Amylolytic enzymes and selected physiological properties of Streptococcus bovis and Streptococcus equinus

Ernest Wendell Boyer

Iowa State University

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AMYLOLYTIC ENZYMES AND SELECTED PHYSIOLOGICAL PROPERTIES OF
STREPTOCOCCUS BOVIS AND STREPTOCOCCUS EQUINUS

by

Ernest Wendell Boyer

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Bacteriology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Ames, Iowa

1969
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INTRODUCTION

The original objective of the present research was to compare the amylolytic enzymes produced by the various streptococcal groups. This task quickly proved to be too large, so the scope of this thesis was limited to a concentrated study of the amylases of two species of the streptococci, *Streptococcus bovis* and *Streptococcus equinus*. As will be discussed on the following pages, the amylases of only one strain of *S. bovis* have received detailed attention; *S. equinus* amylases have been almost completely neglected in the literature.

Deibel (1964) reviewed the literature on the group D streptococci and concluded that they may be separated into two physiological groups, the enterococci and the *S. bovis-S. equinus* group. He also stated that the *S. bovis-S. equinus* group is not distinctive at the species level, and additional study is indicated prior to definitive speciation. *S. bovis* and *S. equinus* both possess Group D antigen, but are separated from the enterococci on the basis of several physiological characteristics. These two species are very similar to each other physiologically; however, some differences have been observed. On the basis of an extensive study of the fermentation pattern of the two groups, Smith and Shattock (1962) suggested retention of the two species. The chief differentiating characteristic is the inability of *S. equinus* to ferment lactose.
Duncan (1960) reviewed the literature on *S. equinus* and reported that *S. equinus* is most commonly found in horse feces; that a distinct variety of *S. equinus* exists in the human intestine which does not ferment lactose but which has a high fermentative power in dextrose broth; and that *S. equinus* occurs occasionally in the cow's intestine.

Walker and Hope (1964) reported that starch-digesting rumen streptococci have been isolated in many laboratories. They stated that it has generally been accepted that *S. bovis* is one of the principal starch-fermenting bacteria in the rumen of sheep and cattle. Seeley and Dain (1960) concluded that starch-hydrolyzing streptococci appear to be broadly distributed among ruminants, at least under conditions of domestication.

Hobson and MacPherson (1952) isolated an α-amylase in the form of stable freeze-dried powders from cell-free filtrates of cultures of a sheep rumen *Streptococcus* species grown in medium containing dissolved starch. MacPherson (1953) isolated and identified amylolytic streptococci from the rumen of the sheep. Twenty-five strains of amylolytic streptococci were isolated from the rumen contents of sheep on a variety of diets. In fermentation reactions the isolates resembled *S. bovis*.

Seeley and Dain (1960) wrote an excellent review of the literature on the starch-hydrolyzing streptococci. They noted
that although *S. equinus* hydrolyzes starch on plates, Orla-Jensen (1943) reported that acid is not formed from starch. Therefore, since *S. equinus* ferments maltose, it would appear that starch hydrolysis by this species results in a non-fermentable intermediate (Seeley and Dain, 1960).

In a study of starch hydrolysis by strains of *S. equinus*, Duncan and Seeley (1962) obtained 52 isolates and determined their amylolytic abilities. All the strains could hydrolyze starch on starch agar plates to some extent when grown in the presence of an easily fermentable carbohydrate, viz., glucose. The organisms did not hydrolyze starch on plates without glucose. The hydrolysis of starch on plates was inhibited when the organisms were grown in an atmosphere of 5% CO₂ and 95% N₂, even if grown in the presence of a fermentable monosaccharide. *S. bovis* readily hydrolyzed starch on plates in the absence of monosaccharides and in atmospheres containing CO₂. When *S. bovis* was incubated with starch in tubes, the yield of reducing sugars was almost quantitative. *S. equinus* did not hydrolyze the starch in tubes to the level of reducing sugars. Duncan (1960) reported, however, that the recovery of reducing sugars was low (approximately 10%) with *S. equinus*. He concluded that the starch-splitting mechanism of *S. equinus* was the same as that of *S. bovis* (α-amylase), the difference being that the enzyme was produced in lesser quantity by *S. equinus*. 
With regard to the observations of Dunican and Seeley (1962) that no starch hydrolysis by S. equinus occurs on starch agar plates unless glucose is present, a reasonable hypothesis would be that glucose is serving as an acceptor molecule for an extracellular transglucosylase. Extracellular bacterial transglucosylases have not been reported in the literature. Thus, one of the objectives of this investigation was to test S. equinus strains for extracellular transglucosylase activity. Up to now, the action patterns and most of the properties of the amylolytic enzymes of S. equinus have not been determined.

Walker (1965b) reviewed the literature on streptococcal transglucosylases. She separated a transglucosylase from the α-amylase of one S. bovis strain by chromatography of the cell extract on DEAE-cellulose. The transglucosylase could synthesize higher maltodextrins from maltotriose, but maltose, isomaltose and panose did not function as donors.

Carlson (1959) found three of 29 S. bovis strains that produced β-amylase. α-Amylase was produced by 26 strains. Therefore, different strains of S. bovis evidently produce different kinds of amylase. Walker (1965a) found two different amylases in one strain of S. bovis. One of these (an α-amylase) was found to be both extracellular and cell-bound. This α-amylase could degrade maize-starch granules. The second, cell-bound amylase, could not degrade maize-starch
granules. Walker and Hope (1964) found that the extracellular amylase of *S. bovis* was at least as active as salivary α-amylase towards maltotriose. Walker (1965a) isolated the cell-bound α-amylase of one *S. bovis* strain from other carbohydrases in the cell extract by chromatography on DEAE-cellulose. She found that the extracellular and the cell-bound α-amylases had similar action patterns on amylose. The main product was maltotriose; smaller amounts of maltose and a little glucose were present at the stage when the iodine stain was 13% of the original value.

Walker (1965a) reviewed the literature on the α-amylases of *S. bovis*. The action patterns and some of the properties of the amylases and transglucosylase of only one strain of *S. bovis* have been determined (Walker, 1965a). Since Walker (1965a) used only one strain of *S. bovis* in all her studies, I decided to compare the amylases of several *S. bovis* strains to determine if different strains of *S. bovis* produce amylases that have different action patterns. And if there is a high degree of difference in the action patterns is there also a high degree of difference in the physiological characteristics? Many authors agree that there are at least two main divisions within the species *S. bovis* as now characterized (Dain et al., 1956; Deibel, 1964). Those belonging to the first division ferment mannitol, produce no slime, and are γ-hemolytic. Those strains which belong to the second division do not
ferment mannitol, do produce slime, and are α-hemolytic. Therefore, a further objective of this study was to discover if the action patterns of the amylases from these two main divisions are different. In like manner, attempts were made to learn more about speciation of *S. bovis* and *S. equinus* by characterizing and comparing their extracellular amylolytic enzymes.

Another objective of this investigation was to find amylases which have unusual properties, products, product distributions and action patterns.
PART I. AMYLOLYTIC ENZYMES AND SELECTED PHYSIOLOGICAL PROPERTIES OF STREPTOCOCCUS BOVIS STRAINS
MATERIALS AND METHODS

Organisms

About 60 *S. bovis* strains were obtained from various investigators in Europe, Australia and the United States. Of these 60 strains, only seven produced large zones of hydrolysis (15-26 mm) on starch plates. The following is a list of the sources of these seven strains. Also, the sources are listed of three strains that produced very small zones (0.3-0.7 mm) on starch plates. The following strains were obtained from Dr. P. N. Hobson, Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland: 2B from the rumen of sheep, 18C2 and 18M2 from the rumen of calves and 2SA. Strains 2B, 2SA, and 18M2 were isolated at the Rowett Research Institute, Aberdeenshire before 1955 (Oxford, 1958). Dr. E. M. Barnes, Cambridge, England furnished strains 105 and 101 which were isolated from a cow. Dr. P. A. Hartman, Dept. of Bacteriology, Iowa State University, Ames, Iowa, 50010, provided strains H12, H24, and 364. Strains H12 and H24 were isolated from the rumen of calves. For purification, pour plates were made with the thallous acetate-tetrazolium agar medium of Barnes (1956) and the minute red colonies that appeared following incubation at 37 C were picked and transferred to slants of Difco Brain Heart Infusion (BHI) agar. Strain ID14 was obtained from
Dr. H. Obiger, Institut für Milch Hygiene, Kiel, Germany; this strain came from Dr. H. Meith's culture collection (present address—Parasitological Institute, Farbwerke Hoechst, 6230 Frankfurt, "M" - Hoechst, Germany).

Screening of Strains for Amylase Production

Difco BHI agar slants were used to maintain the cultures, which were transferred biweekly. The slants were inoculated, incubated at 37 C for 12 hr, and stored in the refrigerator. The inoculum for the starch hydrolysis tests was made by inoculating one loopful from a stock slant into a tube of trypticase carbohydrate broth (Carlson, 1959) and incubating at 37 C for 12 hr. Starch test plates were made with Difco BHI broth, 1.5% agar (Difco), and 0.2% soluble starch powder (Mallinckrodt Chemical Works, St. Louis, Mo.). The starch-containing broth for tube starch-hydrolysis tests was prepared according to the method of Carlson (1959) in screw-cap tubes (10 ml/tube).

The starch plates were inoculated by streaking one loopful of broth inoculum once across the plate. Tube starch-hydrolysis tests were also inoculated with a loopful of inoculum broth. The plates were inverted and incubated for eight days at 37 C. The starch broth cultures were incubated for 10 days at 37 C. After incubation, color was developed on the
plates and in the starch broth tubes with an iodine solution (3.0% KI and 0.3% I₂ -- Manning and Campbell, 1961). Plates were flooded with iodine solution, and the excess was poured off; the width of the zone of hydrolysis from one edge of the cell band of growth was measured in mm. A sufficient quantity of iodine solution was added to the control starch broth tube to produce a blue color; the same quantity was added to the other tubes, and the resulting color was recorded.

Those strains which produced large zones of hydrolysis were retained for further study.

Physiological Tests

Tests

All tests were done in duplicate at 37°C. The inoculum was 0.1 ml of an 18 hr trypticase carbohydrate broth (Carlson, 1959) culture per 10 ml of medium.

The following methods used were those described by Barnes et al. (1956): 0.04% tellurite sensitivity; growth in the presence of 40% bile; growth at 10°C, 45°C, and 50°C; catalase (Difco BHI agar slant culture); survival at 60°C for 30 min; and growth in 6.5% NaCl.

Arginine hydrolysis The medium of Niven et al. (1942) was used; L(+)-Arginine (Matheson-Coleman & Bell, Norwood, Ohio) served as the substrate.
Litmus milk Difco litmus milk was used.

Growth in 0.1% methylene blue medium The medium of Carlson (1959) was used.

Tyrosine decarboxylase Difco Decarboxylase Medium Base was used with 0.5% L-tyrosine.

Gelatin liquefaction The gelatin plate method (Hg Cl₂ precipitant) was used with basal medium B of Barnes et al. (1956).

Hemolysis The procedure of Medrek and Barnes (1962a) was followed; 3BL Blood Agar Base containing 10% of citrated horse blood was used as the plating medium.

Tetrazolium reduction The tetrazolium-glucose agar (TG agar) described by Barnes (1956) was used.

Aesculin hydrolysis The method of Medrek and Barnes (1962a) was used.

Growth at pH 9.6 The medium of Shattock and Hirsch (1947) was used; growth was recorded after 1 and 3 days.

Carbohydrate fermentation The method of Barnes et al. (1956) was used, except the peptone water was not adjusted to pH 7.2. Ten percent aqueous solutions of all carbohydrates were made and sterilized with a Seitz filter using an EK sterilizing pad. The sources of the carbohydrates were as follows: D-mannitol, L-arabinose, melizitose, D-mannose, D-xylose, trehalose, and fructose — (Difco Lab. Inc., Detroit, Michigan); D(+)lactose and salicin — (Pfanstiehl Lab., Inc.,
Waukegan, Ill.); dextrose, sucrose, maltose monohydrate, raffinose hydrate, and inulin — (Matheson Coleman & Bell, Norwood, Ohio); D(+)galactose — (Sigma Chemical Co., St. Louis 18, Mo.); D(+)melibiose hydrate — (Nutritional Biochemical Corp., Cleveland, Ohio); sorbitol — (Eastman Organic Chemicals, Rochester 3, New York); glycerol — (General Chemical Division Allied Chemical & Dye Corp., New York, N.Y.).

Final pH in dextrose, lactose, and mannose broths The initial and final (after 10 days) pH values were measured with a small (5 ml minimum capacity) glass electrode (Beckman Zeromatic II pH meter).

Growth on Rogosa medium The tube and plate methods of Sims (1964) were used.

Production of Amylase

Screw-cap tubes containing 10 ml of the starch medium of Hobson & MacPherson (1952) were inoculated (one loopful/tube) from a BHI agar slant stock culture and grown for 18 hr at 37 C. This culture was used for inoculating the starch medium.

One hundred ml of starch medium (Hobson and MacPherson, 1952) were placed in 125-ml Erlenmeyer flasks. The medium was inoculated with a 1.0% inoculum. The culture was incubated at 37 C for 40.5 hr and shaken about every 4 hr to suspend CaCO₃. The final pH was measured.
Purification of Amylase

A modification of the method of Walker (1965a) was used for the purification of extracellular amylases. Cells and CaCO$_3$ were removed by centrifuging at 23,300 x g in a Sorvall RC-2 refrigerated centrifuge with a GSA rotor at 3.5 C for 30 min. The supernatant was sterilized by filtration through an EK sterilizing pad in a Seitz filter.

The cell-free filtrate was treated with 507 g/liter of (NH$_4$)$_2$SO$_4$. The pH was maintained between 6.0 and 6.5; the final pH was 6.2. Except for strain 101, the precipitate that settled overnight in the refrigerator was collected on the Sorvall RC-2 centrifuge at 12,100 x g for 30 min at 5 C. The precipitate from strain 101 was not allowed to settle overnight in the refrigerator, but was collected two hours after the addition of (NH$_4$)$_2$SO$_4$. The precipitate was washed with 40% (w/v) (NH$_4$)$_2$SO$_4$ solution and centrifuged at 12,100 x g for 30 min. The brown precipitate was resuspended in a quantity of demineralized H$_2$O equal to the ml of cell-free filtrate divided by 10. This solution was placed in a 23 mm diameter dialysis tubing and dialyzed for 12 hr against 0.01 M potassium phosphate buffer at pH 6.0, at 4 C.
Assay of Amylase

The method of Stark et al. (1953) was used for paper disc tests on starch agar. The assay medium contained 0.066 M potassium phosphate buffer at pH 6.0; 0.2% soluble starch powder (Mallinckrodt); 1.0% agar (Difco); and 0.09% NaCl. Exactly 20 ml were placed in each sterile pressed Pyrex Petri plate; the plates were covered with metal lids.

Each sample was assayed on two plates (4 discs/plate). Each filter paper disc (cat. no. 740 E, 12.7 mm diameter — Schleicher and Schuell Co., Keene, N.H.) was touched to the sample by its edge until it was saturated; then the excess was drained by touching the side of the sample container. After 8 hr incubation in the upright position at 45 C, the discs were removed. The starch agar gel was flooded with an aqueous iodine solution (3.0% KI and 0.3% I₂) and the excess poured off. Diameters of the colorless circles of hydrolysis were read to the nearest 0.2 mm on a Fisher-Lilly zone reader (Fisher Scientific Co., Pittsburgh, Pennsylvania). The average diameter of 8 zones was used.

