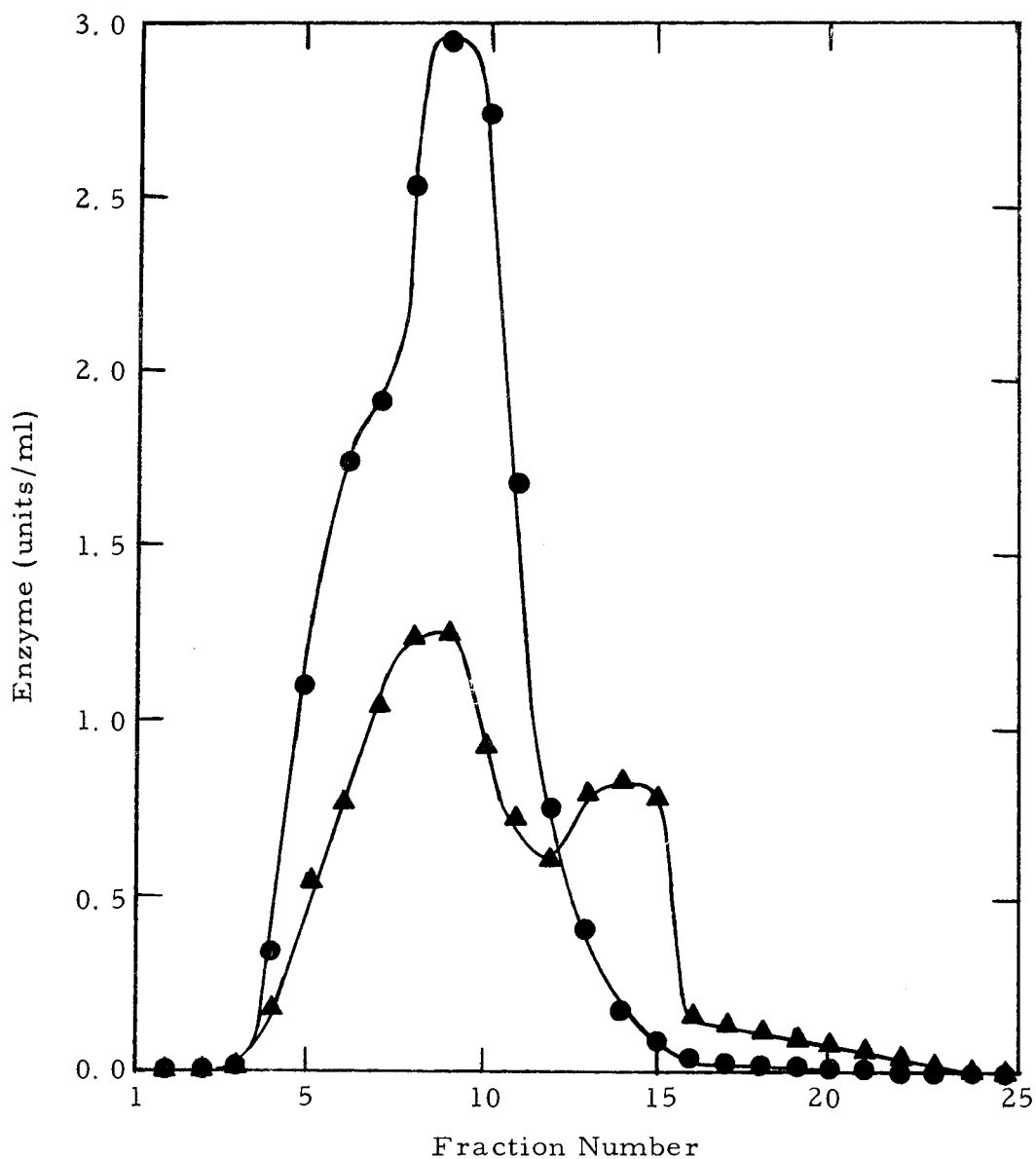coccus lactis 7962 at 5 C.

Figure 7.   Enzyme elution pattern of identical samples of partially
purified   β-galactosidase from Streptococcus lactis 7962
that were salt-stabilized (●) and non-stabilized (▲).
A 2.5 by 38 cm Sephadex G-200 column was used at
5 C with 0. 1 M sodium phosphate buffer, pH 7. 0 as
eluant.   Each fraction contained 100 drops.

seen. In the stabilized sample only one peak (peak I) of enzyme

activity was seen. It appeared therefore that the ammonium sulfate

affected not only a greater recovery of total enzyme activity but also

caused association of peak II enzyme molecules into enzyme units

separating as peak I. Furthermore, about 40 percent of the original

activity of the salt stabilized preparation was recovered from the

salt equilibrated column as compared with about 10 percent in the

case of the non-salt treated sample. (Data presented later will

indicate that peak II represents subunit of the peak I enzyme and

that ammonium sulfate encourages formation of the larger molecular

weight (peak I) form).

The shoulder on the leading edge of peak I in both curves was

caused by turbidity that carried over from the large protein peak

into the tubes sampled for enzyme assay; the enzyme eluted as peak I

and the large protein (A_{280}) peak overlapped in that area. The elu-

tion patterns of the protein in these two separated enzymes were

exactly the same except in the area of peak II (fractions 11 to 15)

where the elution curve of the unstabilized preparation was higher.

Effect of Organic Stabilizers

Cleland's reagent (dithioltheitol) first used by Cleland (1964)

as an enzyme protective agent did afford some protection for the

S. lactis β-galactosidase.

Sucrose (40%, w/v) also protected the enzyme. Data showing these effects are cited below.

Proteinase Activity

Proteinase assays, carried out as described above, were performed on enzyme solutions after the various stages of purification. Evidence for the presence of a proteolytic enzyme in the various samples was found. However, the levels of activity were quite low and it was therefore felt that enzyme instability was not caused by proteolytic enzymes in the cell extracts.

Evidence for Metal Involvement in the Enzyme

The effect of the divalent metal ion chelator ethylenediamine tetraacetic acid (EDTA) on the activity of the enzyme was tested. Figure 8 shows the effect of various concentrations of EDTA on a partially purified preparation at 5 C. It may be seen that there was a rapid period of inactivation and then a stabilization of enzyme activity. The level of inactivation was proportional to the concentration of EDTA in solution. When the level of enzyme activity found at the different EDTA concentrations used was plotted versus the log of chelator concentration, a straight line resulted. This indicated that there were two different effects exerted by EDTA on the enzyme, a rapid inactivation and a stabilization. No explanation was apparent
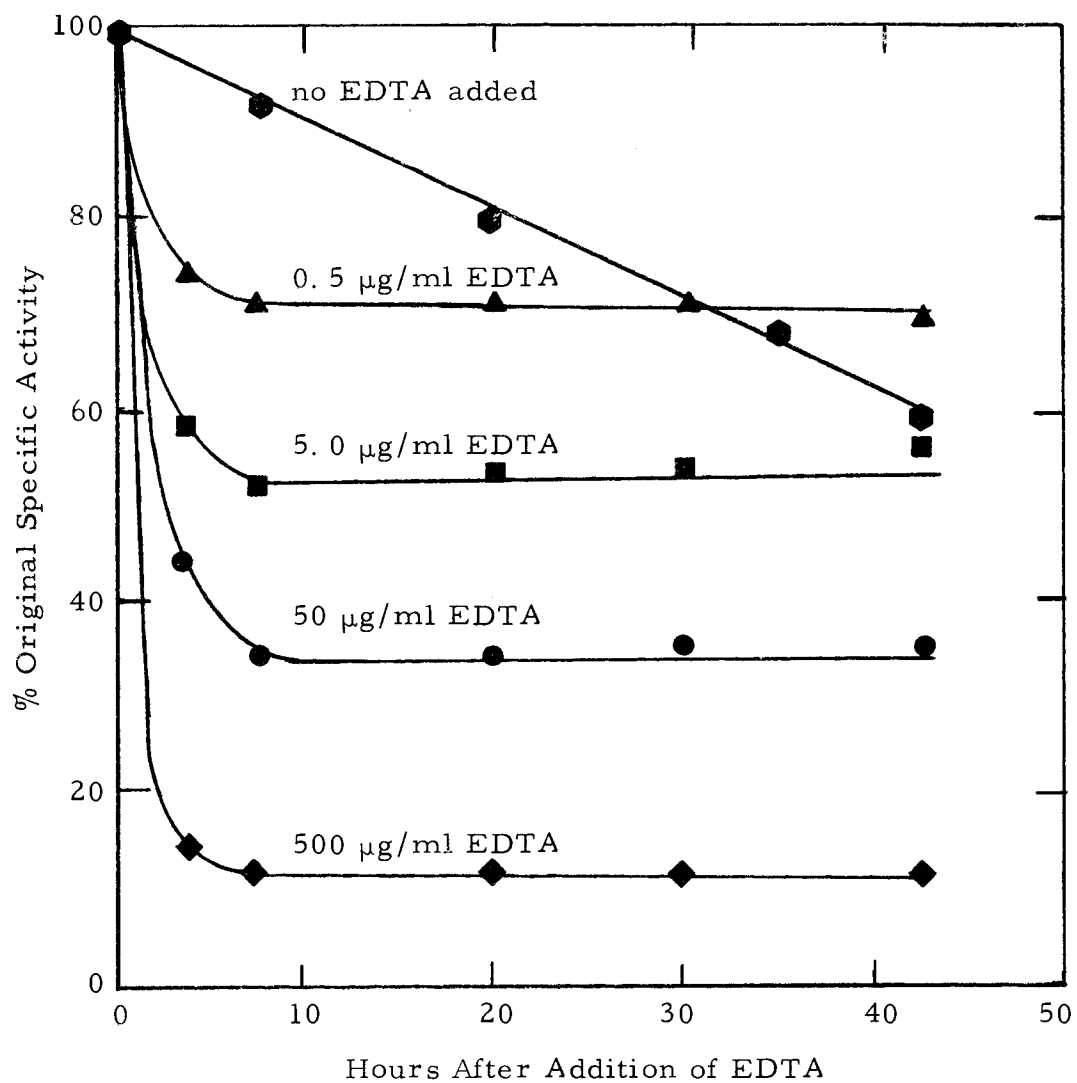
Figure 8.   Effect of various concentrations of EDTA on the
specific activity of partially purified β-galactosidase
from Streptococcus lactis 7962 held at 5 C.

for the stabilization effect but the inactivation indicated that metal

ions were involved in the enzyme structure. When, however, the

EDTA was added (less than 0.5 mg/ml) to that which had been stabi-

lized with 0.85 M ammonium sulfate, the chelator had no effect on

enzyme activity. This may be seen in Figure 9; the salt brought

about changes that make the enzyme insensitive to EDTA.

Calcium also was found to stabilize the enzyme and provided

further evidence that a metal ion, possibly calcium, was a structur-

al part of the enzyme.

Evidence for Sulfhydryl Groups

The effect of the sulfhydryl antagonist para-chloromercuriben-

zoate (PCMB) was tested on the activity of β-galactosidase. Figure

10 shows the inactivation brought about by the addition of 0.02 mg/ml

of PCMB to an unstabilized enzyme preparation. This supported the

idea that -SH groups were involved in the active center of the enzyme.

When a salt-stabilized enzyme preparation was exposed to 0.01 mg/ml

PCMB, no inactivation resulted, hence it was clear that the ammon-

ium sulfate protected the enzyme from the effects of the sulfhydryl

antagonist.

Clelands reagent (0.25 to 5.0 mg/ml) was added to partially

purified enzyme solutions in buffer. The stabilization which resulted

may be seen in Figure 11. The effect of concentration of the reagent
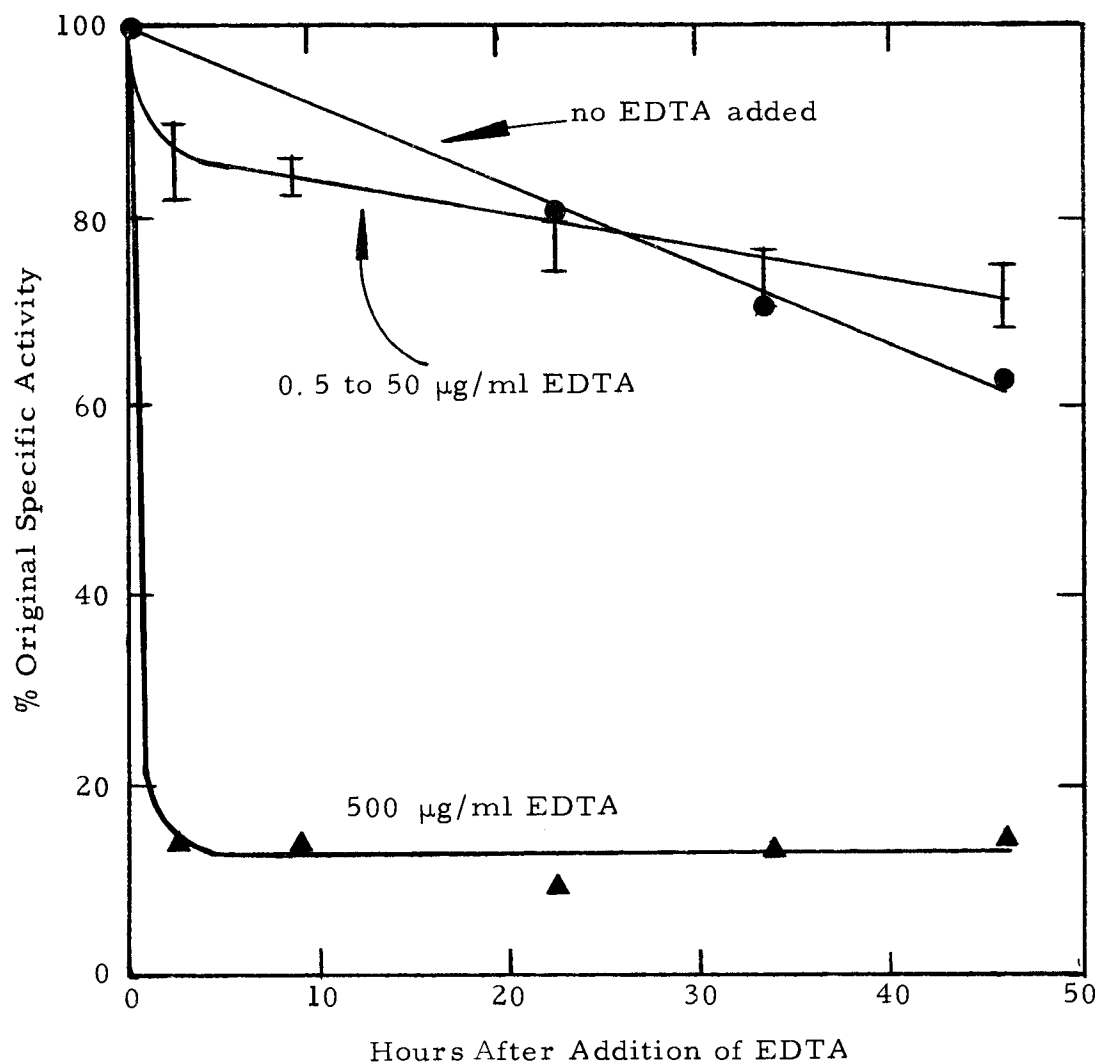
Figure 9.   Effect of various concentrations of EDTA on the specific
activity of partially purified, salt-stabilized   β-
galactosidase from Streptococcus lactis 7962 held at
5 C.
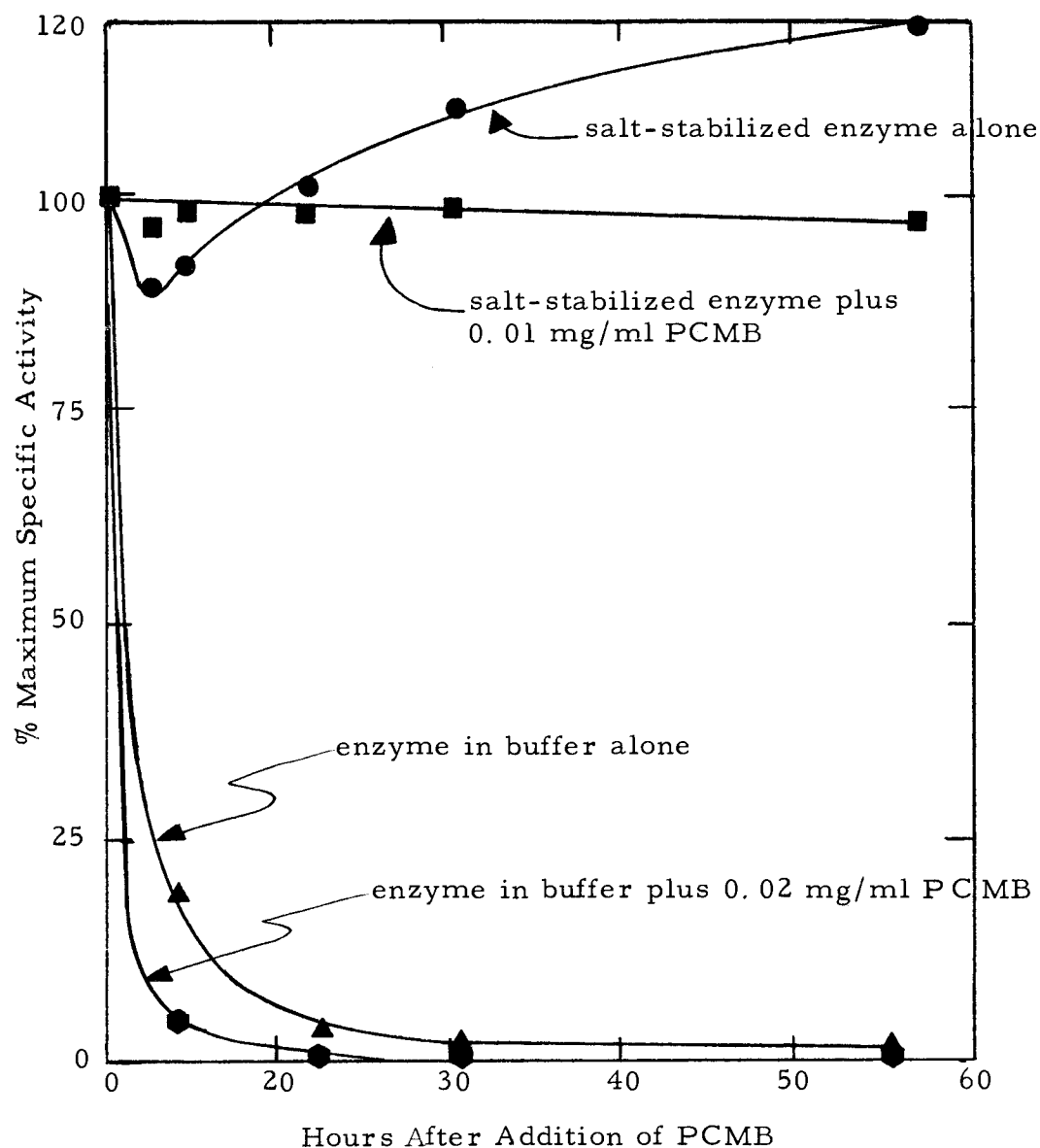
Figure 10. Effect of PCMB on specific activity of salt-stabilized
and non-stabilized β-galactosidase of Streptococcus
lactis 7962 at 5 C. The non-stabilized enzyme was
in 0. 1 M sodium phosphate buffer at pH 7. 0 while the
salt-stabilized enzyme was in the same buffer plus 0. 85
M ammonium sulfate.
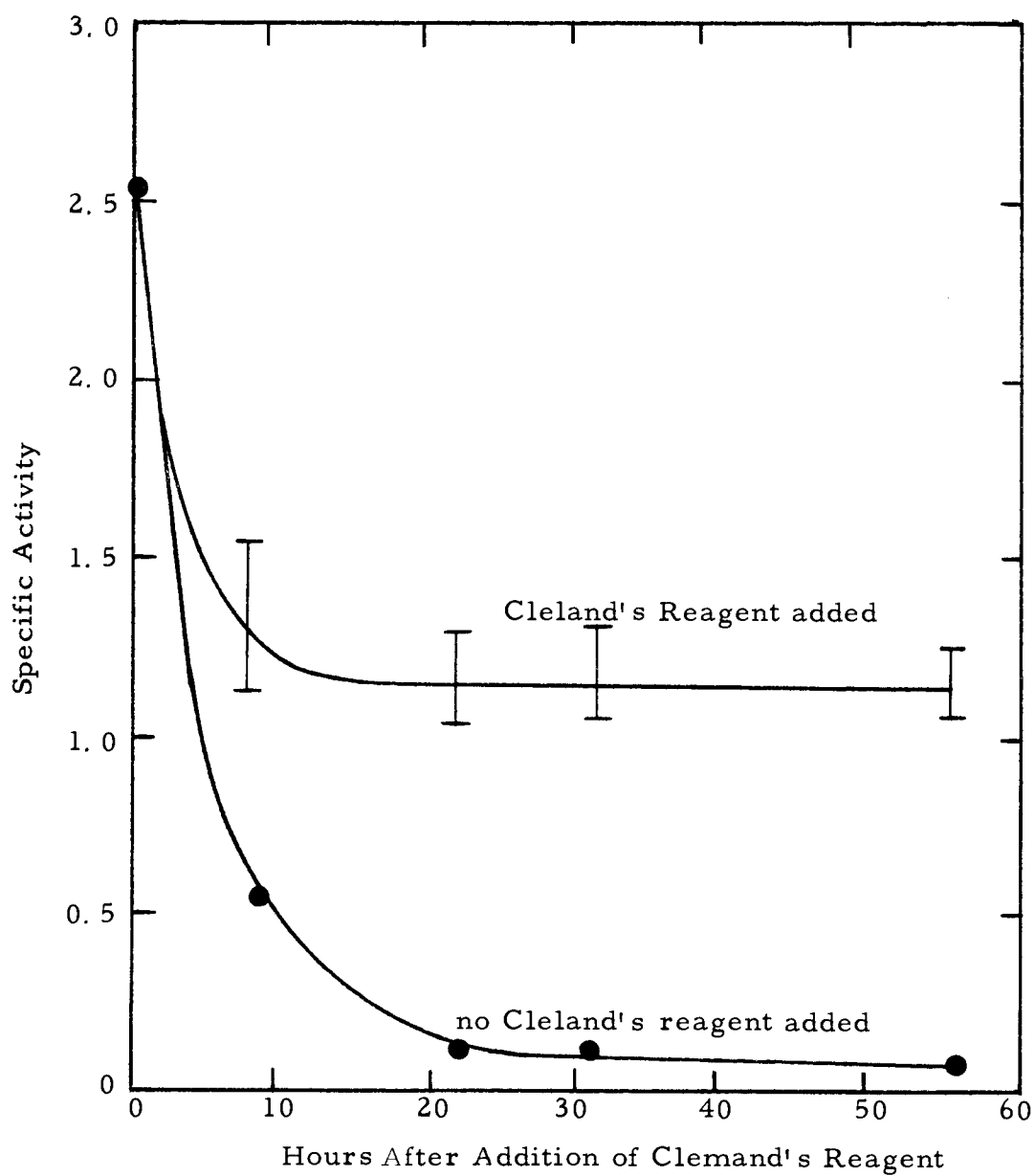
Figure 11. Effect of addition of Cleland's reagent to partially pur-
ified β-galactosidase of <u>Streptococcus</u> <u>lactis</u> 7962.
The concentration of reagent added ranged from 0. 25
to 5. 0 mg/ml.

appeared to be negligible over the range studied. However, it did stabilize the enzyme at about 50 percent of maximum specific activity. These data also suggested the involvement of -SH groups contributing to the activity in the enzyme.

Sulfhydryl titrations were carried out as described above on enzyme eluting in peaks I and II following Sephadex gel filtration. The number (23) of micromoles of -SH per mg of protein was determined and the data recalculated to express the number of SH groups per molecule, assuming a molecular weight of 5×10^5. The calculated value was 11.5 moles of -SH per mole of enzyme.

Molecular Weight Determination

By replotting the data of Dasgupta, Boroff and Rothstein (1966) and adding the V_e data from a typical Sephadex G-200 enzyme separation, molecular weights were determined for both peak I and peak II enzyme fractions. This can be seen in Figure 12. The molecular weights of peak I and peak II fraction were 9.4×10^5 and 5.4×10^5 respectively, since they eluted at a V_e of about 12 and 24 ml respectively. Since the volume of each fraction was 3.2 ml, the V_e marking the beginning of a peak elution was ± 3.2 ml; therefore the molecular weights were rounded off to 10^6 and 5×10^5 for the peak I and peak II enzymes respectively.