The method of Robyt and Whelan (1968), which uses the colorimetric Nelson copper reagents, (Nelson, 1944) was used for reducing sugar assays. Soluble starch -- according to Lintner -- (Merck & Co., Inc., Rahway, N.J.) was used as the
substrate. A sufficient quantity of soluble starch was weighed out so that the final concentration in the solution was 1.0%. A cold water slurry of the starch was added to boiling 0.02 M potassium phosphate buffer at pH 6.00. After cooling, the solution was diluted until the final concentrations were 0.02 M of buffer and 1.0% of starch at a pH of 6.00.

Folin-Wu blood sugar tubes were used. The hydrolysis of the starch was allowed to proceed for 10 min at 37°C. Then, 1.0 ml of the digest was added to 1.0 ml of alkaline copper reagent to stop the reaction. A Bausch and Lomb Spectronic 20 Colorimeter was used at a wavelength of 485 nm.

One amylase unit (U) is a micromole of bonds broken per min per ml of digest.

e.g., if 1.0 mg maltose/ml/10 min is produced.

\[
\begin{align*}
&= 100.0 \text{ mg maltose/ml/min} \\
&= \frac{100.0 \text{ mg maltose/ml/min}}{342.0 \text{ mg maltose/micromole}} \\
&= 0.2925 \text{ micromole/ml/min} \\
&= 0.2925 \text{ U/ml} \\
&= 292.5 \text{ mU/ml}
\end{align*}
\]

Characterization of Amylases

**Determination of disc assay vs. reducing value curve**

First, the reducing sugar assay was used to measure the
concentration of amylase in mU/ml. The enzyme solutions of the seven strains of *S. bovis* were each diluted 1:2, 1:5, 1:15, 1:30, and 1:60. Each of these dilutions was assayed with the paper disc assay on starch agar plates. Then, for each dilution, the \( \log_{10} \) of the reducing value in mU/ml was plotted against the diameter of the zone of hydrolysis on a starch plate.

**Paper-chromatographic analyses**

Whatman no. 3 paper, measuring 40.64 x 31.75 cm was used. The samples were placed 3 cm apart along a line 2.54 cm from the bottom of the paper. The high temperature paper chromatography method of French *et al.* (1965) was used. The solvent system employed was butanol-pyridine-water (3:2:2 parts by volume). A temperature of 70 C was used with three ascents. Eight hours were allowed for each ascent. The solvent solution was changed for each ascent.

The chromatograms were developed by using the silver nitrate dip procedure described by Robyt and French (1963). I found that additional oligosaccharides could be visualized on the chromatogram by shining a bright light through it from the back.

The standard (obtained from Dr. J. F. Robyt, Dept. of Biochemistry and Biophysics, Iowa State University, Ames, Iowa, 50010) contained a mixture of oligosaccharides --
glucose ($G_1$) to $G_{15}$. Twenty µl of standard, (10 mg standard per ml in demineralized H$_2$O), were placed on the paper. The blank was made with boiled enzyme and substrate.

Only the *S. bovis* strain 101 amylase was reacted with amylose. Amylose (Nutritional Biochemical Corp., Cleveland, Ohio) was dissolved in 0.1 M potassium phosphate buffer at a pH of 6.00. Not all of the amylose went into solution. Equal parts of the amylose solution and enzyme solution from *S. bovis* strain 101 were mixed and allowed to react at 37 C. Samples were taken at 5, 10, 20, 30, 60, 120, and 180 min. The reaction was stopped by boiling. Twenty-five µl of each sample were applied to the paper.

The action patterns of all seven strains of *S. bovis* were studied using starch as the substrate. The starch substrate contained 1.0% soluble starch (according to Lintner) in 0.02 M potassium phosphate buffer at pH 6.00. Between 0.5 and 1.5 mU/ml of amylase solution were added per mg of starch substrate at 37 C. Samples were taken at 5, 30, 60, 180, and 600 min. The reaction was stopped by adding 0.1 ml concd HCl to 5 ml of digest. Twenty µl of each sample were applied to the paper.
RESULTS

Screening of Strains for Amylase Production

The *S. bovis* strains that produced zones of hydrolysis are shown in Table 1.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Color of starch broth</th>
<th>Width of starch hydrolysis zone from one edge of cell band of growth (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B</td>
<td>orange</td>
<td>20</td>
</tr>
<tr>
<td>18C2</td>
<td>purple</td>
<td>23</td>
</tr>
<tr>
<td>H24</td>
<td>red-violet</td>
<td>18</td>
</tr>
<tr>
<td>2SA</td>
<td>purple</td>
<td>25-26</td>
</tr>
<tr>
<td>18M2</td>
<td>purple</td>
<td>20</td>
</tr>
<tr>
<td>H12</td>
<td>light-blue</td>
<td>15-18</td>
</tr>
<tr>
<td>101</td>
<td>purple</td>
<td>18</td>
</tr>
<tr>
<td>ID14</td>
<td>blue</td>
<td>0.7</td>
</tr>
<tr>
<td>364</td>
<td>blue</td>
<td>0.3</td>
</tr>
<tr>
<td>105</td>
<td>blue</td>
<td>0.3</td>
</tr>
<tr>
<td>Control</td>
<td>dark-blue</td>
<td>0</td>
</tr>
</tbody>
</table>

*Iodine reagent (3.0% KI and 0.3% I₂) was added. As starch is progressively hydrolyzed, the color with iodine changes as follows: blue-black to purple to red to colorless.*

Physiological Characteristics of *S. Bovis* Strains

The results of the physiological tests of all seven strains indicated that they were *S. bovis* strains as described by Medrek and Barnes (1962b) and Deibel (1964).
All of the strains were catalase negative, did not grow in 0.1% methylene blue, and did not hydrolyze gelatin. All strains grew on 40% bile agar but did not grow on 0.04% potassium tellurite agar. They all grew at 45°C, but not at 10°C or 50°C; neither did they grow in the presence of 6.5% NaCl, or at pH 9.6; only strain 2B survived 60°C for 30 min. None was positive for tyrosine decarboxylase or arginine hydrolysis.

The results of the following tests, which differed between strains, are given in Table 2: litmus milk, hemolysis on horse blood agar plates, tetrazolium reduction, and aesculin hydrolysis. All of the strains except strain 101 grew in Rogosa agar tubes. However, all strains, including strain 101 grew on Rogosa agar plates when incubated at 37°C in 5% CO₂ in air. According to Sims (1964), only *S. bovis* grows on Rogosa medium while other streptococci fail to grow at all. However, I found that *Streptococcus durans* also grew in Rogosa agar tubes. Additional data, which will be discussed in a later section of this thesis, are included in Table 2.

All strains fermented D(+)galactose, salicin, D(+)melibiose, D(+)lactose, dextrose, D-mannose, sucrose, maltose, raffinose, inulin, and fructose. None of the strains fermented melizitose, D-xylose or glycerol. The fermentation of L-arabinose, D-mannitol, sorbitol, and trehalose differed between strains (Table 2).
Table 2. Differences in physiological characters and amylase action pattern
Groups of S. bovis strains

<table>
<thead>
<tr>
<th>Carbohydrates fermented</th>
<th>( \text{Change in pH}^f )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{pH units} )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Amylase action pattern Group no.</th>
<th>L-Arabinose</th>
<th>D-Mannitol</th>
<th>Sorbitol</th>
<th>Fructose</th>
<th>( \gamma )-Hemolysis</th>
<th>Litmus milk</th>
<th>Peptidase</th>
<th>No growth in Bogosha agar</th>
<th>Colony size, color, and hours ( ^e )</th>
<th>( D(+) ) Lactose</th>
<th>Dextrose</th>
<th>D-Mannose</th>
</tr>
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<tbody>
<tr>
<td>2B</td>
<td>1</td>
<td>sA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>minute, red, 48 2.82 2.91 2.81</td>
<td>2.45 3.04 2.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18C2</td>
<td>1</td>
<td>sA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>minute, red, 48 2.45 3.04 2.85</td>
<td>2.45 3.04 2.85</td>
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<tr>
<td>H24</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>minute, red, 48 2.41 3.05 2.92</td>
<td>2.72 3.04 2.94</td>
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<tr>
<td>2SA</td>
<td>2</td>
<td>sA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>minute, red, 48 2.57 2.97 2.94</td>
<td>2.99 3.05 3.04</td>
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</tr>
<tr>
<td>18M2</td>
<td>3</td>
<td>sA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>minute, red, 48 2.57 2.97 2.94</td>
<td>2.99 3.05 3.04</td>
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<tr>
<td>H12</td>
<td>4</td>
<td>AC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>minute, red, 48 2.57 2.97 2.94</td>
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<tr>
<td>101</td>
<td>5</td>
<td>AC</td>
<td>+</td>
<td>+</td>
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<td>small, white, 48 2.72 3.04 2.94</td>
<td>3.12 3.19 3.15</td>
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</table>

\( ^a \) Starch was the substrate; Group 4 had both an amylase and a transglucosylase.

\( ^b \) The other strains were \( \alpha \)-hemolytic on horse blood.

\( ^c \) A, acid; sA, slight acid; C, clot.

\( ^d \) The method of Sims (1964) was used.

\( ^e \) On tetrazolium-glucose agar; after indicated hours of incubation.

\( ^f \) Final pH subtracted from the initial pH; the initial pH was 7.92 for \( D(+) \) lactose, 7.91 for dextrose, and 7.87 for D-mannose.
Production of Amylase

Fermentations were conducted as described in the Materials and Methods section. The terminal pH values at the time of harvest were as follows: 2B — 5.21, 18C2 — 5.16, H24 — 5.17, 2SA — 5.16, 18M2 — 5.20, H12 — 5.10, and 101 — 5.04. Strains H12 and 101 produced the most acid during the fermentation; at least these strains caused the greatest alteration of the pH of the medium (Table 2).

Purification of Amylase

The following concentrations of amylase (U/ml) were obtained after dialysis: 2B — 1.97, 18C2 — 2.54, H24 — 3.89, 2SA — 5.26, 18M2 — 2.60, H12 — 3.07, and 101 — 0.52. The concentration of amylase from strain 101 was the lowest, probably, because the \((\text{NH}_4)_2\text{SO}_4\) precipitate was not allowed to settle overnight in the refrigerator.

Characterization of Amylases

Determination of disc assay vs. reducing value curves

Saccharogenic/dextrinogenic curves for amylase preparations obtained from the seven strains of \textit{S. bovis} are shown in Fig. 1. There is a linear relationship between the \(\log_{10}\) of
Fig. 1. Saccharogenic/dextrinogenic (S/D) curves for the amylases of seven strains of *S. bovis*. The enzyme solutions were assayed with the reducing sugar method (mU/ml) and diluted 1:2, 1:5, 1:15, and 1:30. The diluted enzymes were assayed with the disc assay method for 8 hr at 45° C.
the amylase activity (reducing value in mU/ml) and the diameter of the zone of hydrolysis at the higher dilutions. Also, at the higher dilutions, each amylase has a different curve. The curves are essentially parallel to each other at the higher dilutions. The curve for strain 101 is shifted to the right and below the other curves.

Paper chromatographic analyses

When the amylase from strain 101 was reacted with amylose the five-minute sample yielded glucose ($G_1$), maltose ($G_2$), maltotriose ($G_3$), maltotetraose ($G_4$), and maltopentaose ($G_5$); maltotriose was the predominant end product, followed, in order of decreasing concentration, by maltose, glucose and maltotetraose, and maltopentaose. Upon further amylolysis, greater quantities of maltotriose and glucose appeared; a substantial amount of maltotetraose was still present. At 30 min, most of the maltotetraose had been hydrolyzed to maltotriose and glucose. The quantities of glucose, maltose, and maltotriose increased with time up to two hours, and the quantities of maltotetraose and maltopentaose decreased with time; these latter two saccharides were not detected after two hours of digestion. After three hours of hydrolysis, maltotriose was the major end product, followed by a substantial quantity of maltose and some glucose.
The seven amylase action pattern chromatograms are shown in Fig. 2-6. Five general amylase action patterns (Groups 1-5) were observed among the seven *S. bovis* strains, as follows:

**Amylase action pattern (Group 1)** The amylase action pattern of Group 1 is shown in Fig. 2. The general action pattern for both strains 2B and 18C2 was as follows: Glucose (G₁) to maltoheptaose (G₇) were present through hydrolysis times of 5 to 780 min; G₁ predominated. There were about equal quantities of G₅, G₆, and G₇ with slightly more G₅ than G₆ and G₇. Isomaltose (Rₚ halfway between G₂ and G₃) was formed. More G₇ was produced by these two strains than by any of the other strains examined. An unidentified compound (G₂ₐ) with an Rₚ value slightly less than that of maltose was produced.

Although strains 2B and 18C2 had similar action patterns, there were some differences. Strain 2B produced more G₁ and isomaltose at 780 min than strain 18C2. Whereas the quantity of G₁ gradually increased from 5 to 780 min with strain 2B, the quantity of G₁ remained constant with strain 18C2. Whereas, at 780 min, strain 2B produced about equal quantities of G₂, G₂ₐ, isomaltose, and G₃ with slightly less G₄; strain 18C2 produced almost as much G₃ as G₁ with about half as much G₂ and G₄ as G₃.

**Amylase action pattern (Group 2)** The amylase action pattern of Group 2 is shown in Fig. 3. The general action
Fig. 2. Group 1 amylase action patterns. Time sequence chromatographic analysis of *S. bovis* strains 2B (left) and 18C2 (right) amylase action on starch. The conditions of the digests were 1.0% starch; 0.02 M potassium phosphate buffer, pH 6.0 — 0.58 mU/ml enzyme per mg starch for strain 2B and 0.76 mU/ml enzyme for strain 18C2. Aliquots (20 μl) from various digest times were spotted. The chromatograms were obtained by the techniques of French et al. (1965) and photographed on a x-ray viewer.

Left chromatogram — (A) G1-G15 standards; (B, C, D, E, F) 5, 30, 60, 180, and 780 min. Right chromatogram — (A, B, C, D, E) 5, 30, 60, 180 and 780 min. G1-G15 refers to glucose, maltose, maltotriose, etc., respectively.
Fig. 3. Group 2 amylase action patterns. Time sequence chromatographic analysis of *S. bovis* strains H24 (left) and 2SA (right) amylase action on starch. The conditions of the digests were identical to those of Fig. 2 -- 1.00 mU/ml amylase per mg starch for strain H24 and 1.01 mU/ml amylase for strain 2SA. Left chromatogram -- (A) 0.1-0.5 standards; (B,C,D,E,F) 5, 30, 60, 180, and 600 min. Right chromatogram -- (A,B,C,D,E) 5, 30, 60, 180, and 600 min.
Fig. 4. Group 3 amylase action pattern. Time sequence chromatographic analysis of *S. bovis* strain 18M2 amylase on starch. The conditions of the digest were identical to those of Fig. 2 -- 0.76 mU/ml amylase per mg starch. (A) G₅₋G₁₅ Standards; (B,C,D,E,F) 5, 30, 60, 180, and 780 min
Fig. 5. Group 4 action pattern. Time sequence chromatographic analysis of *S. bovis* strain H12 amylase on starch. The conditions of the digest were identical to those of Fig. 2 -- 1.50 mU/ml amylase per mg starch. (A) G_1-G_{15} Standards; (B, C, D, E, F) 5, 30, 60, 180, and 600 min
Fig. 6. Group 5 amylase action pattern. Time sequence chromatographic analysis of *S. bovis* strain 101 amylase on starch. The conditions of the digest were identical to those of Fig. 2 — 1.00 mU/ml amylase per mg starch. (A) G1-G15 Standards; (B,C,D,E,F) 5, 30, 60, 180, and 780 min.
pattern for both strains H24 and 2SA was as follows: \( G_1 \) to \( G_6 \) were present from 5 to 600 min with \( G_1 \) predominating. The quantities of \( G_1 \) to \( G_6 \) gradually increased from 5 to 600 min; however, they increased at different rates. Isomaltose and \( G_{2a} \) were produced. At 600 min, the oligosaccharides present in order of decreasing quantities were \( G_1, G_3, G_2, G_4, G_5, \) and \( G_6. \) Whereas \( G_2, G_3, \) and \( G_4 \) were present in substantial quantities, \( G_5 \) and \( G_6 \) were present in low quantities.

The main difference between the action patterns of Groups 1 and 2 was that relative to the quantity of \( G_1 \), more \( G_2, G_3, \) and \( G_4 \) were produced with Group 2 than with Group 1 amylases.

The action patterns of strains H24 and 2SA differed from each other in that strain 2SA produced more \( G_1 \) to \( G_6 \) including \( G_{2a} \) and isomaltose than stain H24. Also, at 5 min, strain 2SA produced less \( G_1 \) and, at 600 min, more \( G_1 \) than strain H24. Compared to strain 2SA, strain H24 produced a very small quantity of isomaltose and \( G_{2a}. \) Strain 2SA produced more isomaltose and \( G_{2a} \) than \( G_5 \) and less than \( G_4. \)

**Amylase action pattern (Group 3)** The amylase action pattern of Group 3 is shown in Fig. 4. This group was different from Groups 1 and 2 in that \( G_1 \) did not predominate at 780 min. Also, no \( G_{2a} \) or isomaltose were present.

At 780 min the following oligosaccharides were present in order of decreasing quantities: \( G_3, G_4, G_1, G_2, G_5, \) and \( G_6. \) Production of the lower maltosaccharides was slight until a
hydrolysis time of about 60 min. At this time, G₃ predominated; G₁, G₂, and G₄ also were present, as were traces of G₅ and G₆. These relative quantities remained unchanged up to 780 min.

Amylase action pattern (Group 4) The amylase action pattern of Group 4 is shown in Fig. 5. The action pattern of strain H12 is very different from the action patterns discussed previously. At 5 min, G₁ to G₇ were present; the quantity of G₁ was much greater than the others. The quantity of G₁ increased gradually up to 60 min then it decreased to almost nothing at 600 min. At 30 min, the quantities of G₂ to G₆ were about equal. At 60 min, the relative quantity of G₄ increased. Then, G₂ to G₅ increased up to 600 min. G₈ started appearing for the first time at 180 min and G₉ appeared at 600 min. At 600 min, the maltosaccharides were present in the following order of decreasing quantity: G₄, G₃, G₂, G₁, G₅, G₆, G₇, G₈, and G₉. No isomaltose was present. G₂ₐ was present up to 180 min but not at 600 min. The fact that the quantity of G₁ decreased from 60 to 600 min while the quantities of G₅ and G₄ increased is evidence that a transglucosylase is present in addition to amylase.

Amylase action pattern (Group 5) The amylase action pattern of Group 5 is shown in Fig. 6. The action pattern of strain 101 was different from the other groups in that, at 780 min, almost equal amounts of G₁ to G₄ were present with slightly more G₃.
At 780 min, small quantities of $G_5$ to $G_8$ were present in the following order of decreasing quantities: $G_5$, $G_6$, $G_7$, and $G_8$. Very small quantities of $G_{2a}$ and isomaltose were present at all sample times. At 5 min, the quantity of $G_7$ was much greater than the quantity of other oligosaccharides. The quantity of $G_7$ remained constant from 5 min to 780 min. The quantities of $G_2$ to $G_4$ increased greatly from 5 min to 780 min; the quantities of $G_5$ to $G_8$ increased slightly during this time period.

Comparison of Amylase Action Pattern Groups with the Change in pH Produced by Each Strain in D(+)-Lactose, D-Mannose, and Dextrose Broths

Fig. 7 shows the results of comparing the five amylase action pattern Groups with the terminal pH values produced by each strain when grown in D(+)-lactose, D-mannose, and dextrose broths. In Group 1, the change in pH produced by strains 2B and 18C2 in D-mannose and dextrose was about the same. The difference in the amount of acid produced by strain 2B in D-mannose and dextrose was about the same as the corresponding difference for strain 18C2. The same difference pattern was obtained for Group 2, but the total quantity of acid produced in D-mannose and dextrose was greater. For Group 3, the quantity of acid produced in D-mannose was the same as in Group 2 but the amount produced in dextrose was less than in Group 2. For Group 4, nearly equal amounts of acid were
Fig. 7. Comparison of the acid production patterns (relative pH changes in 1% D(+)lactose, D-mannose, and dextrose broths) with the amylase action pattern Groups
DECREASE IN pH OF THE MEDIUM (pH UNITS)

101 112 H12 18M2 3S 2SA H24 H22 28

D(-) LACTOSE
D-MANNOSE
DEXTROSE

DECREASE IN pH OF THE MEDIUM (pH UNITS)

AMYLAISE ACTION PATTERN GROUPS

0 2 4 6 8 10
produced in D-mannose and dextrose; the terminal pH values were just slightly different than those recorded after growth in D(+)lactose broth. In Group 5, the total amount of acid produced in all three carbohydrate broths was higher than the amount produced by any of the other Groups; approximately equal pH changes were noted in all three carbohydrate broths.

The quantity of acid produced in D(+)lactose did not correlate with the amylase action pattern Groups, but helped to differentiate Groups 4 and 5 from the other three Groups.
DISCUSSION

The color reactions obtained in the starch broth cultures correlated to some degree with the widths of the zones of starch hydrolysis on agar plates. Strains which produced zones of 15 to 26 mm in width extending from the edge of the area of growth, yielded purple, orange and red-violet colors in starch broth. Strains that produced small or no zones of hydrolysis yielded blue and dark blue colors, respectively, in starch broth. Thus, the color reactions can be used to some extent in a screening program. Also, unusual colors are indicative of amylases which have different action patterns. Some evidence for this is the fact that the amylases of strain 2B, which produced an orange color in starch broth, and strain H24, which produced a red-violet color, had different action patterns. However, the lack of a unique color does not mean that the amylase action pattern will not be unique.

Strains ID14, 364, and 105 produced very small zones on starch plates. Carlson (1959) also found a few *S. bovis* strains that were weakly hydrolytic in starch broth. According to Crowley's (1950) interpretation, a doubtful or weak reaction is considered to be due to β-amylase activity. Other explanations for these weak reactions are that the amylase might be cell-bound or produced in very small quantities. These strains
should be studied further by means of paper chromatography to determine whether or not they have β-amylases.

Walker (1965a) studied thoroughly the amylases of only one strain of *S. bovis*. The results of the present study show that none of the amylase action patterns of the seven strains of *S. bovis* was the same. Thus, there is no really "typical" *S. bovis* amylase. Each of these amylases should be characterized further; it would be interesting to examine differences in serological reaction, amino acid composition, active site, and other properties of these amylases.

Those *S. bovis* strains which are closely related physiologically have amylases with similar action patterns. For example, the only physiological differences between strains 2B and 18C2 are that strain 2B produces more acid in D(+)lactose and slightly less acid in dextrose and D-mannose. Also strain 2B grows slower and has a smaller size colony. The amylases of 2B and 18C2 yield very similar action patterns. Another example of this is seen when strain H24 is compared with strain 2SA. The only differences are that whereas strain H24 ferments L-arabinose, reduces tetrazolium in 21 hr, and produces less acid in D(+)lactose broth, strain 2SA hydrolyzes aesculin and does not reduce tetrazolium in 48 hr. Oxford (1958) found that strain 2SA behaved a little differently from strains 2B and 18M2 in that it produced small yields of dextran in the absence of added CO$_2$. Strains H24 and 2SA are alike in
that they both ferment trehalose, produce slight acid in litmus milk, and produce equal quantities of acid in dextrose and D-mannose broths. And these two strains produce amylases that have similarities in their action patterns on starch.

The correlation between the degree of pH change produced in dextrose and mannose broths and the action patterns of the amylases produced was a chance observation that can not be explained. A possible reason might be that strains producing larger quantities of acid produce amylases that must be able to function at the lower pH values. Thus, this amylase might have a lower pH optimum and probably a different amino acid sequence that might cause it to yield a different action pattern. Many experiments could be designed to test this hypothesis.

It is not surprising that the strain which produces the most acid in dextrose, D-mannose, and D(+)lactose also produces the most acid in the starch broth used for the production of amylase.

It is interesting that there is no acid and clot in litmus milk unless the pH becomes 4.93 or lower in D(+) lactose broth.

Almost everyone who has worked extensively with many S. bovis strains recognizes that there are at least two main divisions within the S. bovis species as now characterized (Dain et al., 1956; Deibel, 1964). Division I comprises those
strains which ferment mannitol, produce no slime, and are \( \gamma \)-hemolytic; the others do not ferment mannitol, do produce slime, and are \( \alpha \)-hemolytic. One of the strains in this study belongs to Division I -- strain 101. Compared to the other six strains, strain 101 ferments more carbohydrates, is the only strain that ferments sorbitol, does not grow in Rogosa agar tubes, and produces the most acid in D(+)lactose, dextrose, and D-mannose broths. It is not surprising that this strain has an action pattern which differs greatly from all the others. Sims (1964) reported that Rogosa agar is a simple test for differentiating \( S. \ bovis \) from other streptococci. Sims (1964) used 11 stock strains of \( S. \ bovis \) one of which was a mannitol-fermenting strain. He found that good growth of \( S. \ bovis \) occurs in 1-4 days while other streptococci fail to grow at all. In this study all the \( S. \ bovis \) strains grew on the Rogosa agar plates in 5\% CO\(_2\) in air and all of the strains except strain 101 grew in the Rogosa agar tubes. The fact that strain 101 grows on Rogosa agar plates in 5\% CO\(_2\) in air and not in Rogosa agar tubes provides another differentiating test for strain 101. Since a certain concentration of CO\(_2\) is necessary for growth in Rogosa medium (Sims, 1964), the lack of slime production by strain 101 probably accounts for its inability to trap the CO\(_2\) that it produces.
The strain 101 used in this study has the same physiological characters as strain Cl01 described by Medrek and Barnes (1962b). Also strain 101 is probably similar to the strain that Walker (1965a) used to study the extracellular and intracellular amylases of \textit{S. bovis}. The action pattern on amylose of the extracellular amylase of \textit{S. bovis} strain 101 used in this study is similar to the results obtained by Walker (1965a). However, there are some differences. Whereas, at the early stages of hydrolysis, Walker's (1965a) strain produced only maltotriose, strain 101 produced \( G_1 \) to \( G_2 \) with \( G_3 \) predominating. However, \( G_1 \) predominated at the early stages when starch was the substrate for strain 101 amylase.

The action pattern of strain 101 amylase on starch was different from that on amylose. The final products with amylose in order of decreasing quantity were \( G_3 \), \( G_2 \), and \( G_1 \); with starch they were \( G_3 \), (\( G_1 = G_2 = G_4 \)), \( G_5 \), \( G_6 \), \( G_7 \), and \( G_8 \).

The saccharogenic/dextrinogenic (S/D) curves obtained by plotting the \( \log_{10} \) of the reducing value against the diameter of the zone of hydrolysis on a starch agar plate are different for the amylases from different strains of \textit{S. bovis}. Especially at the lower dilutions of the enzymes, the curves are shifted to the right or left, i.e., for a given reducing value, the diameter of the zone of hydrolysis on a starch plate is different for different \textit{S. bovis} strains. This is an indication that
the amylases are different and that they may have different action patterns.

The slopes of many of the S/D curves are the same; however, the slopes of some of the curves differ slightly. More measurements would have to be made to determine if the slopes differ significantly. No reason can be given for why the curves are linear at low dilutions and linear at high dilutions but not linear for all concentrations. Maybe the cause of this is the difference in viscosity of the concentrated and dilute enzyme solutions, because the concentrated solutions were absorbed into the discs slowly compared to the dilute solutions. It might have been better to pipette 0.07 ml/disc rather than dip the disc (Stark et al., 1953). Over a certain range, the amount of enzyme per disc, the concentration of the starch and the concentration of the amylase, respectively, are linearly related to the diameter of the zone of hydrolysis (Stark et al., 1953).

The evidence that strain H12 produces a transglucosylase is seen in the paper chromatogram of the action pattern on starch. The glucose spot becomes smaller after one hour of digestion, and the quantities of maltotriose and maltotetraose increase. Assuming this extracellular transglucosylase is like the intracellular transglucosylase of S. bovis described by Walker (1965b), the glucose is probably acting as the
acceptor molecule for the transglucosylase which can use maltotriose as a donor. Walker's (1965b) transglucosylase can degrade higher maltodextrins or starch by transferring one or more glucosyl residues from the non-reducing end of the donor to the non-reducing end of the acceptor. Walker (1965b) found no evidence that the transglucosylase of *S. bovis* was extracellular. It was cell-bound, and was released by treatment of the cells with lysozyme and by suspension of the spheroplasts in dilute buffer. Another, different intracellular transglucosylase was found in *Streptococcus mitis* by Walker (1966).

Up to this time, transglucosylases have been found only intracellularly in bacteria. Thus, the transglucosylase of *S. bovis* strain H12 is the first extracellular bacterial transglucosylase to be described. More experiments with strain H12 would reveal more information about the nature of its extracellular transglucosylase and amylase. An attempt should be made to separate these two enzymes so they can be characterized. Sawai and Hehre (1962) found a transglucosylase-amylase (produced by *Candida tropicalis* yeast) which catalyzes both hydrolytic and transglucosylative degradation of amylaceous materials.
The amylases from all seven strains should be characterized by determining their Ca$^{++}$ and Cl$^{-}$ ion requirements, pH and temperature optima, action patterns on amylose, blue value-reducing value curves (Kung et al., 1953), and degree of multiple attack (Robyt and French, 1967). Walker and Hope (1964) determined the optimum pH (5.8 at 39°C) for one strain of \emph{S. bovis}. Hobson and MacPherson (1952) determined the blue value-reducing value curve, pH optimum (5.5 to 6.5 at 35°C), and temperature optimum (48°C at pH 7.0) for a \textit{Streptococcus} amylase, probably related to \emph{S. bovis}. Also, they found that this amylase is not activated by calcium or chloride ions, but dilute solutions of this amylase are stabilized by the presence of small concentrations of calcium ions.

This study demonstrates that, when doing a dextrinogenic-type amylase assay, it is necessary to do the assay in the presence and absence of dextrose to determine if a transglucosylase is present along with an amylase.

Some of the \emph{S. bovis} amylases produce more glucose than any one maltodextrin at all stages of hydrolysis. This indicates that a 7-amylase might be present. According to Walker and Hope (1964) glucose appears at an early stage in the reaction when the amylase can attack all α-(1→4)-glucosidic linkages at random, regardless of their proximity to end groups. Also, those amylases of \emph{S. bovis} strains that produce
large quantities of glucose early in the hydrolysis of starch probably have a higher degree of multiple attack than those that produce very small quantities of glucose at early hydrolysis times.

The glucose concentration remains constant at all stages of hydrolysis with some of the *S. bovis* amylases; however, this qualitative observation should be checked by quantitative paper chromatography. Only some of the *S. bovis* amylases produce isomaltose and $G_{2a}$. According to Robyt and Whelan (1968), isomaltose has only been isolated from a digest of waxy-maize starch (amylopectin) with a mixture of fungal enzymes. They suggested that an $\alpha$-amylase may yet be found that will liberate the branch point as isomaltose. Thus, the $\alpha$-amylases of the *S. bovis* strains in the present study that yield isomaltose from starch are the first reported bacterial $\alpha$-amylases that liberate the branch point as isomaltose. With respect to the predominating product in the final starch hydrolysis digest, *S. bovis* amylases yield either glucose, maltotriose, maltotetraose (when transglucosylase is also present), or almost equal quantities of maltotriose, maltose, glucose, and maltotetraose with just slightly more maltotriose.