Figure 12. Molecular weight standard curve determined on a 2. 5 by
40 cm Sephadex G-200 column using proteins of known
molecular weights (Dasgupta, Boroff and Rothstein, 1966)
Data from similar separations of Streptococcus lactis
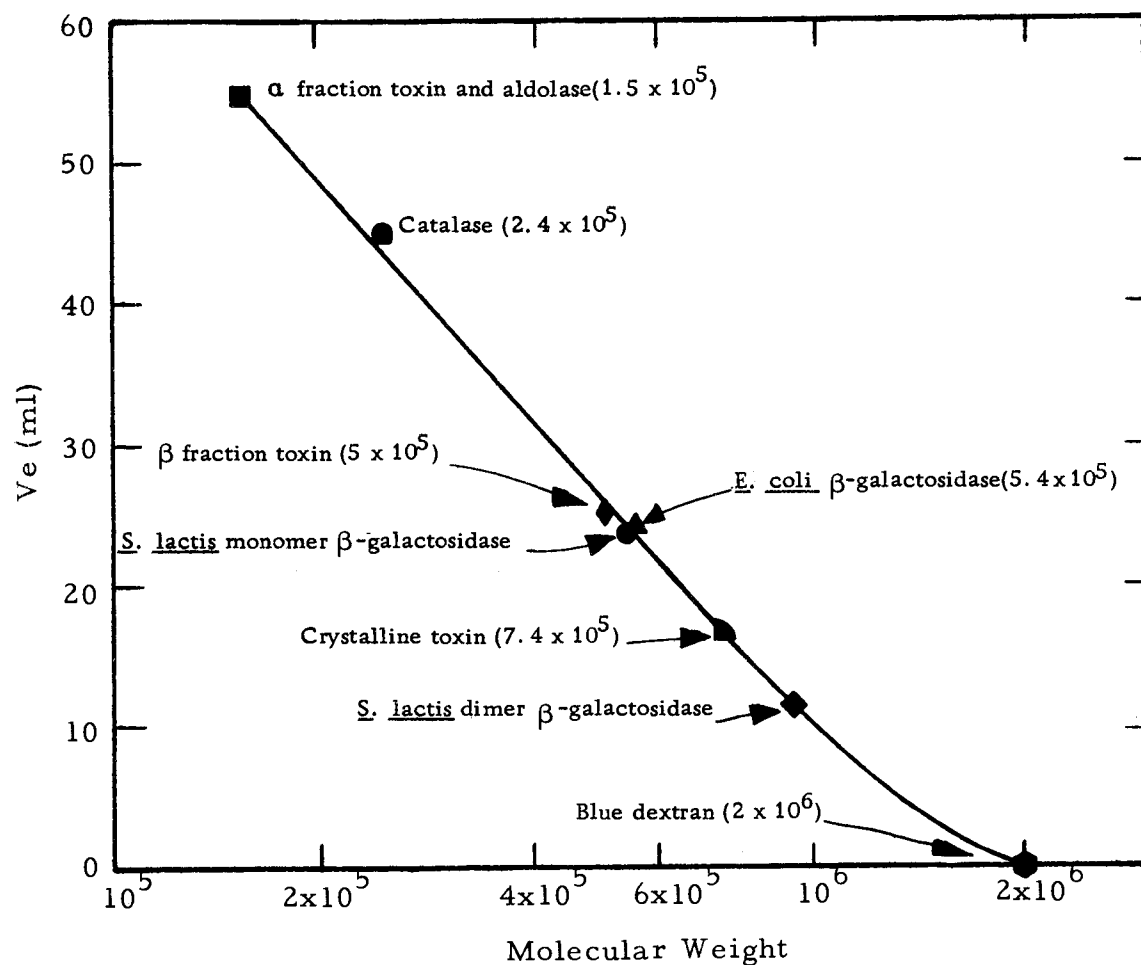7962 β-galactosidase were added to the graph.

Amino Acid Composition

Examination of initial data obtained from the amino acid ana-

lyzer indicated that the S. lactis 7962 β-galactosidase did not contain

any half cystine residues; histidine was the amino acid present in the

least amount. The absence of cystine was contradictory to the data

implicating -SH groups in the enzyme. Therefore, the performic

acid oxidation procedure of Moore (1963) for conversion of cysteine

to cysteic acid was carried out and then a larger sample was examined

on the analyzer. These results indicated that there was six times as

much histidine as cysteic acid present in the enzyme. These data

may be seen in column five of Table 2. The values represent a mul-

tiple of the molecular weight of the peak II enzyme. The figures in

column four then were each multiplied by the molecular weight of the

appropriate amino acid to obtain the data seen in column five. The

total of the column five figures then represented a minimum multi-

ple of the molecular weight and the determined molecular weight of

the peak II fraction was 11 times greater than the column five total

figure. In other words, there was 11 times the number of each of the

amino acids indicated in column four. The values in column three

are presented to allow comparison of the amino acid composition

data found in this study with data for the β-galactosidase of Escher-

ichia coli ML 308 and Aerobacter formicans PG-L seen in columns

Table 2. Amino acid composition of Streptococcus lactis 7962 β-galactosidase as compared with the same enzyme from Escherichia coli and Aerobacter formicans (Rohlfing and Crawford, 1966).

Amino Acid	Moles of Amino Acid/10^5 gram Protein			Residues/Cysteic acid	Residues/Cysteic acid x MW
	Column 1	Column 2	Column 3	Column 4	Column 5
	E. coli ML 308	A. formicans PG-L	S. lactis 7962	S. lactis 7962	S. lactis 7962
Alanine	63.6	86.7	70.6	35.0	3118.15
Arginine	51.8	68.0	24.5	12.0	2090.40
Aspartic acid	88.8	80.0	80.4	40.0	5324.00
Half cystine	12.2	16.8	2.0	1.0	121.16
Glutamic acid	97.9	90.4	82.8	41.0	6032.33
Glycine	56.1	70.1	58.6	29.0	2177.03
Histidine	26.4	33.2	12.0	6.0	1257.84
Isoleucine	33.1	28.1	40.6	20.0	2623.00
Leucine	83.7	102.4	57.0	28.0	3672.00
Lysine	17.3	13.3	47.2	24.0	3508.56
Methionine	32.3	23.9	15.0	8.0	1193.68
Phenylalanine	30.6	27.8	27.1	14.0	2312.66
Proline	51.1	60.3	NR[a]	NR[a]	NR[a]
Serine	33.2	31.1	35.4	18.0	1891.62
Threonine	39.0	37.4	41.0	21.0	4288.62
Tryptophan	46.3	41.9	NE[b]	NE[b]	NE[b]
Tyrosine	27.6	24.4	15.8	8.0	1449.52
Valine	53.6	49.4	46.7	23.0	2694.45
				TOTAL	43,766.02

[a] The proline peak was not resolved sufficiently to estimate its content.

[b] Tryptophan was not estimated.

one and two (Rohlfing and Crawford, 1966).

Evidence of Conformational Changes

The amino acid sequence of a protein determines its primary structure, while the regular and nonregular folding of the polypeptide chains determines the secondary and tertiary structure, respectively. Conformational changes are defined as any changes in the secondary or tertiary structure. In the present study, the effect of ammonium sulfate in reducing the inhibitory action of EDTA and PCMB on the enzyme appeared to be related to conformational transition.

Urea is well known as a strong perturbant of enzyme conformation and therefore the effect of this compound on enzyme activity was examined. The effect of the urea on both salt stabilized and nonstabilized enzyme is shown in Figure 13. The specific activities of enzyme exposed to 2.0 M and 4.0 M urea was compared to non-urea treated controls (salt stabilized and nonstabilized) at various times. The data were plotted as percent of control specific activity at each time. From the figure it may be seen that salt stabilization did prevent much of the inactivation, especially at the lower urea concentrations. The fact that the urea did inactivate the enzyme at a rate so much higher than the control was further evidence that conformational changes with resulting enzyme damage had occurred.
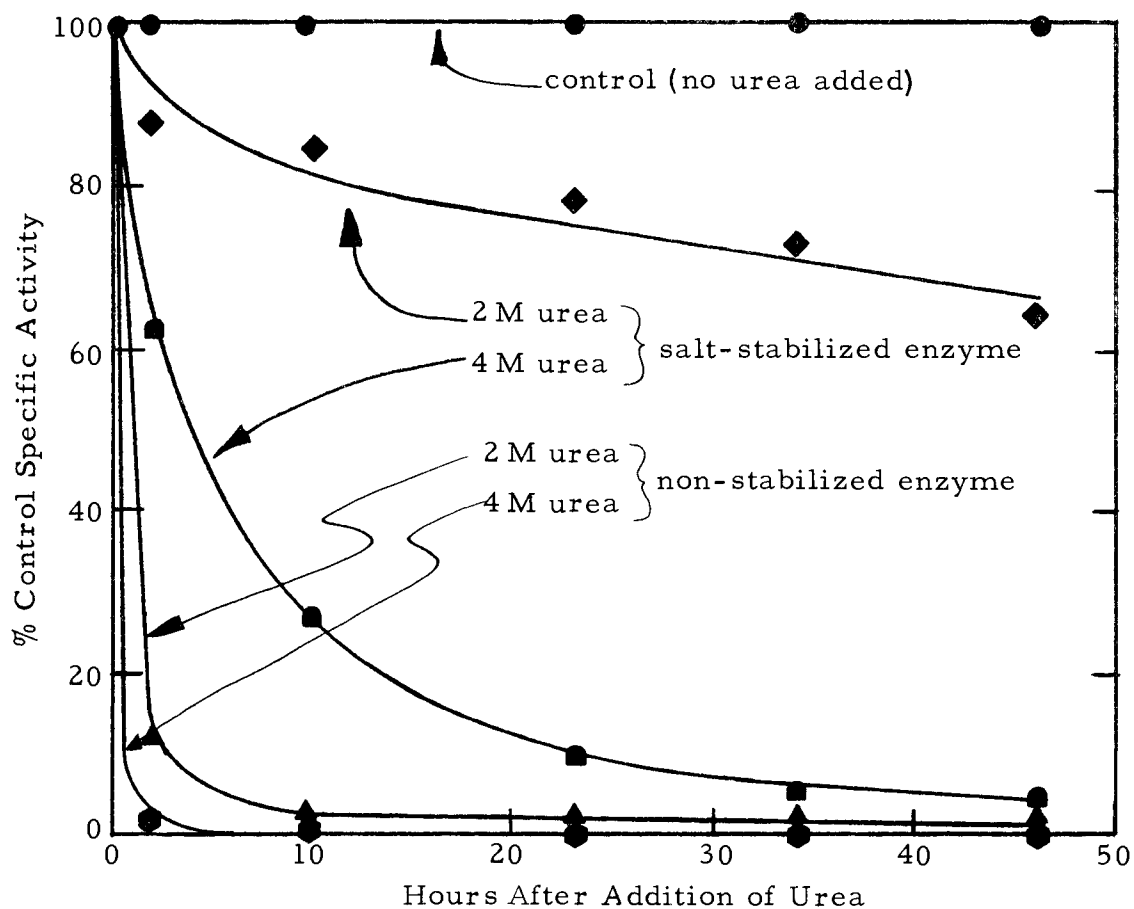
Figure 13.   Effect of 2 and 4 M urea on salt stabilized and non-
stabilized partially purified β-galactosidase of Strepto-
coccus lactis 7962.   Specific activities were calculated
as percent of a control (no urea added and non-stabilized)
at each time.

Blue shift data provided additional aid in determining what changes occurred during the salt-stabilization process since a blue shift also is indicative of conformational enzyme changes. The UV spectra of the enzyme preparations revealed a maximum adsorption at 260 mμ. When the spectrum of a nonstabilized sample was compared with that of a salt stabilized enzyme of equal protein concentration, a definite shift toward the shorter wavelength was noted in the spectrum of the nonstabilized sample (blue shift). This spectral shift resulted in an increase in the A_{250} in the nonstabilized sample. Therefore, blue shift data were obtained by comparing the increase in absorbancy at 250 mμ of the two samples. Figure 14 shows the blue shift that took place when salt-stabilized and nonstabilized preparations were compared. The nonstabilized preparation did exhibit a blue shift while the stabilized sample remained unchanged. Similar results were noted (Figure 14) when nonstabilized enzyme from peak I and peak II were compared; peak I enzyme revealed a larger shift than the peak II fraction and quantitative comparisons could be made since the results were expressed as the change in A_{250}/mg of protein.

Kinetic Properties

When the hydrolysis of ONPG by a crude enzyme extract was followed on the recording spectrophotometer, it was found that at
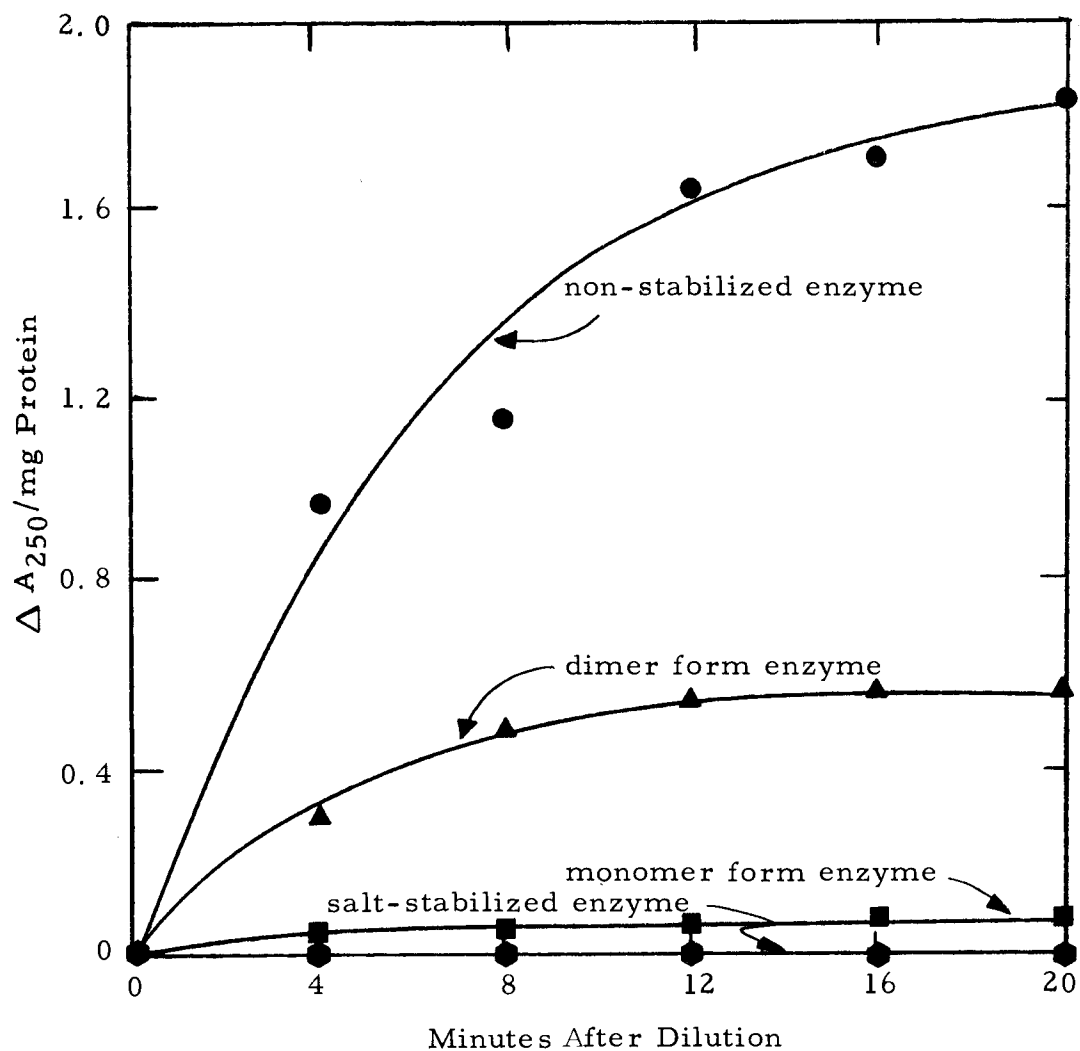
Figure 14. Blue-shift following 1:10 dilution into 0. 1 M phosphate
buffer, pH 7. 0 at 25 C of Streptococcus lactis 7962
β -galactosidase; monomer and dimer forms after gel
filtration and salt stabilized and non-stabilized partially
purified enzyme.

37 C the duration over which linear (first order) kinetics was observed was only about 5 minutes. When three different enzyme concentrations were used, the curves all deviated from linearity at the same time. Following this the reaction rate decreased due to heat inactivation of the enzyme even at high protein concentrations.

Quaternary Properties

In describing the structure of proteins, the physical association of subunits (monomers) to form a larger (polymeric) molecule is defined as quaternization; the monomeric make-up of a polymer is referred to as the quaternary structure. In the present study, four types of data (pH and temperature optima, kinetic and molecular weight information) were obtained to indicate that a quaternary relationship did exist in the S. lactis 7962 β-galactosidase. Optimum pH determinations were done on both the crude extract and the peak I fraction following gel filtration (Figure 15); an optimum for both of 7.0 to 7.1 was obtained. Furthermore, the close fit of the two curves indicated that pH changes affected both fractions in an identical manner.

Temperature optima were determined as described above on enzyme from peaks I and II obtained after gel filtration (Figure 16). Because of the low activity of the enzymes in these samples the reaction was carried out for 30 minutes. Curves for both fractions
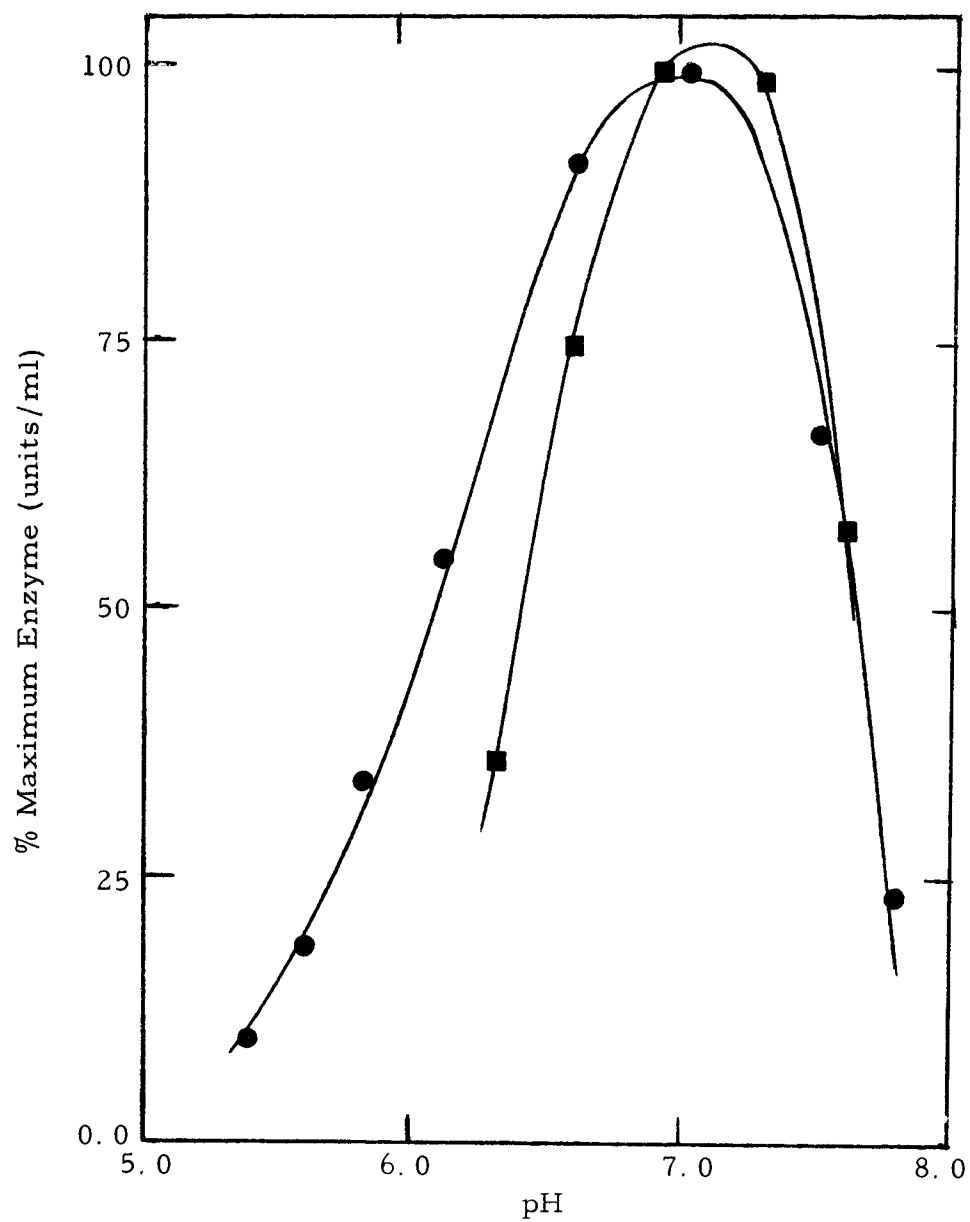
Figure 15. Effect of pH on β-galactosidase activity in crude extract (●) and dimer form following gel filtration (■).
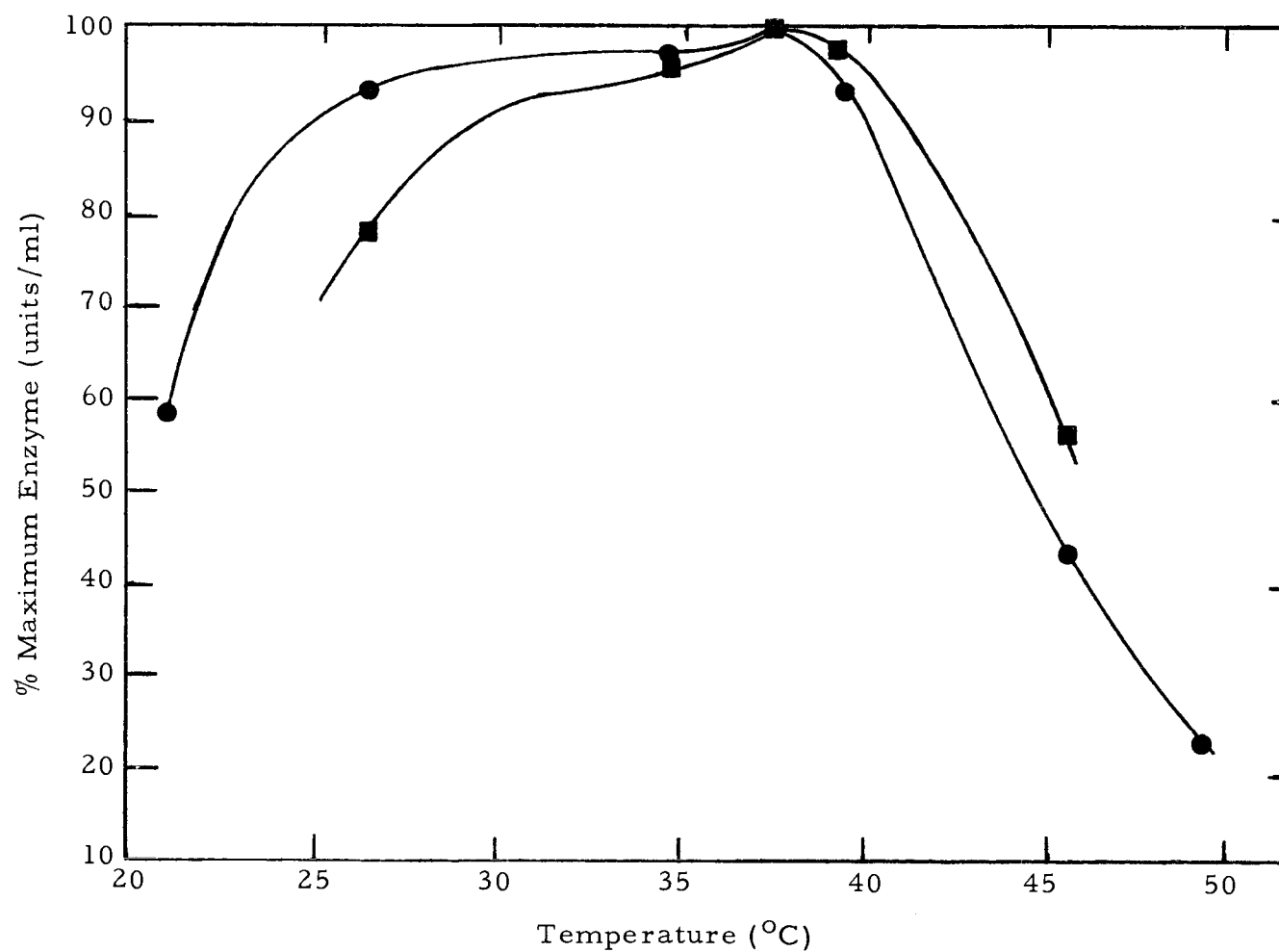
Figure 16. Effect of temperature on Streptococcus lactis 7962 β-galactosidase activity in the monomer (■) and dimer (●) form following gel filtration. The enzyme assays were performed in 0.1 M phosphate buffer, pH 7.0, 5 mM ONPG for 30 minutes.

are similar and reflect identical optimum temperatures (37.5 C).

K_m values determined for enzyme eluting as peaks I and II were

1.10 x 10^{-3} and 1.16 x 10^{-3} M respectively. The average value of

four determinations was 1.06 x 10^{-3} M ONPG.

The data in Figure 7 also support the idea that quaternization occurred in the enzyme under investigation. Here it may be seen that ammonium sulfate treatment causes association of subunits to form a larger enzyme molecule. Sucrose also appeared to affect the enzyme in this manner since sucrose density-gradient centrifugation revealed only one peak of activity (Figure 17).