Although the action patterns of the amylase of *S. bovis* strains are significantly different, they are alike in that the predominant final product(s) are either one of or a combination of the following: $G_1$, $G_2$, $G_3$, and/or $G_4$. Some
similarity would be expected since they come from the same species. This \( G_1 \) to \( G_4 \) pattern of \textit{S. bovis} strains is very different from the predominant final products produced by \textit{Bacillus subtilis} amylase with amylopectin -- \( G_1, G_2, G_3, G_5 \) and \( G_6 \) (Robyt and French, 1963). Maltotriose is usually the most abundant final product with \textit{S. bovis} strains. Maltotriose is also the most abundant product with the intracellular \( \alpha \)-amylase of \textit{Escherichia coli}. However, the other final products with amylopectin are \( G_2 \) and a trace of \( G_5 \) (Chambost \textit{et al.}, 1967). \textit{Bacillus polymyxa} has an \( \alpha \)-type amylase which produces mostly \( G_2 \) and small quantities of \( G_1 \) and \( G_3 \) with amylopectin (Robyt and French, 1964). Taka-amylase A (\( \alpha \)-amylase produced by \textit{Aspergillus oryzae}) produces mostly \( G_2 \) and \( G_3 \) with small quantities of \( G_1 \) and \( G_4 \) as final products on starch (Minoda \textit{et al.}, 1968). Thus, \textit{A. oryzae} produces an \( \alpha \)-amylase which is similar to that of \textit{S. bovis} strains in that the predominant final products on starch include \( G_1 \) to \( G_4 \), but there is a difference in that the proportions of each product are not the same. The acid-stable \( \alpha \)-amylase of \textit{Aspergillus niger} produces mostly \( G_2 \), slightly less \( G_3 \), slightly less \( G_4 \) than \( G_3 \), and very small quantities of \( G_1 \) and \( G_5 \) as final products on starch (Minoda \textit{et al.}, 1968). The main end products of the hydrolysis of amylose produced by human salivary amylase are \( G_2 \) and \( G_3 \) with small quantities of
G₃ and G₄; the same is the case for porcine pancreatic amylase except that larger quantities of G₃ and G₄ are produced (Robyt, 1962). However, porcine pancreatic amylase has a degree of multiple attack of about six which is twice that of human salivary amylase (Robyt and French, 1967). Thus, even though the main end products of hydrolysis of amylases from very diverse sources (including S. bovis strains) are similar (production of G₃ to G₄), the pattern of the early products are not similar. Also, many other characteristics (e.g., pH optimum, temperature optimum, degree of multiple attack, and acid-stability) of amylases from diverse sources are different. It would be interesting to see if different strains of E. subtilis, E. coli, B. polymyxa, A. oryzae, and A. niger produce amylases that have action patterns which differ from each other as much as the amylases of S. bovis strains.

All S. bovis amylases or amylase-transglucosylase mixtures could be used in certain industrial applications. In fact, since S. bovis grows anaerobically, and since aeration is a very expensive item in industrial fermentations, S. bovis strains might be an economical source of new commercial amylases.

Another advantage of using S. bovis for commercial amylase production would be that it is devoid of proteolytic activity (Deibel, 1964). It has been found that preparations of crystalline amylases (particularly those of hog pancreas and
Bacillus subtilis) were still contaminated by traces of proteases even after repeated crystallizations. These protease impurities catalyze the destruction of amylases in the presence of metal-binding agents such as ethylenediaminetetraacetate (EDTA). When these amylases are purified and stored in the presence of a protease inhibitor (diisopropylphosphorofluoridate -- DFP), they are stable even in the presence of EDTA.

Still another advantage of using S. bovis for commercial amylase production is the fact that it has uniquely simple requirements for exogenous amino acids and vitamins (Deibel, 1964). In fact, S. bovis is capable of utilizing ammonium salts as a sole source of nitrogen (Prescott, et al., 1959; Wolin et al., 1959). If S. bovis is cultivated under anaerobic conditions with an increased carbon dioxide tension in a medium containing Tween 80 (thus obviating a biotin requirement), no vitamin requirements are demonstrable (Ford et al., 1958). When incubated under atmospheric conditions, most S. bovis strains require biotin and are stimulated by thiamine, pantothenate, and nicotinate (Niven et al., 1948). Strain variation exists; some are stimulated by thiamin, others require pantothenate, most do not require exogenous purines or pyrimidines (Barnes et al., 1961). Growth with ammonium salts is stimulated by acetate (Prescott et al., 1959) or bicarbonate, and glutamine also serves as a sole source of nitrogen (Wolin
et al., 1959). Wolin and Weinberg (1960) found that growth with ammonium salts, but not with glutamine, requires an unknown substance formed by autoclaving phosphate with glucose. However, the addition of potential electron acceptors or a lowering of the pH value of the medium replaces this requirement. According to Burchall and Wolin (1962), asparagine also satisfies the nitrogen requirement of *S. bovis*, and enzymatic studies indicate that glutamine and asparagine are the most likely primary products of ammonia incorporation.

Many strains of *S. bovis* should be screened by using starch agar plates and determining the amount of acid produced in dextrose, D-mannose, and D(+)lactose broths. Those strains which produce large zones on starch plates and also produce different patterns with respect to the total quantity and relative quantities of acid produced in dextrose and D-mannose as well as D(+)lactose in some cases should be kept for further study.

Walker (1965a) found two cell-bound amylases (one degraded starch granules and the other one did not) and one extracellular α-amylase with the one *S. bovis* strain studied. Her results indicate that one of the cell-bound amylases is similar to the extracellular α-amylase. She did not investigate the possibility that the second amylase was also liberated into the medium. Thus, it is possible that some of the *S. bovis*
strains used in this study have more than one extracellular amylase.

The starch adsorption step that Walker (1965a) used in the purification of the extracellular α-amylase might have removed the second amylase if it were present because some amylases that do not degrade maize-starch granules are not adsorbed on the granules. This is the reason I did not use a starch adsorption step in the purification of the extracellular amylase.

Walker (1965a) raised the question whether the α-amylase in the medium arose from autolysis of the *S. bovis* cells during the 48-hr period of growth. She concluded that the α-amylase in the medium around the cells was liberated without cell lysis. Therefore, the amylolytic enzymes from the seven *S. bovis* strains used in this study are probably extracellular.
PART II. AMYLOLYTIC ENZYMES AND SELECTED PHYSIOLOGICAL PROPERTIES OF *STREPTOCOCCUS EQUINUS*
MATERIALS AND METHODS

Sources of Bacteria

Seven *S. equinus* strains were obtained as follows: NCDO 1090 and 1091 from Dr. Ellen I. Garvie, National Inst. for Research in Dairying, Shinfield, Reading, England; T1, T2, G2, and C1 from David G. Smith, University College London, Dept. of Botany, Gower Street, London, WCI, England; and ATCC 9812 from American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. Four *S. bovis* strains were obtained for comparison purposes as follows: S2 from Dr. E. M. Barnes, Low Temperature Research Station, Downing Street, Cambridge, England; ATCC 9809, 15351, and 15352.

Physiological Tests

The methods used were those described in Part I. Only the following strains were tested: *S. equinus* NCDO 1090 and 1091; *S. bovis* strains S2, ATCC 9809, 15351, and 15352. The other *S. equinus* strains were not tested because their amylolytic enzymes were not studied beyond the preliminary screening stage.
Preliminary Experiments on Agar Plates

Effect of media and dextrose concentration on amylase and transglucosylase production

Nutrient-starch agar plates were made with Difco Nutrient Broth, 1.5% agar (Difco), and 0.2% soluble starch (Mallinckrodt). Two additional media were prepared by adding 0.2% and 1.6% dextrose, respectively, to the nutrient-starch agar medium. BHI-starch agar plates were made by adding to Difco BHI agar (which contains 0.2% dextrose), sufficient starch and dextrose to give final concentrations of 0.2% starch and 1.6% dextrose. The other medium contained 1.0% Tryptone (Difco), 0.5% Yeast Extract (Difco), 0.2% K₂HPO₄, 0.2% soluble starch (Mallinckrodt), and 1.5% agar.

The cultures were maintained as described in Part I, except that they were transferred triweekly. The screening was accomplished by streaking one loopful of BHI broth inoculum (18-24 hr) once down the center of each type of starch plate. The plates were inverted and incubated at 37 C for 96 hr. After staining the plates with Gram's iodine solution, the widths (mm) of the zones of hydrolysis (unstained bands) were measured.

Assay of S. equinus 1090 for transglucosylase

A 1.0%, 18 hr BHI broth culture of strain 1090 was used to inoculate BHI broth plus 0.2% soluble starch. After 26
and 66 hr of incubation at 37 C, the cell-free liquor was collected by centrifugation at 49,500 x g in a Sorvall RC-2B centrifuge with a SM-24 rotor at 3 C. The cell-free liquor was dialyzed against 0.01 M, pH 7.3 potassium phosphate buffer to remove any dextrose. The dextrinogenic method of McCready and Hassid (1943) as modified by Robyt was used to measure the reduction in blue color in the presence and absence of dextrose. The procedure used for the dextrinogenic method was as follows: The stock iodine solution was 0.2% I₂ and 2.0% KI in H₂O. The KI was dissolved in a small quantity of H₂O before adding more H₂O and I₂. One-tenth ml of digest was added to 5 ml of working iodine solution (prepared daily by diluting stock iodine solution 1:100 with H₂O and 1 ml concd HCl/liter and mixed. The blank was made by substituting H₂O or boiled enzyme for the enzyme. The zeroing blank was working iodine solution or H₂O. The blue color was read in a spectrophotometer at a wavelength of 620 nm. The calculations were made as follows:

\[
\text{% Reduction in blue value (BV)} = \frac{D_0 - D_1}{D_0} \times 100
\]

\[D_0 = \text{OD of starch or amylose blank}\]
\[D_1 = \text{OD of digest}\]

\[^1\text{Robyt, J. F., Department of Biochemistry and Biophysics Iowa State University, Ames, Iowa 50010. Dextrinogenic amylase assay method. Private communication. 1966.}\]
Transglucosylase was assayed by measuring the % reduction in blue value in the presence and absence of dextrose and subtracting the % reduction without dextrose (due to amylase) from the % reduction with dextrose (Walker, 1965b). The digest with dextrose contained 0.1% amylose and 0.25% dextrose (Walker, 1965b) in 0.02 M β-glycerophosphate buffer. The amylose (Nutritional Biochemical Corp.) was first dissolved in dimethyl sulfoxide at a concentration of 5% and stirred until dissolved (about 10 hr); then it was heated to boiling. The dissolved amylose was added to the buffer which was then heated to boiling. The pH of the substrate mixture was adjusted to 7.3 with concd H₂SO₄. One-tenth ml of dialyzed cell-free liquor was added to 0.9 ml of substrate mixture. Boiled, dialyzed cell-free liquor was added to the two control tubes; one control tube contained dextrose, the other did not. The OD was measured with a Bausch and Lomb Spectronic 20 Colorimeter at a wavelength of 650 nm. The blue values of the digests were measured after 3 and 16 hr of incubation of the digests at 37 C.

Effect of temperature on transglucosylase production

One loopful of BHI broth inoculum (18-24 hr) of S. equinus 1091 was streaked once down the center of each of several BHI-starch agar plates. The plates were incubated at 30 or 37 C for 96 hr. After staining with Gram's iodine,
the widths (mm) of the zones of hydrolysis (unstained bands) were measured.

**Effect of brand of soluble starch on transglucosylase production**

The Tryptone Yeast Extract (TYE) medium used in this experiment and in the following experiments was composed of 1.0% Tryptone, 0.5% Yeast Extract, 0.2296% $\text{K}_2\text{HPO}_4$, 0.2% starch, 1.5% agar, pH 7.3. Soluble starch -- according to Lintner (Merck) and soluble starch powder (Mallinckrodt) were used.

**Effect of concentration of dextrose or maltose on size of amylolytic zone**

TYE medium with soluble starch -- according to Lintner -- was used to determine the effect of the concentration of dextrose or maltose in starch-containing medium on the width of the zone of degraded starch. The concentrations of dextrose or maltose used were 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.15, 0.20 and 2.00%. *S. equinus* 1090 and 1091 and *S. bovis* S2 (for comparison) were the strains used. One loopful of BHI broth inoculum (14 hr) was streaked once down the center of each plate. The plates were incubated for 30 hr at 37 C, then the size of the zones of hydrolysis were determined.
Kinetics of transglucosylase production on agar plates by S. equinus 1091

The objective of this experiment was to obtain extracellular transglucosylase on the agar plate without starch being present; then to detect the transglucosylase with an overlay solution containing an antibiotic to stop protein synthesis, starch, and an accepting molecule (dextrose or maltose).

The media contained 1.0% Difco Proteose-Peptone No. 3, 0.5% Difco Yeast Extract, 0.2% K$_2$HPO$_4$, 0.2% soluble starch (according to Lintner), 0.15% carbohydrate and 1.5% agar, pH 7.4. The carbohydrates used were salicin, mannose, sucrose, raffinose, fructose, sorbitol, maltose, dextrose, and cellobiose. All carbohydrates were autoclaved with the medium. Another identical set of media was made except that these media contained no starch. One loopful of an 18-24 hr, dextrose-free inoculum (1.0% Proteose-Peptone No. 3, 0.5% Yeast Extract, and 0.2% K$_2$HPO$_4$, pH 7.4) was streaked once down the center of each plate. After 2 and 4 days of incubation at 37 C, the plates were checked for growth and the plates containing starch (controls) were checked with Gram's iodine to determine if there was a zone of hydrolysis when starch and the carbohydrate being tested for acceptor activity were present in the growth medium.

After 76 hr of growth at 37 C, both the growth and protein synthesis were stopped when about 12 ml of overlay solution
were poured on the plates that contained no starch. The overlay basal contained 0.2% soluble starch (according to Lintner), 0.0505% \( \text{KH}_2\text{PO}_4 \), 0.3467% \( \text{K}_2\text{HPO}_4 \), 0.2% dextrose or maltose (acceptor molecules), and 1.0% agar, pH 7.4. The basal was sterilized by autoclaving; enough filter-sterilized, 0.2% chloramphenicol -- Parke, Davis & Co., Detroit, Michigan -- (4.04 ml/100 ml of medium) was added to the overlay basal so that the final concentration in the Petri plate after complete diffusion would be \( 10^{-4} \) M. The overlayed plates were incubated at 37 C for 12 hr in the inverted position; then they were stained with Gram's iodine.

Relative efficiencies of various carbohydrates as acceptors for transglucosylase

This experiment was designed to determine the relative efficiencies of dextrose, maltose, sorbitol, and sucrose as acceptor molecules; also, these determinations were done at different temperatures. All the carbohydrates used in this experiment, with the exception of the starch, were filter-sterilized with a Seitz filter using an EK filter pad. The growth media contained 1.5% Proteose Peptone No. 3, 0.2% \( \text{K}_2\text{HPO}_4 \), 1.5% agar; 0.2% soluble starch (Lintner) was added to some media. The pH was 7.72 before the starch was added and before autoclaving. A 20% filter-sterilized solution of carbohydrate was added after autoclaving in a quantity sufficient to result in a final carbohydrate concentration of 0.15%.
Media were prepared containing sucrose with and without added starch, maltose with and without added starch, and starch alone. The overlay mixture was made by using 0.03 M potassium phosphate buffer, pH 7.6; 0.2% soluble starch (Lintner), and 1.0% agar. It was sterilized by autoclaving. After autoclaving, 20% solutions of filter-sterilized carbohydrates were added in quantities sufficient to result in a concentration of 0.25% in the overlay mixture. Also, enough filter-sterilized, 0.2% chloramphenicol solution was added to result in a final concentration in the Petri plate of $10^{-4}$ M (4.04 ml of 0.2% chloramphenicol per 100 ml of overlay mixture).

The carbohydrates used in the overlay mixtures were sucrose, maltose, dextrose or sorbitol. A control overlay solution was prepared which contained no carbohydrate except starch.

After 9 and 21 hr of incubation at 37°C, the medium with maltose and starch, and the medium with sucrose and starch were stained with Gram's iodine.

The overlay mixtures were applied after 21 hr and incubated at 20, 30, 37, and 42°C. Plates were examined after 2-3 and 5 hr of incubation.

Factors Affecting Production of Amylase and Transglucosylase in Broth by *S. equinus* 1091

**Effect of calcium carbonate and medium**

Three CaCO$_3$-containing media were prepared: (A) 2.0%
Proteose Peptone No. 3, 2.0% maltose, 0.2% K$_2$HPO$_4$, and 3.0% CaCO$_3$, pH 7.58; (B) 1.0% Tryptone, 1.0% Yeast Extract, 2.0% maltose, 0.2% K$_2$HPO$_4$, and 3.0% CaCO$_3$, pH 7.27; and (C) 1.2% Tryptone, 2.0% maltose, 0.2% K$_2$HPO$_4$, and 3.0% CaCO$_3$, pH 7.52. A 1.0% (V/V) BHI broth inoculum (18 hr) was added to 40 ml of medium in 125-ml Erlenmeyer flasks. Samples were taken after 18, 30, 42, 52.5, 66, and 120 hr of incubation at 37 C. The samples were assayed for amylase and transglucosylase activities and the pH values were measured. Also, three media without CaCO$_3$ were prepared. To a basal broth containing 1.0% Tryptone and 1.0% Yeast Extract in 0.03 M potassium phosphate buffer, pH 7.5, were added 1, 3, or 5% maltose (autoclaved separately as a 20.0% solution). These media (40 ml in 125-ml Erlenmeyer flasks) were inoculated with 1.0% (V/V) of an 18-hr, BHI broth culture and incubated at 37 C for 25 hr. The pH was maintained at 6.0 to 7.0 throughout the fermentation by periodically adding 1.0 N NaOH (autoclaved). The cells were removed from the broth by centrifugation in a Sorvall RC-2B with a SS-34 rotor at 48,200 x g for 30 min at 3 C. The cell-free liquor was dialyzed against 11 liters of 0.03 M potassium phosphate buffer, pH 7.3 at 5 C to remove maltose that would serve as an acceptor for transglucosylase. Dialysis tubing -- 6 mm (LaPine Scientific Co., 6001 S. Knox Ave., Chicago, Ill. 60629) -- was used. The 11 liters of buffer were renewed four times in 24 hr.
Effect of age of inoculum

The medium consisted of 1.0% Tryptone, 1.0% Yeast Extract, and 1.5% maltose (autoclaved separately as a 20.0% solution) in 0.03 M potassium phosphate buffer, pH 7.5. The medium was inoculated with 1.0% (V/V) of a 12.8, 17.5, or 24.0-hr, BHI broth culture and incubated at 37 C for 16 hr. The pH was maintained at 6.0 to 7.0 throughout the fermentation by periodically adding 1.0 N NaOH (autoclaved). The cell-free liquor was obtained and dialyzed as described in the previous experiment.

Effect of concentration of maltose

The basal medium described in the previous experiment was used with the addition of 1.0, 1.5, 2.0, 3.0 or 4.0% maltose. The media were inoculated with a 17.5-hr inoculum and the rest of the procedure was identical to that described in the previous experiment.

Effect of length of fermentation

The basal medium to which 1.0% maltose had been added, was inoculated with a 26-hr BHI broth culture; the rest of the procedure was identical to that of the previous experiments except that samples were taken every 2 hr from 8 to 24 hr. The OD of the cultures was monitored at a wavelength of 525 nm; after centrifugation and dialysis, the samples were
assayed for amylase and transglucosylase activities. For control purposes, the pH of one flask was not maintained between 6.0 and 7.0.

**Production of amylase and transglucosylase**

The medium was 1.0% Tryptone, 1.0% Yeast Extract, and 1.5% maltose (autoclaved separately as a 20.0% solution) in 0.03 M potassium phosphate buffer, pH 7.5. Seven liters of the medium were placed in a 9-liter capacity, double sidearm Bellco spinner flask (Bellco Glass Inc., P. O. Box B, Vineland, N. J. 08360). The medium was inoculated with a 17.5-hr BHI broth inoculum -- 1.0% (V/V). The culture was stirred constantly at 37°C for 18 hr. If 1.0 N NaOH was added to the culture before the pH had dropped to 6.0, the fermentation ceased; however, if the pH was 6.0 or lower before the first addition of 1.0 N NaOH, the fermentation was normal. Therefore, after the pH of the culture had dropped to 6.0, 1.0 N NaOH (autoclaved) was added by gravity feed from a reservoir at a rate to maintain the pH between 6.5 and 7.0. A one-inch board was placed between the magnetic stirrer and the spinner flask to insulate the latter from the heat of the stirrer motor. The cells were removed from the broth by centrifuging at 12,500 x g with the Sorvall RC-2B centrifuge with a GSA rotor for 1 hr at 3°C. The cell-free liquor was preserved by adding thymol crystals and storing at 5°C.
Purification of Amylase and Transglucosylase

Assays

Thymol crystals were added as a preservative to all buffers, starch substrates, amylose substrates, enzyme solutions, and digests that were longer than one hour.

Effect of CaCl₂ on amylase and transglucosylase assays

The dextrinogenic method for the assay of amylase and transglucosylase described previously was modified by adding 0.01 M CaCl₂ to the amylose substrate. The cell-free liquor was concentrated to 3% of the original volume with an Amicon ultrafilter using a 30,000 M.W. Diaflo membrane (Amicon Corp., 27 Hartwell Ave., Lexington, Mass. 02173) and diluted 1:3 with 0.02 M 8-glycerophosphate-citric acid buffer, pH 7.3. This solution, containing amylase and transglucosylase activities, was assayed with and without the addition of 0.01 M CaCl₂.

Amylase and transglucosylase assays

The dextrinogenic procedure described previously was used. The digest contained 0.25% dextrose, 0.1% amylose, and 0.0155 M 8-glycerophosphate (Matheson Coleman & Bell) adjusted to pH 6.5 or 7.3 with concd H₂SO₄ -- pH 6.5 was used for transglucosylase and pH 7.3 for preliminary studies with both amylase and transglucosylase. One ml of substrate and 0.1 ml of enzyme were used; duplicate
digests were made. The amylase and transglucosylase activities were proportional to the concentration of the enzyme if the percent reduction in blue value did not exceed 12% after 15 min of reaction time. Therefore, the reaction was stopped after 15 min. The OD was read at 620 nm on a Beckman DB spectrophotometer. A unit of transglucosylase activity was defined as the percent reduction in amylose blue value in 15 min at pH 6.5. All digests were made in duplicate.

The dextrinogenic activity of amylase was sometimes determined using starch as the substrate. The conditions were identical to those mentioned in the preceding paragraph except 0.5% soluble starch (according to Lintner) instead of amylose served as the substrate; in addition, 0.01 M CaCl₂ was included; the buffer was adjusted to pH 7.0. The amylase activity was proportional to the concentration of enzyme if the percent reduction in blue value did not exceed 16% after 10 min of reaction time. Therefore, the reaction was stopped after 10 min. All digests were made in duplicate. The unit of amylase activity was defined as the percent reduction in starch blue value in 10 min at pH 7.0.

The reducing value method used for the assay of amylase was that of Robyt and Whelan (1968). The digests were prepared as described for the dextrinogenic assay using a starch substrate. All reactions were carried out in Folin-Wu blood sugar tubes. The maltose hydrate standard curve, which could
be extrapolated through zero, was linear from 0.1 to 0.4 mg/ml of maltose when the final volume in the Polin-Wu tubes was 25 ml and light absorption was read at a wavelength of 520 nm on a Beckman DB spectrophotometer. When very small quantities of reducing sugar were produced -- early stages in hydrolysis of amylose for blue value-reducing value curves, the method of Robyt and Whelan (1968) was modified by diluting samples and standards in the Polin-Wu tubes to 12.5 ml instead of 25 ml and reading the color at a wavelength of 660 nm (Nelson, 1944) instead of 520 nm. Under these conditions, the maltose hydrate standard curve, which could be extrapolated through zero, was linear from 0.006 to 0.20 mg/ml of maltose. All digests were made in duplicate.

Protein assay. The method of Sutherland et al. (1949) was used except that the protein solution was incubated with the alkaline copper tartrate reagent for 20 min at 37 C instead of 10-15 min at 45 C. Also, the Folin-Ciocalteau phenol reagent 2N (Fisher Scientific Co., Fair Lawn, N.J.) was diluted 1:2 to make it 1 N in acid (Lowry et al., 1951). The OD was measured at 500 nm (for 25-200 μg of protein) on a DB spectrophotometer 30 min (at room temperature) after the phenol reagent was added (Lowry et al., 1951). Crystalline bovine serum albumin (Pentex Inc., Kankakee, Ill. 60901) was used as a standard.
Concentration of enzymes

The thymol crystals used as a preservative in the cell-free liquor were removed by filtration with Whatman No. 12 filter paper. The liquor was then concentrated with an Amicon ultrafilter using a Diaflo membrane. The Model 50 and 401 ultrafilter cells and 50,000; 30,000; or 10,000 M.W. (XM-50, PM-30, and UM-10, respectively) retentive capacity Diaflo membranes were used. The liquor was concentrated to 1% of the original volume using either the 50,000 or 30,000 M.W. membrane, then various buffers (to be described later) were used for "dialysis" in the ultrafilter with the 10,000 M.W. membrane. All of the ultrafiltration was done at 5 C.

Chromatography on DEAE-cellulose

Microgranular preswollen DEAE-cellulose (DE-52 Whatman advanced ion-exchange cellulose -- Reeve Angel, 9 Bridewell Place, Clifton, N.J. 07014) was used. The exchanger was prepared as suggested in "Whatman Technical Bulletin IE2, Advanced Ion-Exchange Cellulose" (Reeve Angel, 9 Bridewell Place, Clifton, N.J. 07014). The exchanger was stirred into a volume of 1.0 M Tris-HCl buffer (Sigma Chemical Co., 3500 Dekalb St., St. Louis 18, Mo.), pH 7.3 and left for 10 min. The supernatant liquor was decanted. This step was repeated eight times. The pH and conductivity (measured with a Melabs Model CCM-1 electrodless conductivity meter -- Melabs, 3300
Hillview Avenue, Standford Industrial Park, Palo Alto, Calif.) remained stable and at the correct values. A chromatographic column (Model No. LC-1/2-23-Chromatronix Inc., 2743 Eighth Street, Berkeley, Calif. 94710) was put upside down when it was filled. The packing was settled to a depth of about 45 cm by placing the column in the upright position and passing 780 ml of 0.01 M Tris-HCl buffer, pH 7.3 through it; the column bed was 1.27 x 45 cm. The cell-free broth (480 ml) was concentrated in dialysis tubing to 21.5 ml with Aquacide 1 (Calbiochem, P.O. Box 54282, Terminal Annex, Los Angeles, Calif. 90063). The concentrated enzyme solution was "dialyzed" with 150 ml of 0.03 M potassium phosphate buffer, pH 7.3, in an ultrafilter (Model 50 cell), using a 50,000 M.W. membrane; it was then "dialyzed" in the ultrafilter with 50 ml of 0.01 M Tris-HCl buffer, pH 7.3, using a 10,000 M.W. membrane. Finally, the enzyme solution was concentrated to 2.2 ml. The enzyme solution (2.2 ml) was applied to the column. The column was eluted with increasing concentrations of Tris-HCl buffer, pH 7.3, the gradient being linear between 0.01 and 0.10 M. The flow rate was 16.2 ml/cm²/hr. A Rinco fraction collector (QM Instrument Co., Greenville, Ill.) was used to collect 5.2-ml fractions. The eluate was monitored at a wavelength of 280 nm with an Isco Ultraviolet Analyzer (Model UA-2, 1 cm flow cell -- Isco, Inc., 5624 Seward Ave., Lincoln, Nebr. 68507).
Acetone fractionation

Eight liters of cell-free liquor were concentrated to 230 ml by ultrafiltration (30,000 M.W. membrane in a Model 401 cell). One-hundred ml of the concentrated liquor were dialyzed in 6 mm dialysis tubing at 5 C against a solution that contained 0.01 M CaCl₂ in 0.00707 M β-glycerophosphate buffer adjusted to pH 6.5 with concd H₂SO₄. After dialysis, the volume of the enzyme solution was 124 ml. The enzyme solution was placed in a stainless steel beaker that was immersed in a NaCl-ice water bath at -5.0 to -7.0 C. A magnetic stirrer was used to stir the enzyme solution while cold (-18 C) acetone (reagent grade, C.P.) was slowly added with a cold (-18 C) 10-ml pipette; the acetone was allowed to run down the side of the beaker. The mixture was stirred for 30 min and the precipitate was collected by centrifuging at 25,400 x g for 20 min at -7 C in a Sorvall RC-2B centrifuge with a GSA rotor. Fractions were collected after 20, 35, 46, 56, 66, 75, and 82% (V/V) acetone had been added. Each precipitate was immediately dissolved in a minimal volume of cold (-5 C) solution of 0.01 M CaCl₂ in 0.00706 M β-glycerophosphate buffer adjusted to pH 6.5 with concd H₂SO₄. To remove residual acetone, each dissolved enzyme fraction was placed in a test tube and subjected to a vacuum for 1 hr while being stirred with a small magnetic stirrer. All fractions were assayed for amylase
and transglucosylase activities. The specific activities of the amylase and transglucosylase were determined for the 20-35% acetone fraction.

A second acetone fractionation was made on the 20-35% fraction of the first acetone fractionation. The 0-20% and 20-60% acetone fractionations were collected. The specific activity was determined for the amylase on the 0-20% acetone fraction and the transglucosylase on the 20-60% fraction.

Another 100-ml aliquot of the concentrated liquor was fractionated with acetone as described in the preceding paragraph, except that fractions were collected after 15, 20, 26, 30, and 35% acetone had been added.

Characterization of Amylase and Transglucosylase

Optimum pH

Optimum pH of amylase in the absence of CaCl₂. The pH optimum for the amylase was first determined in the absence of added CaCl₂. Cell-free liquor that had been concentrated to 3% of the original volume with the ultrafilter (30,000 M.W. membrane) was diluted 1:2 with buffers to obtain the following pH ranges: 0.02 M acetic acid-sodium acetate, pH 3.5-5.5; 0.02 M potassium phosphate, pH 5.5-8.5; 0.02 M boric acid-borax, pH 8.0-9.0; and 0.02 M borax-sodium hydroxide, pH 9.5.
The 15 min reduction in the blue value of amylose was used for the assay.

**Optimum pH of amylase in the presence of CaCl₂** All conditions were the same as in the previous experiment except that 0.01 M CaCl₂ was added to each buffer and to the starch and amylose substrates. β-Glycerophosphate buffer adjusted with concd H₂SO₄ was used instead of potassium phosphate buffer for the pH range 5.5-8.5 because a Ca₃(PO₄)₂ precipitate formed when CaCl₂ was added to potassium phosphate buffer. The optimum pH for amylase activity was also determined using the 0-20% acetone fraction from the second acetone fractionation.

**Optimum pH of transglucosylase** The buffers were the same as described previously except that no CaCl₂ was added. Also no CaCl₂ was added to the amylose substrate. β-Glycerophosphate buffer, pH 5.5-8.5 was used. The liquor that had been concentrated in the ultrafilter to 3% of its original volume using a 30,000 M.W. membrane, and the 26-30% acetone fraction from the first acetone fractionation, were used as enzyme preparations.