Samples stored at different temperatures also provided evidence for association. An enzyme sample was partially purified and then divided into three identical fractions; one was run through the gel filtration column immediately (control), one was held at 27 C for 22 hours, and the third was held at 5 C for 70 hours before addition to the column. Figure 18 shows the elution pattern of the control and the sample that was held at 27 C for 22 hours. During the incubation peak II fraction became much smaller while peak I fraction became larger. The amount of enzyme in each was estimated by plotting both peaks separately for each separation, cutting them out and weighing them on an analytical balance. Rubber gloves were worn to avoid the addition of skin oils or prespiration during the process. The ratios of the weight of peak I to the weight of peak II
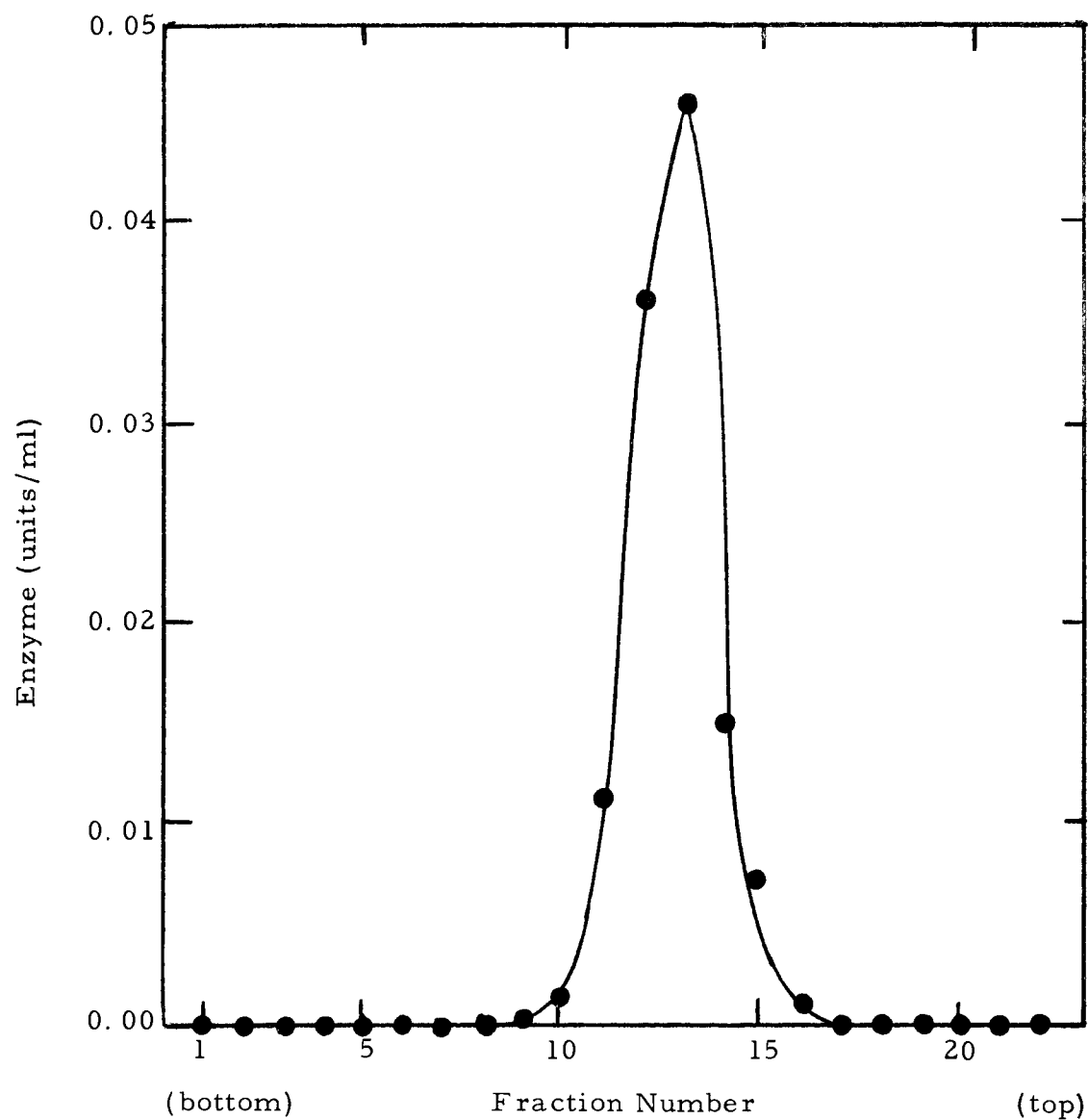
Figure 17. Distribution of <u>Streptococcus</u> <u>lactis</u> 7962 β-galactosidase
following centrifugation in a gradient of sucrose from 20%
to 5% (w/v) for 8.5 hours at 30,000 rpm. Each fraction
contained 3 drops.
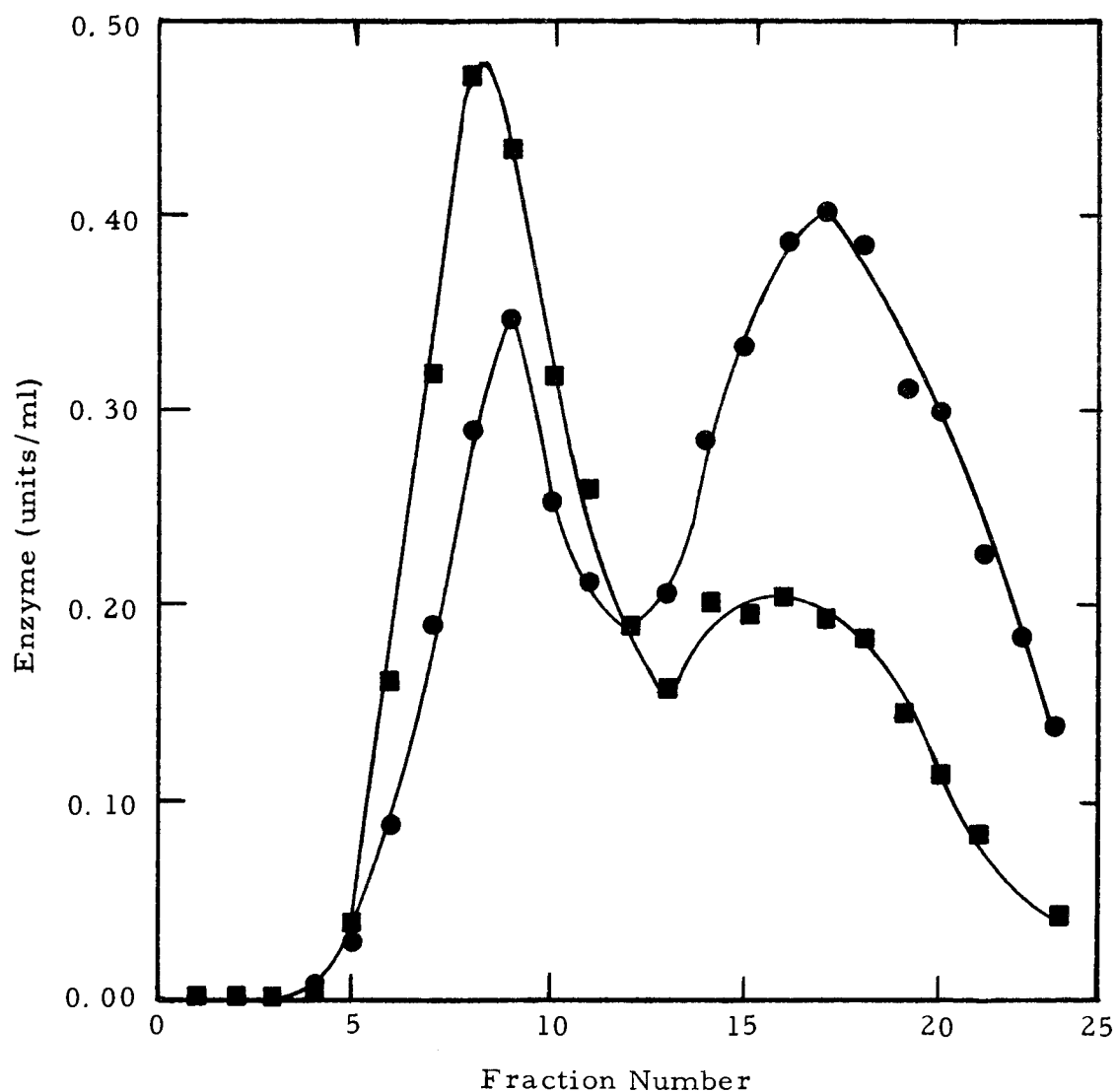
Figure 18. Sephadex G-200 gel filtration pattern of paired β-
galactosidase samples from a 12 hour culture of Strepto-
coccus lactis 7962 done immediately after partial purifi-
cation (●) and after incubation at 27 C for 22 hours (■).
The separations were done at 5 C on a 2. 5 by 38 cm
column. Each fraction contained 100 drops.

were determined for both separations; they were 0.37 for the control

and 1.10 for the sample held at 27 C for 22 hours. This indicated

there was a three-fold change in the ratio during the incubation pro-

cess. The total weight of both peaks decreased from 611.8 mg in the

control to 511.0 mg in the sample held at 27 C for 22 hours (Table 3).

Peak II fractions also were rechromatographed through a

smaller Sephadex G-200 column, following incubation for 22 hours

at 27 C to determine whether or not association would occur in a more

purified enzyme preparation. The enzyme activity dropped below

the level of detectability, even when large preparative separations

were used.

The finding that the increased sonication time did not alter

the relative amounts of enzyme subunit eluting from Sephadex columns

justified the use of sonication as an extraction procedure and estab-

lished that sonication did not, in itself, cause dissociation of the

enzyme. However, other experiments were carried out to bring about

dissociation.

The gel filtration enzyme elution pattern of a partially puri-

fied enzyme preparation was determined after holding for 48 and 168

hours at 5 C. This was done in order to study the effect of incubation

at 5 C on the enzyme quaternary composition. Results indicated

that no dissociation took place.

The gel filtration enzyme elution pattern did change, as to

Table 3. Comparison of the amount of β-galactosidase in monomer and dimer form in identical samples following extraction and purification from Streptococcus lactis 7962 grown for 12 hours and incubated under different conditions. Separations were done with a Sephadex G-200 column. The control sample was separated immediately after purification. The amount of enzyme in each peak is expressed as the weight of paper contained in the total area of each peak (see test).

Peak	Control	27 C for 22 hr	5 C for 70 hr
Dimer (D)	166.2	267.0	258.6
Monomer (M)	445.6	244.0	537.3
Total	611.8	511.0	795.9
$\dfrac{mg\ D}{mg\ M}$	0.37	1.10	0.48

the relative size of the two peaks, as the incubation time was increas-
ed prior to extraction and purification of the enzyme. This was
seen by comparing a normal enzyme elution pattern for an enzyme
extracted from culture grown for 10 hours (Figure 3) with one
grown for 12 hours (control curve from Figure 18). The age, and
hence the physiological state, of the cells from which the enzyme
was extracted was thought to be the cause of this variation. Conse-
quently a growth curve was determined for S. lactis 7962 under the
conditions that were used to grow the cells for enzyme extraction
and gel-filtration (Figure 19). The logarithmic (exponential) growth
phase extended from 6.5 hours until 11 hours after inoculation.
Cells that were grown for 10, 11, 12, and 13 hours were then har-
vested and the enzyme purified and analyzed by gel filtration
(Figure 20). The elution pattern obtained from enzyme extracted
and purified from a 10 hour culture was typical of that from cells in
the logarithmic phase of growth. The elution pattern of enzyme from
cells grown for 12 hours was radically different from the chromato-
graph of log phase cultures in that peak II significantly increased;
by 13 hours both peaks decreased in size. In some separations
that were done on enzyme prepared for cultures grown for about
12 hours, peak II was even larger in size than the one shown in
Figure 20.