**Optimum temperature**

**Amylase temperature optimum** The buffer for the starch substrate and for diluting the enzymes was 0.01 M CaCl₂ in 0.0155 M β-glycerophosphate, adjusted with concd H₂SO₄ to
pH 7.0. Activities were measured at various temperatures between 30 and 55°C. Both the saccharogenic and dextrinogenic assays were used. The enzyme preparation examined was a 1:3 dilution of the 0-20% acetone fraction of the second acetone fractionation.

**Transglucosylase temperature optimum** The buffer for the amylose substrate and for diluting the enzyme was 0.0155 M β-glycerophosphate adjusted to pH 6.5 with concd H₂SO₄. Activities were measured at various temperatures between 30 and 55°C. The enzyme preparation examined was a 1:6 dilution of the 26-30% acetone fraction of the first acetone fractionation.

**Activities of various carbohydrates as acceptors for transglucosylase**

The acceptor activity of carbohydrates that would replace dextrose as acceptor of glucosyl residues was determined. The amylose substrate was modified by adding 2% carbohydrate instead of 0.25% dextrose. The pH was 6.5 and the reaction temperature was 35°C. A 1:7 dilution of the 26-30% fraction of the first acetone fractionation was used. The relative activities were determined after 10 min of reaction for dextrose, D-mannose, L-sorbose, maltose, sucrose, αα-trehalose, and sorbitol.
Effect of wheat α-amylase inhibitor on amylase and transglucosylase

The amylose substrate with a pH of 6.5 was used. A 1:3 dilution of the 20-60% acetone fraction from the second acetone fractionation was made in β-glycerophosphate buffer, pH 6.5 and divided into two portions. Some dry wheat α-Amylase Inhibitor (Nutritional Biochemicals Corp., Cleveland, Ohio) was added to one portion. The percent reduction in amylose blue value in 15 min was determined.

Characterization of Amylase Free of Transglucosylase

Production and purification

Filter-sterilized maltose was used as the energy source in the production of eight liters of amylase solution. The cell-free liquor was concentrated to 1% of original volume using a 30,000 M.W. membrane and dialyzed with the ultrafilter (10,000 M.W. membrane) with two liters of 0.01 M CaCl₂ in 0.00706 M β-glycerophosphate adjusted to pH 6.5 with concd H₂SO₄. The final volume was 80 ml. Amylase, free of detectable transglucosylase activity was found in the 0-15% acetone fraction of the first acetone fractionation. This fraction was used in the following experiments at a dilution of 1:100.
Calcium and chloride ion requirements

An amylose substrate was made up in 0.0155 M β-glycerophosphate buffer, pH 7.0. Additional substrates were made by adding one of the following: 0.01 M calcium acetate; 0.01 M NaCl and 0.01 M calcium acetate; 0.01 M CaCl₂, 0.01 M CaCl₂ and 0.01 M EDTA (trisodium salt); or 0.01 M NaCl and 0.01 M EDTA. The amylase was diluted 1:100 in 0.0155 M β-glycerophosphate, pH 7.0, before addition to the substrate. The percent reduction in blue value was determined after reaction times of 5, 10, 15, 20, 25, and 30 min.

Paper-chromatographic analysis of amylase

The method was identical to that described in Part I of this thesis with the following exceptions: One part of a 1:100 dilution of the enzyme was added to 10 parts of substrate. The digest contained 0.1% amylose and 0.01 M CaCl₂ in 0.0155 M β-glycerophosphate adjusted to pH 7.0 with concd H₂SO₄. Samples were taken at various times up to 97 hr; the reaction was stopped by boiling. Each sample was assayed with the dextrinogenic and saccharogenic methods for the blue value-reducing value curve and the percent conversion to apparent maltose was calculated. Each sample was desalted with Amberlite MB-3 -- ion exchange resin -- (Mallinckrodt Chemical Works) before it was spotted on paper. One-hundred µl of each sample and 30 µl of standard were used.
Blue value-reducing value curves

The method was described in the section on paper-chromatographic analysis. The blue value-reducing value curve was plotted. In order to compare the blue value-reducing value curve of the mixture of amylase and transglucosylase, a new lot of cells was grown up and processed the same way as for the first reducing value curve except that the concentrated liquor was dialyzed with dialysis tubing (6 mm) instead of the ultrafilter. The first and second acetone fractionations did not yield pure amylase but the 15-20% fractions contained more amylase than transglucosylase. The blue value-reducing value curves were determined for dialyzed and non-dialyzed, ultrafilter-concentrated liquor and for the 15-20% acetone fraction of the second acetone fractionation.
RESULTS

Physiological Characteristics of S. Equinus and S. Bovis Strains

The results of the physiological tests indicated that the bacteria under study were indeed S. equinus and S. bovis as described by Deibel (1964) and Smith and Shattock (1962).

All of the strains were catalase negative, did not grow in 0.1% methylene blue, at 50°C, on 0.04% potassium tellurite agar, at pH 9.6, in the presence of 6.5% NaCl or at 10°C. None was positive for tyrosine decarboxylase or arginine hydrolysis. All strains hydrolyzed aesculin. Only S. bovis 15352 did not grow on 40% bile. All strains grew at 45°C except S. equinus 1091. All strains were α-hemolytic on horse blood except S. bovis 9809 which was γ-hemolytic. S. equinus strains 1090 and 1091 and S. bovis strain S2 produced small white colonies on tetrazolium-glucose agar; S. bovis 9809 reduced tetrazolium and S. bovis 15351 and 15352 did not grow on tetrazolium-glucose agar. Only S. bovis 9809 did not survive 60°C for 30 min. All strains produced slight acid in litmus milk except S. equinus 1090 which produced no change and S. bovis 9809 which formed an acid curd. Only S. equinus 1091 reduced litmus. S. bovis S2 grew well in Rogosa agar tubes;
S. equinus 1091 and S. bovis 9809 did not grow; the remainder of the strains grew poorly.

All strains fermented dextrose, D-mannose, sucrose, and maltose. None of the strains fermented melizitose, D-xylose, or glycerol. The S. equinus strains did not ferment lactose or inulin. Fermentation of the other carbohydrates differed between strains (Table 3).

Preliminary Experiments on Agar Plates

Preliminary experiments

Effect of media and dextrose concentration on amylase and transglucosylase production The results of the experiment to determine the effect of different media and dextrose concentrations on amylase and transglucosylase production are given in Table 4. The four S. bovis strains grew whereas the seven S. equinus strains did not grow on nutrient agar plates containing 0.2% soluble starch. However, all S. equinus strains grew on TYE agar. All seven S. equinus strains grew on nutrient agar-starch plates containing 0.2% or 1.6% dextrose, but none produced a zone of starch hydrolysis on this medium. This was also the case for all strains on BHI plus 1.6% dextrose except for S. equinus 1090 which produced a zone of 12 mm. All of the S. equinus strains produced a zone of starch hydrolysis on BHI-starch agar, which contains 0.2% dextrose. Strain 1090
Table 3. Differences in carbohydrate fermentation patterns of strains of *S. equinus* and *S. bovis*

<table>
<thead>
<tr>
<th>Streptococcus culture</th>
<th>D(+)Galactose</th>
<th>Salicin</th>
<th>D-Mannitol</th>
<th>D(+)Melibiose</th>
<th>L-Arabinose</th>
<th>Sorbitol</th>
<th>Raffinose</th>
<th>Inulin</th>
<th>Trehalose</th>
<th>Fruuctose</th>
<th>D(+)Lactose</th>
<th>Dextrose</th>
<th>D-Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. equinus:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCDO 1090</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
<td>2.66</td>
<td>2.33</td>
</tr>
<tr>
<td>NCDO 1091</td>
<td>+</td>
<td>s+</td>
<td>s+</td>
<td>s+</td>
<td>s+</td>
<td>s+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
<td>3.56</td>
<td>3.31</td>
</tr>
<tr>
<td><strong>S. bovis:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 9809</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>s+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>3.14</td>
<td>3.21</td>
<td>2.93</td>
</tr>
<tr>
<td>ATCC 15351</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>2.84</td>
<td>2.78</td>
<td>2.76</td>
</tr>
<tr>
<td>ATCC 15352</td>
<td>s+</td>
<td>+</td>
<td>+</td>
<td>s+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>2.52</td>
<td>2.77</td>
<td>2.64</td>
</tr>
<tr>
<td>S2</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.84</td>
<td>3.22</td>
<td>2.81</td>
</tr>
</tbody>
</table>

*Change in pH*\(^a\) *(pH units)*

\(^a\)Final pH subtracted from the initial pH; the initial pH was 7.92 for D(+)lactose, 7.91 for dextrose, and 7.87 for D-mannose.

\(^b\)s+ = Ferments carbohydrate very slowly.
Table 4. Effect of type of medium and dextrose concentration on amylase and transglucosylase production by *S. bovis* and *S. equinus* strains

<table>
<thead>
<tr>
<th>Streptococcus culture</th>
<th>TYE</th>
<th>Nutrient (0.2% dextrose)</th>
<th>Nutrient (1.6% dextrose)</th>
<th>BHI (0.2% dextrose)</th>
<th>BHI (1.6% dextrose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Width of zone on starch agar plate (mm)</td>
<td>Dextrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No dextrose</td>
<td>Dextrose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. bovis:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>38</td>
<td>28</td>
<td>17</td>
<td>5</td>
<td>36</td>
</tr>
<tr>
<td>ATCC 9809</td>
<td>34</td>
<td>26</td>
<td>nz,g,</td>
<td>nz,g,</td>
<td>27</td>
</tr>
<tr>
<td>ATCC 15351</td>
<td>nt</td>
<td>18</td>
<td>11</td>
<td>s</td>
<td>32</td>
</tr>
<tr>
<td>ATCC 15352</td>
<td>nt</td>
<td>15</td>
<td>12</td>
<td>5</td>
<td>34</td>
</tr>
<tr>
<td>S. equinus:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCDO 1090</td>
<td>14</td>
<td>ng</td>
<td>nz,g</td>
<td>nz,g</td>
<td>18</td>
</tr>
<tr>
<td>NCDO 1091</td>
<td>nz,g</td>
<td>ng</td>
<td>nz,g</td>
<td>nz,g</td>
<td>10,t</td>
</tr>
<tr>
<td>T1</td>
<td>6</td>
<td>ng</td>
<td>nz,g</td>
<td>nz,g</td>
<td>13</td>
</tr>
<tr>
<td>T2</td>
<td>4</td>
<td>ng</td>
<td>nz,g</td>
<td>nz,g</td>
<td>10</td>
</tr>
<tr>
<td>G2</td>
<td>10</td>
<td>ng</td>
<td>nz,g</td>
<td>nz,g</td>
<td>11</td>
</tr>
<tr>
<td>C1</td>
<td>8</td>
<td>ng</td>
<td>nz,g</td>
<td>nz,g</td>
<td>10</td>
</tr>
<tr>
<td>ATCC 9812</td>
<td>6</td>
<td>ng</td>
<td>nz,g</td>
<td>nz,g</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\)Symbols: nt -- not tested; nz -- no zone; g -- growth; ng -- no growth; s -- some zone; t -- transglucosylase.
produced a zone about twice the size of the others. When TYE with no dextrose was used, all *S. equinus* strains produced a zone except strain 1091. This evidence suggested that, of the cultures examined, only strain 1091 produced only an extracellular transglucosylase. This evidence does not indicate that the other strains do not produce a transglucosylase in addition to amylase. Since *S. equinus* 1090 produced the largest zones of starch hydrolysis on starch plates and since *S. equinus* 1091 seemed to produce only a transglucosylase, these two strains were used in further experiments.

Under certain conditions, *S. bovis* and *S. equinus* strains, including *S. equinus* 1091 produced giant colony variants (two to three times normal size) on BHI starch agar plates and not on TYE starch agar plates. More giant colonies appeared on the BHI agar plates when more dextrose was added to the BHI agar medium. On BHI agar plates, sometimes, a few giant colonies would appear on the plates during the first 24 hr of incubation; more giant colonies appeared with longer incubation times. When the giant colonies were picked and streaked, both normal and giant colonies appeared. Pure variants of normal or giant colonies could not be obtained. Therefore, if purity checks were done on BHI starch agar plates, the appearance of giant colonies was not necessarily an indication of contamination.
With all strains tested, increased dextrose concentration resulted in a decrease in the size of the zone of hydrolysis or a complete repression of amylolytic activity. Different strains of *S. bovis* differed greatly in their susceptibility to repression of amylase production by increased dextrose concentrations. Of the seven *S. equinus* strains tested, only one (strain 1090) was not completely repressed by 1.6% dextrose.

**Assay of *S. equinus* 1090 for amylase and transglucosylase production** The results of the assays of *S. equinus* 1090 for amylase and transglucosylase production are shown in Table 5. These results show that strain 1090 produced much more amylase than transglucosylase. Also more transglucosylase was produced with a longer fermentation time.

**Temperature optimum** The zone of transglucosylase activity with *S. equinus* 1091 after four days of growth on a BHI starch plate was much larger at a growth temperature of 37 C than at 30 C.

**Effect of brand of soluble starch on transglucosylase production** The results showed that if soluble starch — according to Lintner — (Merck) was used, *S. equinus* 1091 produced a zone when 0.2% dextrose was used in a TYE medium. However, if soluble starch (Mallinckrodt) was used, no zone was produced. Thus, only soluble starch — according to Lintner — was used for future experiments.
Table 5. Assay of *S. equinus* 1090 for amylase and transglucosylase

<table>
<thead>
<tr>
<th>Length of fermentation (hr)</th>
<th>Age of digest (hr)</th>
<th>Reduction in blue value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amylase plus transglucosylase</td>
</tr>
<tr>
<td>26.0</td>
<td>3</td>
<td>4.29</td>
</tr>
<tr>
<td>26.0</td>
<td>16</td>
<td>13.10</td>
</tr>
<tr>
<td>65.5</td>
<td>16</td>
<td>11.10</td>
</tr>
</tbody>
</table>

Effect of concentration of dextrose or maltose on size of amylolytic zone

The results of the experiment to determine the effect of the concentration of dextrose or maltose on the sizes of the amylolytic zones on starch-agar plates are shown in Fig. 8. *S. bovis* S2 was used in this experiment for comparison purposes. With *S. bovis* S2, the width of the zone was inversely proportional to the \( \log_{10} \) of the concentration of dextrose over the range of 0 to 2.0% dextrose. When no dextrose was present, the zone was 17 mm; when 2.0% dextrose was present, the zone was 9 mm. The response of *S. bovis* S2 to maltose was quite different; all zones were approximately 16 mm for concentrations of maltose between 0 and 0.2%; a zone size of 13.5 mm was obtained when the maltose concentration was increased to 2.0%. With
Fig. 8. Effect of the concentration of dextrose or maltose in starch-agar plates on the width of the zone of hydrolysis for *S. bovis* S2, *S. equinus* 1090, and *S. equinus* 1091.
MALTOSE – S. bovis S2

DEXTROSE – S. bovis S2

DEXTROSE AND MALTOSE – S. equinus 1090

MALTOSE – S. equinus 1091

DEXTROSE – S. equinus 1091

WIDTH OF ZONE OF HYDROLYSIS (mm)

CONCENTRATION OF DEXTROSE OR MALTOSE (%)
S. equinus 1090 the zone was 8.5 mm at all concentrations of maltose and dextrose from zero to 2.0%. The response of S. equinus 1091 was quite different. The zone increased in size from zero to 8 mm as the concentration of the dextrose increased from zero to 0.15%, then it decreased from 8 mm to zero mm as the dextrose concentration increased from 0.15% to 2.0%. Thus, a small amount (up to 0.15%) of dextrose was needed to act as an acceptor for transglucosylase activity. But if an excess amount of dextrose was present, the synthesis of the transglucosylase was repressed. In contrast to the action of dextrose, the maltose effect was very different. With no maltose, there was no zone even though there was growth; however, zones of 8 to 9 mm were obtained at maltose concentrations of 0.01 to 2.0%. It appeared that maltose was inducing the transglucosylase and also was acting as an acceptor molecule.

**Kinetics of transglucosylase production by S. equinus 1091 on agar plates** The results of the experiment to determine the kinetics of transglucosylase production by S. equinus 1091 on agar plates are summarized in Table 6. In this experiment, a starch-agar basal medium was supplemented with 0.15% of various carbohydrates and the plates were inoculated with S. equinus 1091. After 48 hr of incubation, the plates were stained with Gram's iodine. Zones of starch hydrolysis were noted only on plates that contained added
Table 6. Kinetics of transglucosylase production by *S. equinus* 1091 on agar plates

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>48 hr and 76 hr zone with starch</th>
<th>Chloramphenicol Dextrose overlay</th>
<th>Maltose overlay</th>
<th>Control no Chloramphenicol (Maltose overlay)</th>
<th>Day 5 zone with starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salicin</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Salicin +</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td></td>
<td>10 12</td>
</tr>
<tr>
<td>Mannose +</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Sucrose +</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>8 12</td>
</tr>
<tr>
<td>Raffinose +</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td></td>
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<tr>
<td>Fructose</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Sorbitol +</td>
<td>+</td>
<td>++</td>
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<td></td>
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</tr>
<tr>
<td>Maltose</td>
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<tr>
<td>Maltose +</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Dextrose</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td></td>
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</tr>
<tr>
<td>Dextrose +</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellobiose</td>
<td>-</td>
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<td>++</td>
<td></td>
<td>7 13</td>
</tr>
<tr>
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</tr>
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<td>None</td>
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<td>+</td>
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<td></td>
<td>10</td>
</tr>
<tr>
<td>None + starch</td>
<td>-</td>
<td>+</td>
<td>some</td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>
sorbitol (sorbitol was probably oxidized to glucose since _S. equinus_ I09I ferments sorbitol), maltose, or dextrose. After 76 hr of growth, an overlay mixture (starch agar basal -- dextrose or maltose acceptor molecule -- with added chloramphenicol was poured on a set of plates and the plates were incubated at 37 C for 12 hr. When the plates were flooded with iodine, zones of starch degradation were observed with both dextrose and maltose as acceptor molecules and on both starch and non-starch growth plates containing salicin, sucrose, raffinose, sorbitol, maltose (by far the largest zone), dextrose, cellobiose, and also on the plate that had no carbohydrate and no starch added to the underlay (control). This experiment proved that a low level of transglucosylase was produced constitutively, that maltose greatly enhanced the production of transglucosylase, that sorbitol (probably oxidized to glucose) was an acceptor molecule for the transglucosylase, that the enzyme was produced when the following carbohydrates at a concentration of 0.15% were autoclaved with the medium: salicin, sucrose, raffinose, sorbitol, maltose, dextrose, cellobiose; and that the enzyme was not produced when the following carbohydrates at a concentration of 0.15% were autoclaved with the medium: mannose and fructose.

After four days of growth, the following carbohydrate-starch plates showed a zone without any overlay: salicin, raffinose, and cellobiose. The following did not show any zone without any overlay at four days: mannose, sucrose,
and fructose. This means that autoclaved mannose, and fructose permanently repressed the synthesis of transglucosylase. However, when a maltose overlay without chloramphenicol was used as a control, a large zone was produced with all of the carbohydrates except mannose and sucrose, and new colonies started growing and producing zones at a distance from the center streak. Evidently something in the autoclaved mannose and sucrose media prevented the synthesis of transglucosylase. This was in contrast to the case with fructose, for whereas autoclaved fructose prevented the synthesis of transglucosylase, it did not prevent the synthesis of transglucosylase after the maltose overlay without chloramphenicol was added.

Relative efficiencies of various carbohydrates as acceptors for transglucosylase When yeast extract was omitted from the growth medium, a carbohydrate other than starch was necessary for cell multiplication. After nine hr of incubation, the medium containing maltose and starch had a zone of five mm of starch hydrolysis whereas the medium containing sucrose and starch had none. After 21 hr of incubation, however, the medium with maltose and starch had a zone of 10 mm and the medium with sucrose and starch had a zone of six mm. Thus, filter sterilized sucrose can and autoclaved sucrose can not (Table 6) serve as acceptor molecule. Therefore, the ability of sucrose as an accepting molecule was tested along with dextrose, maltose, and sorbitol. The higher
the temperature at which the transglucosylase was allowed to react, the larger were the zones of hydrolysis. The activity was quite low at 20°C. Dextrose, maltose, sorbitol, and sucrose served as acceptor molecules for the transglucosylase produced by S. equinus 1091. The overlay mixture with no carbohydrate in addition to starch produced no zone after 2 hr of incubation. When the activity of the transglucosylase produced on the maltose plus starch medium after 21 hr of growth was checked at 30°C for 5 hr, there was a difference in the size of the zone produced with the different carbohydrate acceptors. The rank in order of decreasing zone size was dextrose, sorbitol, maltose, sucrose. However, the rank was as follows when the medium contained sucrose plus starch: sorbitol, maltose, sucrose, and dextrose. This suggested that there might be a sucrase present when sucrose is the carbon source. This sucrase might have a higher affinity for the dextrose than the transglucosylase.

When the medium that contained maltose and no starch was assayed for transglucosylase activity, the same pattern was observed as that described above for the maltose plus starch. This proves that it is not necessary to have starch present in the growth medium to obtain production of the transglucosylase. The control overlay mixture resulted in no zone if the duration of the assay was short (2-3 hr, depending on the
amount of enzyme present). If the assay was read early, the overlays with dextrose, maltose, sorbitol, or sucrose resulted in good zones and the overlay with no carbohydrate in addition to starch resulted in no zone. However, since the growth medium contained maltose or sucrose, if the assay was read late (10 hr) the overlays with no carbohydrate had a zone as large as the overlays with carbohydrate -- this could also be due to the late production of amylase. At 14 hr, all of the carbohydrate overlays and the non-carbohydrate overlays (controls) had the same size zones. This was probably due to the maltose diffusing into the area occupied by the transglucosylase or due to late production of amylase.

Factors Affecting Production of Amylase and Transglucosylase in Broth by _S. Equinus_ 1091

Effect of calcium carbonate and medium

The best growth (lowest pH values) was obtained in the TYE medium. No transglucosylase activity was obtained with the three media that contained CaCO$_3$; however, both amylase and transglucosylase activities were observed in the cell-free filtrates when CaCO$_3$ was omitted from the media and the pH was maintained between 6.0 and 7.0. The best maltose concentration of those examined for both amylase and transglucosylase production was 1.0% (Table 7).
Table 7. Effect of concentration of maltose on absolute and relative quantities of amylase and transglucosylase produced by *S. equinus* 1091

<table>
<thead>
<tr>
<th>Concentration of maltose (%)</th>
<th>Blue value reduction in 7.5 hr(%)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (amylase plus transglucosylase)</td>
<td>Transglucosylase</td>
</tr>
<tr>
<td>1</td>
<td>60.5</td>
<td>17.0</td>
</tr>
<tr>
<td>3</td>
<td>20.9</td>
<td>9.9</td>
</tr>
<tr>
<td>5</td>
<td>17.9</td>
<td>9.1</td>
</tr>
</tbody>
</table>

The results in Table 7 show that there was a differential induction of amylase and transglucosylase that was a function of the concentration of maltose in the medium. The lower the concentration of maltose, the lower was the percent amylase and higher the percent transglucosylase.

Effect of age of inoculum

The results (Table 8) show that more transglucosylase was produced when the age of the inoculum was 17.5 hr than when the inoculum was 13.0 or 24.0 hr old. Also, the relative amounts of amylase and transglucosylase produced were a function of the age of the inoculum. The younger the age of the inoculum, the higher was the percent of amylase and the lower the percent of transglucosylase.
Table 8. Effect of age of BHI broth inoculum on absolute and relative quantities of amylase and transglucosylase produced by S. equinus 1091

<table>
<thead>
<tr>
<th>Age of inoculum (hr)</th>
<th>Blue value reduction in 1 hr (%)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total (amylase plus transgluco-</td>
<td>Transglu-</td>
</tr>
<tr>
<td></td>
<td>cosylase)</td>
<td>Amylase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cosylase</td>
</tr>
<tr>
<td>13.0</td>
<td>10.50</td>
<td>2.64</td>
</tr>
<tr>
<td>17.5</td>
<td>11.79</td>
<td>2.64</td>
</tr>
<tr>
<td>24.0</td>
<td>7.18</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Effect of concentration of maltose

The results are shown in Table 9. More transglucosylase was produced when the maltose concentration was 1.5% than when higher or lower levels of maltose were added to a basal medium.

Effect of length of fermentation.

The results are shown in Fig. 9. Amylase and transglucosylase were produced throughout the logarithmic growth phase; quantities of both enzymes decreased during the stationary phase of bacterial growth. The optimum harvest time for transglucosylase was at the end of the logarithmic phase; amylase activity seemed to reach a peak slightly earlier. When maximum growth was attained, acid production ceased and no additional 1 N NaOH had to be added. It was found that
Table 9. Effect of concentration of maltose on the quantity of transglucosylase produced by *S. equinus* 1091

<table>
<thead>
<tr>
<th>Concentration of maltose (%)</th>
<th>Length of fermentation (hr)</th>
<th>Transglucosylase activity (blue value reduction in 45 min) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>13</td>
<td>3.0</td>
</tr>
<tr>
<td>1.5</td>
<td>16</td>
<td>15.4</td>
</tr>
<tr>
<td>2.0</td>
<td>16</td>
<td>4.4</td>
</tr>
<tr>
<td>3.0</td>
<td>17</td>
<td>4.2</td>
</tr>
<tr>
<td>4.0</td>
<td>19</td>
<td>10.1</td>
</tr>
</tbody>
</table>

when 1 N NaOH was not added, the maximum optical density was only 1.4 and there was no amylase or transglucosylase activity at the end of the logarithmic phase of growth.

Purification of Amylase and Transglucosylase

Effect of calcium chloride on amylase and transglucosylase assays

When CaCl$_2$ was present in the digest and in the diluent, the amylase activity was increased six-fold and the transglucosylase activity was not changed. Therefore, 0.01 M CaCl$_2$ was used in the starch substrate solutions and in the diluent buffers for amylase assays. CaCl$_2$ was omitted from the transglucosylase assays because, if CaCl$_2$ was present, the
Fig. 9. Effect of length of fermentation on amylase and transglucosylase production
the amylase activity in solutions that contained both enzymes was increased to such an extent that it masked the transglucosylase activity.

**Chromatography on DEAE-cellulose**

The results are shown in Fig. 10. The transglucosylase was eluted with 0.01-0.02 M and the amylase with 0.07-0.09 M Tris-HCl buffer. The amylase and transglucosylase fractions in Tris-HCl buffer became completely inactivated after 5 days of storage at 5°C.

**Acetone fractionation**

When the fractions were collected after 20, 35, 46, 56, 66, 75 and 82% acetone had been added (first acetone fractionation), almost all of the amylase and transglucosylase were in the fractions from 0-50% acetone with peaks at 35% (data not shown).

The results of the first acetone fractionation of another preparation — when the fractions were collected after 20, 26, 30, 35, 40, 45, and 50% acetone had been added — are shown in Fig. 11. The 0-20% acetone fraction was almost free of transglucosylase and the 26-30% acetone fraction was rich in transglucosylase.
Fig. 10. Separation of extracellular amylase and transglucosylase of *S. equinus* 1091 on DEAE-cellulose. All conditions were as stated in the Materials and Methods section. Fractions 10, 21, 33, and 39 (peaks A, B, C, and D) were assayed for amylase and transglucosylase by determining the % reduction in the blue value of amylase in 10 hr. The activities (% reduction in blue value) were (A) amylase -- none, transglucosylase -- 10.7%; (B) amylase -- 0.8%, Transglucosylase -- 2.4%; (C) amylase -- 0.3%, transglucosylase -- 5.1%; and (D) amylase -- 14.8%, transglucosylase -- none.
Fig. 11. Acetone fractionation of the extracellular amylase and transglucosylase of *S. equinus*.
The results of the purification procedures -- described in the "Acetone fractionation" section of Materials and Methods -- are summarized in Tables 10 and 11.

Characterization of Amylase and Transglucosylase

**Optimum pH**

The results of the effect of pH on amylase and transglucosylase activities and the effect of CaCl\(_2\) on the pH optimum for amylase are shown in Fig. 12. These data were obtained using amylose as the substrate. When starch was used as the substrate for the amylase in the presence of CaCl\(_2\), the results were identical to those shown in Fig. 12. The pH optimum for amylase in the presence of CaCl\(_2\) is 7.0. The pH optima for amylase in the absence of CaCl\(_2\) are 4.75 and 8.0. The pH optimum for transglucosylase is 6.0.

**Optimum temperature**

The effect of temperature on amylase and transglucosylase activities is shown in Fig. 13. The optimum temperature for amylase at pH 7.0 was 38 C. The optimum temperature for transglucosylase at pH 6.5 was 37 C.

The amylase temperature curve was sharp and steep from the low to the high temperatures when the dextrinogenic assay
Table 10. Purification summary of the extracellular amylase of *S. equinus* 1091

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol (ml)</th>
<th>(mg/ml)</th>
<th>Total mg</th>
<th>(u/ml)</th>
<th>Total units</th>
<th>Specific activity (u/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free liquor</td>
<td>7,553</td>
<td>6.10</td>
<td>46,100</td>
<td>(30.5)</td>
<td>230,000</td>
<td>(5.0)</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>230</td>
<td>14.75</td>
<td>3,400</td>
<td>170</td>
<td>39,100</td>
<td>11.5</td>
<td>17.0</td>
<td>2.3</td>
</tr>
<tr>
<td>CaCl₂ Dialysis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>124</td>
<td>5.78</td>
<td>1,650</td>
<td>120</td>
<td>34,200</td>
<td>20.8</td>
<td>14.9</td>
<td>4.2</td>
</tr>
<tr>
<td>First acetone ppt, 20-35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
<td>6.30</td>
<td>188</td>
<td>671</td>
<td>20,100</td>
<td>107.0</td>
<td>8.8</td>
<td>21.3</td>
</tr>
<tr>
<td>Second acetone ppt, 0-20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2</td>
<td>6.79</td>
<td>31 1,630</td>
<td>7,510</td>
<td>240.0</td>
<td>3.3</td>
<td>48.0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Since the cell-free liquor contained maltose, the dextrinogenic assay could not be used due to the presence of transglucosylase; therefore, an arbitrary value of 5 was used for the specific activity of the amylase because the transglucosylase had a specific activity of 4.6 (see Table 11).

<sup>b</sup>Only 100 ml of the 230 ml of liquor were dialyzed; therefore, a multiplication factor of 2.3 was used to calculate the total protein and total units.

<sup>c</sup>Most of the loss occurred during storage of enzyme solution between steps.
Table 11. Purification summary of the extracellular transglucosylase of *S. equinus* 1091

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total mg</th>
<th>Amylase</th>
<th>Specific activity (u/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free liquor</td>
<td>7,553</td>
<td>6.10</td>
<td>46,100</td>
<td>28</td>
<td>211,000</td>
<td>4.6</td>
<td>100</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>230</td>
<td>14.75</td>
<td>3,400</td>
<td>411</td>
<td>94,500</td>
<td>27.9</td>
<td>44.7</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt; Dialysis</td>
<td>124</td>
<td>5.78</td>
<td>1,650</td>
<td>106</td>
<td>30,200</td>
<td>18.3</td>
<td>14.6</td>
</tr>
<tr>
<td>First acetone ppt, 20-35%</td>
<td>13</td>
<td>6.30</td>
<td>188</td>
<td>975</td>
<td>29,200</td>
<td>155.0</td>
<td>13.8</td>
</tr>
<tr>
<td>Second acetone ppt, 20-60%</td>
<td>7.5</td>
<td>9.49</td>
<td>164</td>
<td>1200</td>
<td>20,800</td>
<td>127.0</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*a* Half of the transglucosylase activity was lost before dialysis while the liquor was stored for one month at 5 C.