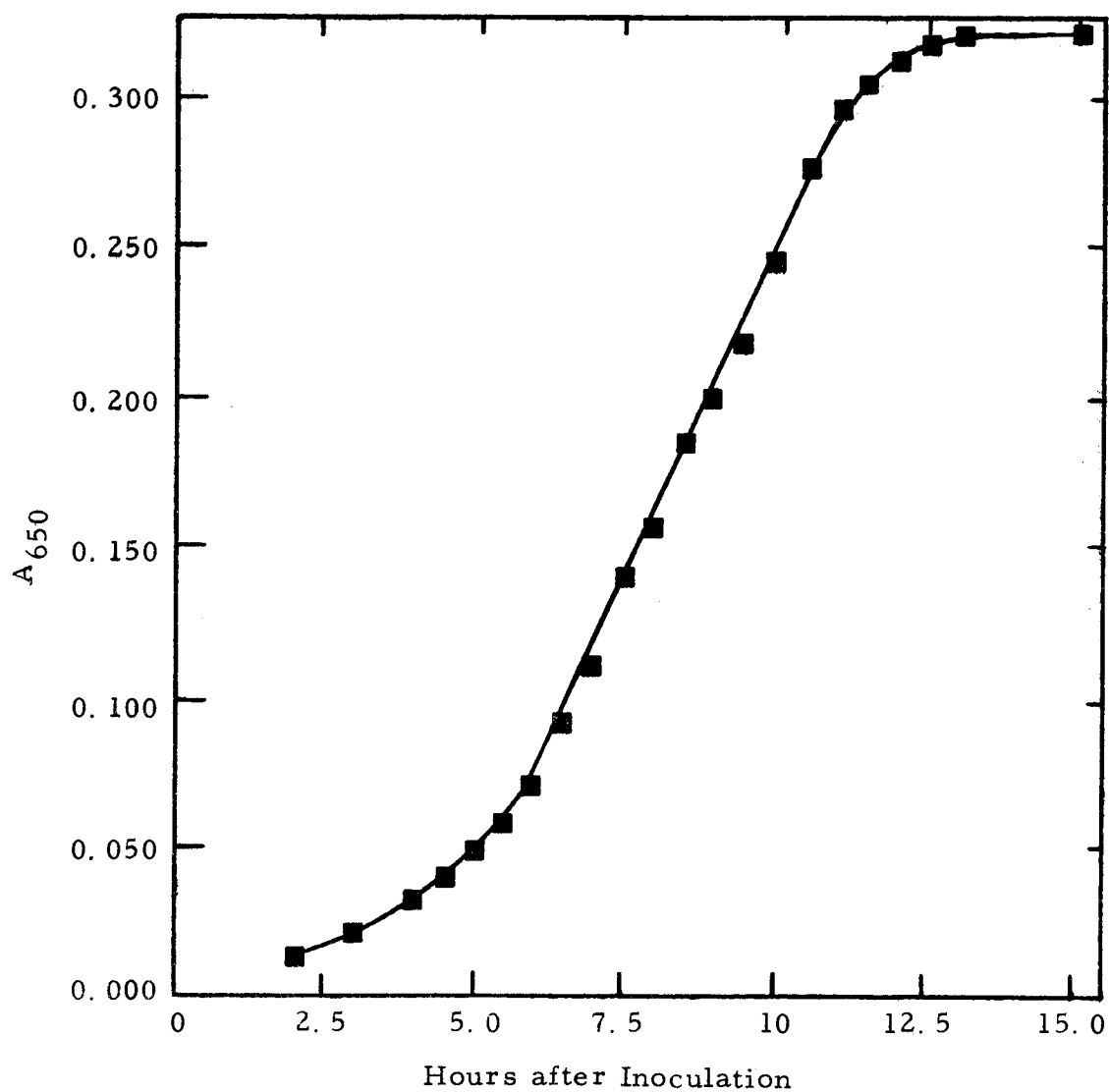Figure 19. Growth curve of <u>Streptococcus</u> <u>lactis</u> 7962 at 37 C starting with a 1% inoculum.
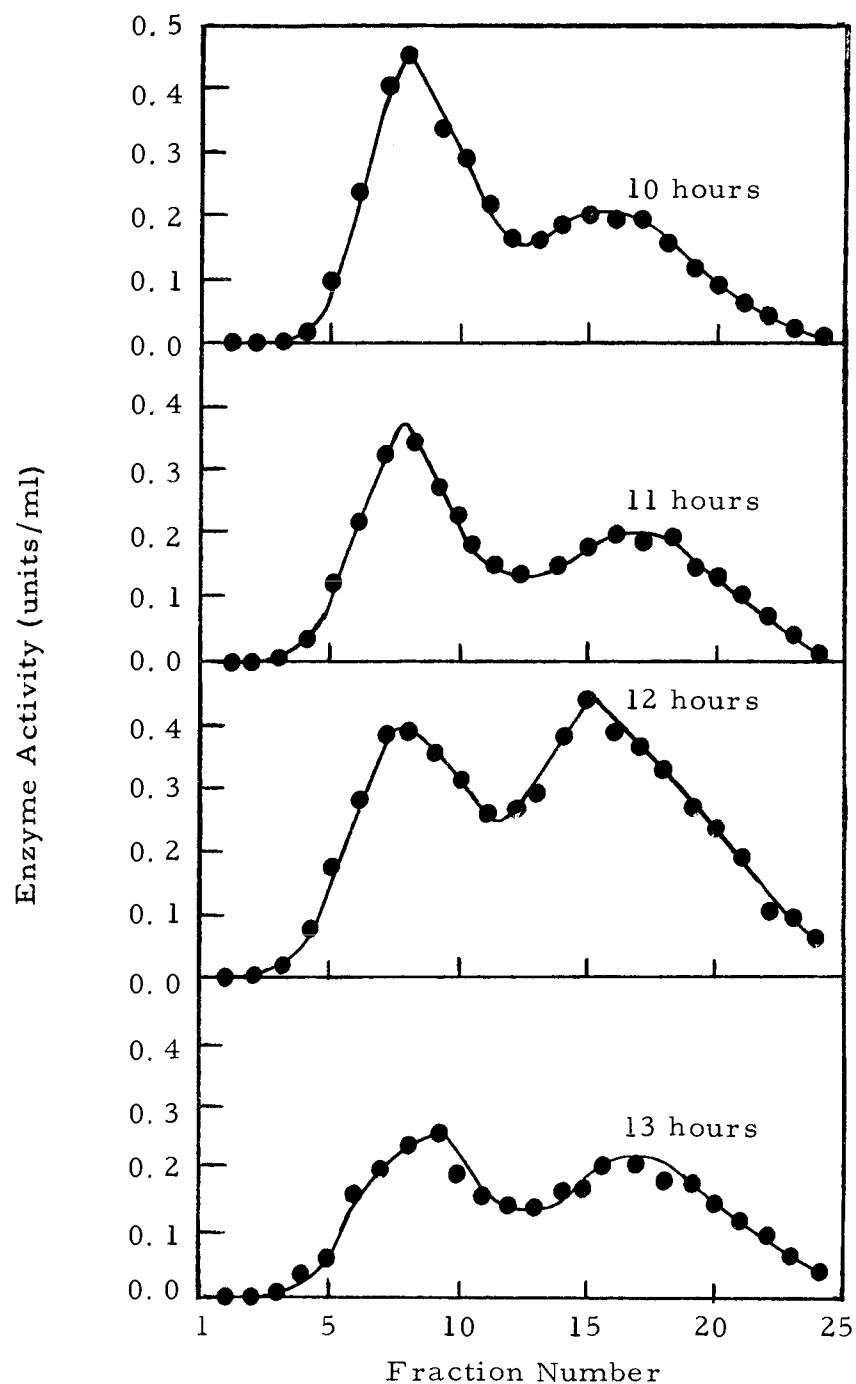
Figure 20. Sephadex G-200 gel filtration patterns of the β - galactosidase extracted and partially purified from Streptococcus lactis 7962 grown in 500 ml of lactic broth for various times starting with a 1% innoculum. The separations were done at 5 C on a 2. 5 x 38 cm column and each fraction contained 100 drops.

Evidence for Enzyme Activation

The top two chromatographs of Figure 21 show the data seen

in Figure 18 supporting the conclusion that association of subunits

took place as described above. The bottom curve (Figure 21) shows

the G-200 gel filtration pattern of the third sample that was stored

at 5 C for 70 hours. Identical results were seen after incubation

at 27 C for 48 hours. The size of each peak was determined as be-

fore and compared to show the change that took place in the enzyme

elution curves (Table 3). The total of both peaks after 70 hours at

5 C increased to a value 155 percent greater than the sample stored

at 27 C for 22 hours. The ratio of peak I to peak II after 70 hours at

5 C returned nearly to the value of the control. Of interest was the

observation that peak I on the chromatograph of the sample held for

70 hours at 5 C was the same size as the peak I of the sample held

at 27 C for 22 hours while peak II doubled in size. Thus the increase

in the total enzyme recovered following gel filtration of the sample

held for 70 hours at 5 C was accounted for in peak II. This implied

that the same association process must have taken place back in the

samples held for 70 hours at 5 C and at 27 C for 48 hours that took

place at 27 C for 22 hours, but in addition a process occurred that

resulted in an increase in the concentration or the activity of the

enzyme subunit fraction of the sample.
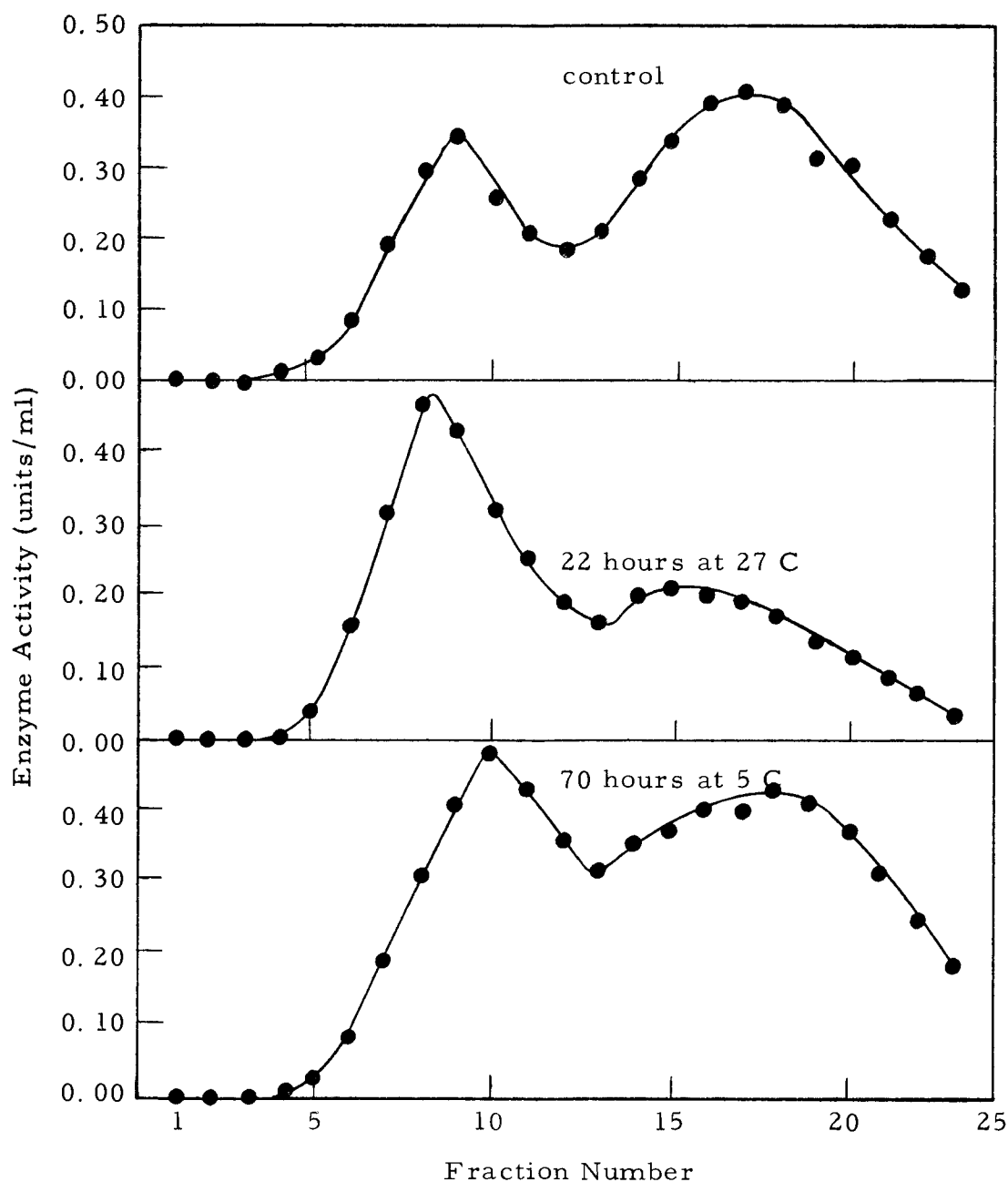
Figure 21. Sephadex G-200 gel filtration patterns of the β-galacto-
sidase of Streptococcus lactis 7962 extracted and
partially purified after growth for 12 hours (control)
and of identical samples that were incubated following
purification for 22 hours at 27 C and for 70 hours at
5 C. Separations were done at 5 C on a 2.5 by 38 cm
column. Each fraction contained 100 drops.

DISCUSSION

Purification of the Enzyme

The lability of β-galactosidase from S. lactis 7962 can be

seen from the purification data. This lability was a major factor in

the rather low increase in specific activity of the enzyme after salt

fractionation (four fold) and after gel filtration (40 fold). However,

the separation of the enzyme from nonenzyme protein was quite good

following gel filtration. While the subunit enzyme peak amounted

to only 1.0 percent of the total protein the crude extract, the sulf-

hydryl titration and the cysteic acid analysis data on this enzyme

protein agreed very well. Thus two independent methods of analysis

revealed the presence of sulfhydryl groups in the enzyme. These

groups are apparently essential for enzyme activity as revealed by

the information obtained with the sulfhydryl antagonists.

Properties of the Enzyme

The finding that the S. lactis 7962 β-galactosidase was un-

stable in buffer but stable in high concentrations of ammonium sul-

fate is not unique among enzymes (Metzenberg et al., 1957). Other

workers also have reported on this stimulating and/or stabilizing

effect of salt but in each case enzymes other than β-galactosidase

were involved (Joyce and Grisolia, 1961; Windnell and Tata, 1966).

This indicated either that the β-galactosidase described here was unique in this respect or that this enzyme from other sources has not been investigated in this respect. In addition to ammonium sulfate, other salts have been found that produce this effect (Landridge and Mortia, 1966; Ravel, Sund and Shive, 1959). For example, the effect of various neutral salts on macromolecular conformation has been studied by Von Hippel and Wong (1964) and the ammonium and sulfate ions both ranked quite high in the order of affording stability. Therefore it was expected in the present study that other combinations of the ammonium and sulfate ions would afford some protection to the enzyme. This was found not to be the case so the effect was found to be unique for ammonium sulfate. While no direct evidence was obtained in this research to explain the mechanism of salt stabilization, it is possible that it caused an increase in solute interactions resulting in conformational changes in the protein leading to enhanced stability.

The effect that ammonium sulfate exerted on the enzyme quaternization was dramatic. Gel filtration data showed that after the enzyme became salt stabilized the enzyme in peak II associated (quaternized) to form the enzyme in peak I.

Since the chemical characterization (pH and temperature optima and K_m) of the enzyme in peak I and peak II was identical and since they were interconvertable, a close relation between them

was thought to exist. However this was not to imply the existence

of isozymes or multiple molecular species. This general idea is

discussed by Kaplan (1963) who maintains that multiple enzyme forms

must be shown to be dissimilar by critical analysis; the criteria of

molecular weight and electrophoretic mobility are not sufficient to

define separate molecular species of a given enzyme. In view of

this and the relationship found between the molecular weights of the

two forms (10^6 and 5×10^5), it was concluded that peak I was a dimer

of peak II (the monomer); hence the idea of an association-dissociation

relationship (quaternization) between the two forms of the enzyme was

established. These data clearly showed that quaternization took place

as opposed to the existence of two separate β-galactosidases within

one microorganism as found by Lester and Byers (1965).

Ammonium sulfate brought about modifications in the enzyme

that changed it from a sensitive to an insensitive state with regard

to the action of EDTA and PCMB. The sensitivity of the nonsalt-

treated enzyme to EDTA and PCMB did establish that both metal ion

and -SH groups were involved in the function of the enzyme. Salt

addition caused changes such that both these essential groups became

inaccessible or incapable of reacting with EDTA and PCMB. This

suggests the possibility that the metal and the -SH groups are inter-

nal enzyme structures and the salt changes the molecular conforma-

tion, making them unavailable. Another possibility is that the labile

groups are located near or on the surface of the monomer and are

enveloped during dimerization.

Another manifestation of the structural changes in the enzyme

caused by ammonium sulfate was detected by the blue-shift procedure.

Changes in the ultraviolet absorption spectrum of a protein have been

described and interpreted as conformational changes in the secondary

and tertiary structure (Beaven, Holiday and Jope, 1950; Yanari and

Bovey, 1960). These changes were attributed to transitions in the

hydrophobic bonding of the protein (Williams and Foster, 1959).

When the salt stabilized and the nonstabilized enzyme preparations

employed in the present study were compared, the former showed no

blue shift while the latter showed a large shift. It was suggested

from this finding that ammonium sulfate exerted a stabilizing effect

on the conformation of the protein and that hydrophobic bonding is

involved in this conformation. When the blue shift data of the non-

stabilized monomer and dimer peaks were compared following gel

filtration, a greater shift per mg of protein was found in the dimer

peak, indicating that there was more conformational complexity in

that form than in the monomer. Hydrophobic bonding also possibly

is involved in the association of monomers.

The addition of urea to the β-galactosidase of E. coli has been

reported to result in dissociation of the polymeric native enzyme to

form identical, enzymatically inactive monomers (Wallenfels, Sund and Weber, 1963; Zipser, 1963a). In addition to causing dequaternization of protein, urea also causes disruption of the tertiary structure (Fasella and Hammes, 1964). Since the monomers of S. lactis β-galactosidase were enzymatically active it was assumed that the urea treatment caused tertiary as well as quaternary disruption of the enzyme as evidenced by the greater inactivation brought about by the urea treatment. Salt stabilization did protect the enzyme to some degree but the urea perterbation was the stronger force of the two. This finding was also reported by Di Sabatio and Kaplan (1965) who have used neutral salts to protect enzyme protein against urea denaturation. Therefore, it seemed likely that in the present study urea accelerated the inactivation rate as a result of tertiary perterbation or denaturation; ammonium sulfate minimized this effect perhaps by stabilizing the tertiary, secondary, and quaternary structures of the enzyme.

From the amino acid composition data of the β-galactosidase enzymes of E. coli, A. formicans and S. lactis, it can be seen that there was wide variation as well as some similarities between these organisms. Rohlfing and Crawford (1966) have used these types of data to show the relatedness of microorganisms. The enzyme β-galactosidase has been studied from numerous sources and the various characteristics of these enzymes differ widely; the only

characteristics shared were the cleavage of the same substrates and large size. The K_m of β-galactosidase from different sources varied all the way from 1.4×10^{-2} M ONPG in the snail (Barnett, 1965) to 1.3×10^{-4} M ONPG, for E. coli strain K - 12 (Lederberg, 1960) and 9×10^{-5} M ONPG for P. aerogenoidese strain BEK (Anderson and Rickenberg, 1960). The K_m value that was determined for the S. lactis 7962 enzyme (1.06×10^{-3} M ONPG) was within the range of that reported for the same enzyme from other sources. The pH optima varied from 2.2 in the snail (Barnett, 1965) to 7.0 in S. lactis as well as several other sources.

The quaternary structure of the β-galactosidase from E. coli has been described as being composed of four identical monomers (Wallenfels, Sund and Weber, 1963; Zipser, 1963a). Many other enzymes exhibiting one or more types of quaternary structure have been described, such as a lactic dehydrogenase (Cahn et al., 1962), lactic and malic dehydrogenase (Chilson, et al., 1966), aspartate transcarbamylase (Gerhart and Schachman, 1965). Discussion has already been made of the point that the β-galactosidase of S. lactis 7962 was composed of two, probably identical, monomers of molecular weight 5×10^{5} that form the dimer molecular weight of 10^{6}. This was demonstrated by incubation of an enzyme preparation at 27 C in buffer as well as in 0.85 M ammonium sulfate following salt stabilization. Since hydrophobic bonds are readily formed at room

temperature (Kauzmann, 1959) and since it was shown that quaternization occurs at 27 C, such bonding was likely involved in the process seen here as has been suggested in other polymeric proteins (Waugh 1959). This was also suggested from some of the blue shift data as discussed earlier.

Recently Cowman and Swaisgood (1966) reported that a proteinase of S. lactis underwent an in vitro temperature - dependant dequaternization that was similar to the quaternization reported here. When the enzyme from a 12-hour culture of S. lactis 7962 was partially purified and analyzed by gel filtration, the enzyme elution pattern that resulted showed peak II much larger in relation to peak I than when the enzyme was prepared from log phase cells. When the enzyme was allowed to quaternize at 27 C (Figure 18) the peak size pattern that resulted was typical for log phase enzyme (Figure 3). This indicated that under these conditions the quaternization process was apparently limited by an equilibrium between the two peaks both in vitro and in vivo. After about 12 hours of growth this in vivo equilibrium became changed, probably due to the radical metabolic modifications which took place at the time (12 hr) representing the transition between the log and stationary phases of growth (Figure 19). Since both forms of the enzyme were enzymatically identical relative to the properties listed earlier, the role of equilibrium changes in cellular regulation is uncertain. The possibility exists

that only one form of the enzyme is involved in allosteric interactions

(Gerhart and Schachman, 1965), thus implicating the in vivo equilibri-

um in cellular regulation. Cowman and Swaisgood (1966) gave evi-

dence for such an equilibrium in vitro and Appel, Alpers and Tomkins

(1965) also have suggested that this quaternization-dequaternization

process occurs in vivo with the β-galactosidase of E. coli. Further-

more, Goodman and Picket (1966) pointed out that different forms of

an enzyme within a microorganism could result from this process

starting with nonidentical monomers. This idea was also proposed

earlier by Cahn, et al. (1962) for chicken lactic dehydrogenase.

In the present study there appeared to be no specific ion or compound

required for quaternization since it occurred in buffer; this is in

contrast to an amylase where zinc ions were required for the

association of the monomers into the dimer form (Kakiuchi and Kato,

1964).

The quaternary structure of the S. lactis 7962 dimer enzyme

must be rather strong since sonication did not cause the appearance of

the monomer form. The only evidence that was seen of accumulation

of monomer was observed in enzyme from cultures about 12 hours

old (Figure 20) or between log and stationary phases of growth. It

might be assumed, but no data was collected regarding this point,

that the lactose concentration in the medium had dropped below the

level that was critical to maintain β-galactosidase synthesis after

12 hours of growth. Therefore, repression of enzyme synthesis would have occurred. However, in view of the accumulation of the monomer form of enzyme at 12 hours another process was thought to take place along with repression. This accumulation of monomer, at a time when repression was presumed to be exerted, might be explained by inhibition of quaternization in the absence of lactose, resulting in the build-up of monomer enzyme. This mechanism implied that the substrate (lactose) was required for quaternization, at least in vivo. Once repression had taken place, the enzyme concentration of both forms decreased, as seen in the enzyme elution pattern for 13-hour old culture (Figure 20).

The appearance of increasing amounts of monomer with prolonged incubation at either 27 C or 5 C as seen in Figure 21 (bottom curve) brought up the question of enzyme activation. Protein synthesis was not likely taking place in the purified cell-free extract. Also, the possibility of ribosomal enzyme liberation was not likely. In this regard, Webster, Englehardt and Zinder (1966) have shown that proteins can be "masked" relative to a function such as enzyme activity by the presence of a N-formylmethionine on the N-terminal end of a poly-peptide chain. They also suggested that cleavage of the N-formal group could result in an active protein. This type of activation could have occurred under these conditions. The observation that the amount of dimer form of enzyme remained about the

same after short and prolonged incubation further complicated the

picture. If the newly "activated" monomers were normal they should

quaternize to form dimers, especially at 27 C, and produce a larger

dimer concentration. Limited quaternization such as this apparently

does occur with the prolonged incubation but inactivation must have

occurred allowing the dimer peak of enzyme to remain at the same

level. Since the dimer peak of enzyme did not increase in propor-

tion to the increase in the monomer form under these conditions,

the newly activated monomers could have been defective.

In consideration of all these facts the mechanism of inactiva-

tion of S. lactis 7962 β-galactosidase is probably not initiated at the

quaternary level of enzyme structure since prolonged incubation did

not cause dequaternization. It appears rather that enzyme inactiva-

tion is caused by changes in the tertiary level of protein structure.

SUMMARY

Upon the extraction and purification of the β-galactosidase

from S. lactis 7962 some of its enzymatic and chemical properties

were determined. The enzyme proved to be quite unstable in buffer

solutions at all temperatures but was stabilized by 0.85 M ammonium

sulfate. This treatment also changed the quaternary structure of

the enzyme by causing an association of the monomer form to the

dimer form. Determination of the K_m (10^{-3} M ONPG), temperature

optimum under specified conditions (37 C) and pH optimum (7.0)

supported the idea that the two forms were really interchangeable.

From the molecular weights of the two forms, it appeared that

the dimer was composed of two, probably identical, monomers.

Metal ions (possibly calcium) and -SH groups were essential for

enzyme activity and the salt treatment caused these groups to be

inaccessable to specific inactivating agents. Ammonium sulfate

also caused stabilization of the enzyme structure. Amino acid

analysis indicated similarities yet differences between the β-

galactosidase of S. lactis and E. coli and A. formicans.

Incubation of the enzyme in buffer at 27 C led to the con-

version of monomer to dimer form, resulting in a ratio which was

the same as found in enzyme prepared from log phase cells. This

indicated an equilibrium between the two forms existed

and that hydrophobic bonds were involved in dimer formation.

Enzyme prepared from bacteria in the late log phase (12 hours) exhibited a monomer-dimer composition radically different from enzyme extracted from middle log phase bacteria. Hence something appeared to be interfering with the quaternization as the culture aged. If allowed to incubate at either 27 or 5 C after quaternization had occurred, more monomer was found without the concommitant loss of dimer, indicating monomer activation had occurred.

BIBLIOGRAPHY

Alpers, D. H. and G. M. Tomkins. 1965. The order of induction and deinduction of the enzymes of the lactose operon in Escherichia coli. Proceedings of the National Academy of Sciences 53:797-803.

Anderson, J. M. and H. V. Rickenberg. 1960. β-galactosidase of Paracolorbacterium aerogenoides. Jounal of Bacteriology 80:297-304.

Appel, S. H. , D. H. Alpers and G. M. Tomkins. 1965. Multiple molecular forms of β-galactosidase. Journal of Molecular Biology 11:12-22.

Auema, P. J. 1964. Purification and some properties of β-galactosidase of Bacillus subtilis. Biochimica et Biophysica Acta 89:495-502.

Barnett, J. E. G. 1965. β-galactosidase in the snail Helix aspersa. Biochemical Journal 96:72.

Beaven, G. H. , E. R. Holiday and E. M. Jope. 1950. The ultra-violet absorption spectra of aromatic amino acids in proteins and related compounds. Discussions of the Faraday Society 9:406-417.

Ben-Hamida, F. and D. Schlessinger. 1965. Stability of β-galactosidase messenger ribonucleic acid in Escherichia coli. Journal of Bacteriology 90:1611-1616.

Baoyer, P. O. 1954. Spectrophotometric study of the reaction of protein sulfhydryl groups with organic metcurials. Journal of the American Chemical Society 76:4331-4337.

Cahn, R. D. , N. O. Kaplan, L. Levine and E. Zwilling. 1962. Nature and development of lactic dehydrogenases. Science 136:962-969.

Calbiochem. n. d. The use of insoluble protein-dye conjugates for measuring proteolytic activity. Los Angeles, Calbiochem, 2 p.

Chilson, O. P, G. B. Kitto, J. Puddles and N. O. Kaplan. 1966. Reversible inactivation of dehydrogenases. Journal of Biological Chemistry 241:2431-2445.

Citti, J. E., W. E. Sandine and P. R. Elliker. 1965. β-galactosidase of Streptococcus lactis. Journal of Bacteriology 89:937-942.

Clausen, C. R. and M. Nakamura. 1963. β-galactosidase of Shigella sonnei. Nature 197:570-573.

Cleland, W. W. 1964. Dithiolthieitol, a new protective reagent for SH groups. Biochemistry 3:480-482.

Cohn, M. 1957. Contributions of studies on the β-galactosidase of Escherichia coli. Bacteriological Reviews 21:140-168.

Cohn, M. and J. Monod. 1951. Purification et proprietes de la β-galactosidase (lactase) d' Escherichia coli. Biochimica et Biophysica Acta 7:153-173.

Cowman, R. A. and H. E. Swaisgood. 1966. Temperature-dependent association dissociation of Streptococcus lactis intracellular proteinase. Biochemical and Biophysical Research Communications 23:799-803.

Craven, G. R., E. Steers and C. B. Anfinsen. 1965. Purification, composition and molecular weight of the β-galactosidase of Escherichia coli K12. Journal of Biological Chemistry 240:2468-2477.

Dasgupta, B. R., D. A. Boroff and E. Rothstein. 1966. Chromatographic fractionation of the crystaline toxin of Clostridium botulinum type A. Biochemical and Biophysical Research Communications 22:750-756.

Deal, W. C., W. J. Rutter and K. E. Van Holde. 1963. Reversible dissociation of aldolase into unfolded subunits. Biochemistry 2:246-251.

Deere, C. J., A. D. Dulaney and I. D. Michelson. 1938. The lactase of Escherichia coli mutable. Journal of Bacteriology 37:357-363.

Di Sabatio, G. and N. O. Kaplan. 1965. The denaturation of
lactic dehydrogenase. II. The effect of urea and salts.
Journal of Biological Chemistry 240:1072-1076.

Fasella, P. and G. C. Hammes. 1964. A correlation between
the protein structure and catalytic activity of aspartate
aminotransferase. Biochimica et Biophysica Acta 92:
630-632.

Frieden, C. 1963. Glutamate dehydrogenase. V. The relation of
the enzyme structure to the catalytic function. Journal of
Biological Chemistry 238:3286-3299.

Gerhart, J. C. and H. K. Schachman. 1965. Distinct subunits for
the regulation and catalytic activity of aspartic transcarb-
amylas. Biochemistry 4:1054-1062.

Gilbert, W. and B. Müller-Hill. 1966. Isolation of the lac repres-
sor. Proceedings of the National Academy of Sciences 56:
1891-1898.

Goodman, R. E. and M. J. Pickett. 1966. Delayed lactose fer-
mentation by Enterobacteriaceae. Journal of Bacteriology
92:318-327.

Got, R. , A. Marnay, P. Jarrige and J. Font. 1964. Beta-
galactosidase of Helix pomatia. Nature 204:686-687.

Hagarty, C. P. 1939. Physiological youth as an important factor
in adaptive enzyme formation. Journal of Bacteriology
37:145-152.

Haldane, J. B. S. 1957. Graphical methods of enzyme chemistry.
Nature 179:832.

Hu, A. S. , R. G. Wolfe and F. J. Reithel. 1959. The preparation
and purification of β-galactosidase from Escherichia coli
ML308. Archives of Biochemistry and Biophysics 81:
500-507.

Jacob, F. and J. Monod. 1961. On the regulation of gene activity.
In: Cellular regulatory mechanisms. Cold Spring Harbor,
N. Y. , Biological Laboratory. p 193-211 (Cold Spring Harbor
Symposium on Quantitative Biology Vol. 26).

Joyce, B. K. and S. Grisolia. 1961. Variations in malic dehydrogenase activity with liophilization, dialysis and conditions of incubation. Journal of Biological Chemistry 236:725-729.

Kakiuchi, K. S. and A. Kato. 1964. Association and dissociation of Bacillus subtilis amylase molecule. Journal of Biochemistry 55:103-109.

Kaplan, N. O. 1963. Symposium on multiple molecular forms of enzymes and control mechanisms. I. Multiple forms of enzymes. Bacteriological Reviews 27:155-169.

Kauzman, W. 1959. Some factors in the interpretation of protein denaturation. Advances in Protein Chemistry 14:1-63.

Kepes, A. 1963. Kinetic analysis of early events in induced enzyme synthesis. In: Synthesis and structure of macromolecules. Cold Spring Harbor, N. Y., Biological Laboratory. p 325-327 (Cold Spring Harbor Symposium on Quantitative Biology Vol. 28).

Kuby, S. A. and H. A. Lardy. 1953. Purification and kinetics of β-D-galactosidase from Escherichia coli, strain K-12. Journal of the American Chemical Society 75:890-896.

Landman, O. 1957. Properties and induction of β-galactosidase in Bacillus megatherium. Biochimica et Biophysica Acta 23:558-569.

Langridge, P. and R. Y. Morita. 1966. Thermolability of malic dehydrogenase from the obligate psychrophile Vibrio marinus. Journal of Bacteriology 92:418-423.

Laurent, T. C. and J. Killander. 1964. A theory of gel filtration and its experimental verification. Journal of Chromatography 14:317-330.

Lederberg, J. 1950. The β-D-galactosidase of Escherichia coli strain K-12. Journal of Bacteriology 60:381-392.

Leive, L. 1965. Some effects of inducer on synthesis and utilization of β-galactosidase messenger RNA in actinomycin-sensitive Escherichia coli. Biochemical and Biophysical Research Communications 20:321-327.

Lester, G. and A. Byers. 1965. Properties of two β-galactosidas-es of Neurospora crassa. Biochemical and Biophysical Research Communications 18:725-734.

Lowry, O. H., N. J. Rosebough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193:256-275.

McClatchy, J. K. and E. D. Roseblum. 1963. Indication of lactose utilization in Staphlococcus aureus. Journal of Bacteriology 86:1211-1215.

McFall, E. and J. Mandelstam. 1963. Specific metabolic repres-sion of three induced enzymes in Escherichia coli. Biochem-ical Journal 89:391-398.

Metzenberg, R. L., L. M. Hall, M. Marshall and P. P. Cohen. 1957. Studies on the biosynthesis of carbamyl phosphate. Journal of Biological Chemistry 229:1019-1026.

Moore, S. 1963. On the determination of cysteine as cysteic acid. Journal of Biological Chemistry 238:235-237.

Moses, V. and C. Prevost. 1966. Catabolite repression of β-galactosidase synthesis in Escherichia coli. Biochemical Journal 100:336-353.

Müller-Hill, B., H. V. Rickenberg and K. Wallenfels. 1965. Observations on the kinetics of induction of β-galactosidase in Escherichia coli. Biochemical and Biophysical Research Communications 18:751-756.

Nakata, D. and B. Magasanik. 1964. Roles of inducer and catabolite repressor in the synthesis of β-galactosidase by Escherichia coli. Journal of Molecular Biology 8:105-127.

Neilands, J. B. 1954. Studies on lactic dehydrogenase of heart. III. Action of inhibitors. Journal of Biological Chemistry 208:225-230.

Ornstein, L. and B. J. Davis. n.d. Disc electrophoresis. Bethesda Md. Canal Industrial Corp. (Canalco) 22p (reprint).

Ravel, J. M. , R. F. Sund and W. Shive. 1959. Protection and reactivation of carbamyl phosphate synthetase with sulfate. Biochemical and Biophysical Research Communications 1:186-190.

Reithel, F. J. 1963. The dissociation and association of protein structures. Advances in Protein Chemistry 18:124-226.

Rickenberg, H. V. 1960. Occurance of β-galactosidase in the genus Shigella. Journal of Bacteriology 80:421-422.

Rohlfing, S. R. and I. P. Crawford. 1966. Purification and characterization of the β-galactosidase of Aeromones formicans. Journal of Bacteriology 91:1085-1097.

Sandine, W. E. , P. R. Elliker and H. Hays. 1962. Cultural studies on Streptococcus diacetilactis and other members of the lactic streptococcus group. Canadian Journal of Microbiology 8:161-174.

Steers, E. , G. R. Craven and C. B. Anfinsen. 1965. Evidence for nonidentical chains in the β-galactosidase of Escherichia coli K-12. Journal of Biological Chemistry 240:2478-2484.

Szabó, G. and J. Rózsa. 1965. β-galactosidase activity of Saccharomyces fragilis. Acta Microbiologica of the Academy of Science of Hungary. 12:91-102.

Tacguet, A. , F. Tinson, B. Polspall, P. Roos and B. Devoulder. 1966. Étude de la β-D-galactosidase des mycobactéries. Annales de l' Institut Pasteur 111:86-89.

Vakil, J. R. and K. M. Shahani. 1962. Different pathways of lactose metabolism of Streptococcus lactis and their sensitivity to antibiotics. Journal of Dairy Science 45:655.

von Hippel, P. H. and K. Y. Wong. 1964. Neutral salts: The generality of their effects on the stability of macromolecular conformation. Science 145:577-580.

Wallenfels, K. , H. Sund and K. Weber. 1963. Die untereinheiten der β-galaktosidase aus Escherichia coli. Biochemische Zeitschrift 338:714-727.

Wallenfels, K. , M. L. Zarnitz, G. Laule, H. Bender and M. Keser, 1959. Untersuchungen über milchzuckersplatende enzyme III. Biochemiche Zeitschrift 331:459-485.

Waugh, D. F. 1959. Proteins and their interactions. Reviews of Modern Physics 31:84-93.

Webster, R. E. , D. L. Englehardt and N. D. Zinder. 1966. In vitro protein synthesis: chain initiation. Proceedings of the National Academy of Sciences 55:155-161.

Westhead, E. W. , L. Butler and D. D. Boyer. 1963 Binding sites, reactivation phenomena and possible -S-S- groups of rabbit muscle aldolase. Biochemistry 2:927-934.

Williams, E. J. and J. F. Foster. 1959. An investigation of bovine plasma albumin by differential ultraviolet spectroscopy. Journal of the American Chemical Society 81:865-870.

Windnell, C. C. and J. R. Tata. 1966. Studies on the stimulation by ammonium sulfate on the deoxyribonucleic acid-dependant ribonucleic acid polymerase of isolated rat liver nuclei. Biochimica et Biophysica Acta 123:478-492.

Yanari, S. and F. A. Bovey. 1960. Interpretation of the ultraviolet spectral changes of proteins. Journal of Biological Chemistry 235:2818-2826.

Zipser, D. 1963a. A study of the urea produced subunits of β-galactosidase. Journal of Molecular Biology 7:113-121.

Zipser, D. 1963b. Studies on ribosomal-bound β-galactosidase of Escherichia coli. Journal of Molecular Biology 7:739-751.