*b* Only 100 ml of the 230 ml of liquor were dialyzed; therefore, a multiplication factor of 2.3 was used to calculate the total protein and total units.

*c* Most of the loss occurred during storage of enzyme solution between steps.
Fig. 12. Effect of pH on amylase and transglucosylase activities and effect of CaCl₂ on the pH optimum for amylase. The percent reduction in amylose blue value in 15 min at 37 C was determined.
The graph illustrates the activity (as % reduction in blue value) of TRANSGLUCOSYLASE and AMYLASE with and without CaCl2. The pH range is from 3 to 10. The graph shows distinct peaks for each condition, indicating the optimal pH ranges for the enzymes with and without the presence of CaCl2.
Fig. 13. Effect of temperature on amylase activity at pH 7.0 and transglucosylase activity at pH 6.5. Also effect of method of assay (blue value -- BV vs. reducing value -- RV) on the shape of the amylase temperature curve.
was used; however, when the saccharogenic assay was used the curve was relatively flattened at the low and high temperatures.

**Activities of various carbohydrates as acceptors for transglucosylase**

The results obtained with *S. equinus* are compared in Table 12 to the results that Walker (1965b) obtained for the intracellular transglucosylase of *S. bovis*. With *S. equinus* 1091 transglucosylase, the efficiency of a given carbohydrate as an acceptor decreased as the degree of similarity to dextrose decreased. D-mannose and L-sorbose are monosaccharides. L-sorbose looks almost as much like dextrose as does D-mannose. Maltose, sucrose, and α-trehalose are disaccharides. Sorbitol differs from dextrose in that it has a hydroxyl instead of an aldehyde group on the C1 carbon atom.

**Effect of wheat α-amylase inhibitor on amylase and transglucosylase**

The results of the experiment to determine the effect of wheat α-amylase inhibitor on amylase and transglucosylase activities are shown in Table 13. The amylase activity was inhibited by the α-amylase inhibitor and the transglucosylase activity was not inhibited.

**Characterization of Amylase Free of Transglucosylase**

**Calcium and chloride ion requirements**

The results of the experiment to determine the effect of calcium and chloride ions on amylase activity are given in Table 14. Both calcium and chloride ions are necessary for
Table 12. Acceptor activity of carbohydrates that will replace dextrose as acceptor of glucosyl residues in the reaction between amylose and transglucosylase

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Activity (%)</th>
<th>Extracellular transglucosylase of S. equinus 1091 (10 min reaction)</th>
<th>Intracellular transglucosylase of S. bovis (4 hr reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>63</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>L-Sorbose</td>
<td>50</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>30</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>29</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>αα-Trehalose</td>
<td>14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)(Walker, 1965b)

Table 13. Effect of wheat α-amylase inhibitor on amylase and transglucosylase

<table>
<thead>
<tr>
<th>Addition of α-amylase inhibitor</th>
<th>Reduction in blue value (%)</th>
<th>Amylase</th>
<th>Transglucosylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>15.2</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12.4</td>
<td>9.6</td>
<td></td>
</tr>
</tbody>
</table>
Table 14. Effect of calcium and chloride ions on amylase activity

<table>
<thead>
<tr>
<th>Ion</th>
<th>Activity (%)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca acetate + NaCl</td>
<td>100.0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>98.4</td>
</tr>
<tr>
<td>CaCl₂ + EDTA</td>
<td>98.4</td>
</tr>
<tr>
<td>NaCl</td>
<td>96.7</td>
</tr>
<tr>
<td>NaCl + EDTA</td>
<td>45.2</td>
</tr>
<tr>
<td>Ca acetate</td>
<td>8.5</td>
</tr>
<tr>
<td>No Ca⁺⁺ or Cl⁻</td>
<td>0</td>
</tr>
</tbody>
</table>

^aCalculated from the percent reduction in amylose blue values in 15 min at 37°C, pH 7.0.

activation of the amylase. The EDTA did not inhibit the amylase when it was used with CaCl₂; therefore, the reduced activity observed when NaCl is used with EDTA is caused by the chelation of the calcium ions by EDTA.

Paper-chromatographic analysis of amylase

The action pattern of the amylase is shown in Fig. 14. The percentages used in the following discussion of the action pattern refer to the percent conversion to maltose. At 5.8% (B), a small quantity of maltose and maltotriose appeared; however, at 10.3% (C), G₅-G₁₁ were all present in small quantities.
Fig. 14. Time sequence chromatographic analysis of *S. equinus* 1091 amylase action on amylose. The conditions of the digest were 0.1% amylose in 0.0155 M β-glycerophosphate buffer, pH 7.0, containing 0.01 M CaCl₂. Aliquots (100 µl) from various digest times were spotted. The chromatogram was obtained by the technique of French et al. (1965) and photographed on a x-ray viewer. (A) G₁-G₁₅ Standards; (B,C,D,E,F,G,H,I,J) — (time -- % conversion to maltose) — (B) 0.5 hr -- 5.8%, (C) 1 hr -- 10.3%, (D) 2 hr -- 17.2%, (E) 5 hr -- 31.7%, (F) 12 hr -- 51.0%, (G) 19 hr -- 60.2%, (H) 35 hr -- 68.5%, (I) 75 hr -- 76.5%, (J) 97 hr
At 17.2% (D), the quantities of G\textsubscript{2}-G\textsubscript{11} all increased but the quantities of G\textsubscript{2} and G\textsubscript{3} increased at a greater rate; G\textsubscript{4} also increased at a faster rate than G\textsubscript{5}-G\textsubscript{11} but at a slower rate than G\textsubscript{2} and G\textsubscript{3}. At 31.7% (E), the quantities of G\textsubscript{2}-G\textsubscript{11} increased but the relative quantities of one to another were the same as at 17.2%. At 51.0% (F), the quantities of G\textsubscript{5}-G\textsubscript{11} decreased slightly but the quantities of G\textsubscript{2}-G\textsubscript{4} increased and a trace of G\textsubscript{1} appeared for the first time. At 60.2% (G), all of the G\textsubscript{5}-G\textsubscript{11} had disappeared and the quantity of G\textsubscript{4} had decreased; the quantities of G\textsubscript{1}-G\textsubscript{3} increased. The 60.2% sample was the only sample that was completely desalted. This was due to the fact that more Amberlite MB-3 was used which also resulted in decreasing the size of the maltose and maltotriose spots; therefore, it is likely that the quantity of G\textsubscript{2} and G\textsubscript{3} increased even though this is not obvious from the chromatogram. With the remaining samples (H, I, J), the quantity of G\textsubscript{4} decreased very slightly and the quantity of G\textsubscript{1} increased very slightly; the quantities of G\textsubscript{2} and G\textsubscript{3} increased slightly. Finally, (J) the predominant products were G\textsubscript{2} and G\textsubscript{3} with more G\textsubscript{2} than G\textsubscript{3}; there are small quantities of G\textsubscript{4} and G\textsubscript{1} present with more G\textsubscript{4} than G\textsubscript{1}. The amylase has a low degree of multiple attack in view of the fact that it produces almost as much high M.W. oligosaccharides as G\textsubscript{2}-G\textsubscript{4} at early hydrolysis times and the quantity of high M.W.
oligosaccharides does not decrease until after the point of 55% conversion to apparent maltose.

**Blue value-reducing value curves**

The curves resulting from plotting the blue value vs. the percent apparent conversion to maltose are shown in Fig. 15. The shape and position of the curve for the pure amylase indicate that the amylase is an α-amylase (Hobson and MacPherson, 1952) and has a low degree of multiple attack (Robyt and French, 1967). With the curve for the non-dialyzed mixture of amylase and transglucosylase, the first part of the curve drops very sharply; this is caused by the presence of maltose in the non-dialyzed material which acts as an acceptor for the glucose carried by the transglucosylase—the transglucosylase activity causes an initial sharp drop in blue value without an increase in reducing value. The small increase in reducing value is due to the amylase. On the other hand, when the material is dialyzed, the maltose is removed and the transglucosylase is not as active; therefore, the curve does not drop as sharply. The initial part of the curve of the partially purified amylase (material from the second acetone fractionation which still contained a small quantity of transglucosylase) dropped more sharply than the initial part of the pure amylase curve but not as sharply as the material that had not been treated with acetone. Thus,
Fig. 15. Comparison of the drop in iodine color (blue value) with the increase in reducing value for the hydrolysis of amylase by (A) pure *S. equinus* 1091 amylase, (B) amylase-transglucosylase mixture with maltose, (C) Dialyzed amylase-transglucosylase mixture (maltose acceptor removed), and (D) amylase with a trace of transglucosylase present (from second acetone ppt)
REDUCING VALUE AS % APPARENT MALTOSE

BLUE VALUE (AT 620nm)

0  4  8  12  16  20  24  28  32  36  40  44  48
0  10  20  30  40  50  60  70  80  90  100
the shape of the curve is an indication of the degree to which the amylase is contaminated with transglucosylase.

The bottom half of the curve is also different when the amylase is contaminated with transglucosylase. The pure amylase curve is lower than and to the left of the curves obtained with transglucosylase contaminated amylase preparations. The greater the quantity of transglucosylase the higher and more shifted to the right is the bottom half of the curve.
DISCUSSION

The results of the carbohydrate fermentation tests indicated that the two strains of *S. equinus* were very different from each other.

*S. equinus* 1091 fermented six more carbohydrates and produced a much greater quantity of acid in dextrose and D-mannose than *S. equinus* 1090. In fact, *S. equinus* 1091 produces a much larger quantity of acid in dextrose and D-mannose than any of the *S. bovis* and *S. equinus* strains examined. Therefore, it is likely that *S. equinus* 1091 (high transglucosylase producer) belongs to that distinct variety of *S. equinus*, mentioned by Duncan (1960), that exists in the human intestine and has a high fermentative power in dextrose broth. Also strain 1091 produced a much greater quantity of transglucosylase but half as much amylase as strain 1090. However, strain 1091 produced as much amylase as the other five strains of *S. equinus* examined. Strain 1090 was the only strain of the seven examined that was not repressed in amylase production by 1.5% or 2.0% dextrose on a tryptone yeast extract agar plate.

*S. equinus* 1091 is much more closely related than *S. equinus* 1090 to *S. bovis*. *S. equinus* 1091 is most closely related to those *S. bovis* strains that belong to Division I (mannitol fermenting and ß-hemolytic). *S. equinus*
S. bovis 1091 is like S. bovis 9809 (Division I) in that they both do not ferment D(+)galactose, do ferment sorbitol and trehalose, do not grow in Rogosa agar tubes and produce the largest quantity of acid in dextrose and D-mannose.

S. bovis 9809 is very closely related to S. bovis 101 (Division I and described in Part I of this thesis). They are alike in that they both are $\beta$-hemolytic, produce acid and clot in litmus milk, ferment D-mannitol, sorbitol, and trehalose, and produce the same final pH in lactose and dextrose broths. They are different in that strain 9809 does not ferment D(+)galactose, produces a higher final pH in D-mannose broth, and reduces tetrazolium. Since S. bovis 9809 is so closely related to S. bovis 101 it probably would have a very similar amylase action pattern.

This study lends support to the view that S. bovis and S. equinus are very closely related but there are still enough differences to justify classifying them as two distinct species.

The fact that S. bovis strains grew and S. equinus strains did not grow on nutrient agar starch plates could be the basis of a new S. bovis-S. equinus differentiation test. Evidently, S. equinus does not produce enough amylase to use starch as a nutrient. However, this does not seem to be the case when S. equinus is grown on TYE agar.

Since S. equinus 1090 produces almost twice as much amylase as the other S. equinus strains examined and is the
only strain the amylase of which is not completely repressed by 1.6% dextrose, its amylase action pattern should be determined. Its cell-free filtrates should be fractionated on DEAE-cellulose columns to discover whether separate amylase and transglucosylase peaks can be obtained.

According to Magasanik (1961), the inhibitory effect of glucose on the synthesis of enzymes whose activity does not contribute to growth contributes to the economy of the cell by restricting the formation of enzymes to those it requires in its particular environment. Of the strains examined in the present study, S. bovis 9809 and S. equinus 1091 exhibited the largest "glucose effect" — exhibited the most efficiency; also S. bovis 9809 and S. equinus 1091 were able to tolerate lower (unfavorable) pH values in dextrose and mannose broths than those S. bovis and S. equinus strains that exhibited less of a "glucose effect" and produced higher pH values in dextrose and mannose broths. Therefore, S. bovis 9809 and S. equinus 1091 are better equipped for survival than the other S. bovis and S. equinus strains examined because they are the most economical (do not synthesize unneeded amylase and transglucosylase when excess glucose is present) and are able to use more glucose as an energy source (tolerate more lactic acid); also they ferment more kinds of carbohydrates.

Calcium carbonate (3.0%) did not prevent the production of amylase by S. bovis strains but it did prevent the production
of amylase and transglucosylase by S. equinus strains. Tsuchiya et al. (1950) reported that, in Aspergillus niger fermentations calcium carbonate lowered the maltase yield at a concentration of 0.05 M and drastically inhibited the production of both α-amylase and maltase at the 0.1 M level.

The following suggestions might be helpful to others working with DEAE-cellulose chromatography of S. equinus enzymes. If Tris-HCl buffer is used, the fractions should be dialyzed to remove the Tris-HCl buffer and then concentrated to prevent enzyme inactivation that occurs when protein is stored in a dilute solution. Also, it might be worthwhile to try using β-glycerophosphate buffer, pH 8.0; the enzymes might be more stable in this buffer than in Tris. The elution might be accomplished with a decreasing pH gradient and an increasing molarity gradient or an increasing molarity gradient alone. It is likely that a high degree of purification of the amylase could be obtained if the 0-20% acetone fraction of the second acetone precipitation was chromatographed on DEAE-cellulose. This would also probably be the case for the transglucosylase if the 20-60% acetone fraction of the second acetone precipitation was chromatographed on DEAE-cellulose. Since more than one of the peaks resulting from DEAE-cellulose chromatography had amylase activity, it is possible that further chromatographic studies on DEAE-cellulose would reveal the presence of S. equinus 1091 α-amylase isozymes.
The pH for optimum activity of *S. equinus* 1091 α-amylase (pH 7.0 at 37 C in the presence of CaCl$_2$) is higher than that for *S. bovis* α-amylase. Various investigators have reported that the pH optimum for activity of *S. bovis* α-amylase is pH 5.8 at 39 C (Walker and Hope, 1964), pH 6.0 at 39 C (Walker, 1965a), and pH 5.5-6.5 at 35 C (Hobson and MacPherson, 1952).

The optimum pH values for *S. equinus* α-amylase in the absence of CaCl$_2$ are 8.0 and 4.75. There is more activity at pH 8.0 than at 4.75. The presence of two pH peaks in the absence of CaCl$_2$ invites further study to elucidate reasons for this phenomenon. Narayanan and Shanmugasundaram (1967) found that the amylase of *Fusarium vasinfectum* has three pH optima: equally high peaks of activity were obtained at pH 4.4 and pH 8.0; a peak of about 1/3 as much activity was found at pH 6.0. The *F. vasinfectum* enzyme was reported not to require calcium ions; the effect of chloride ion was not tested.

The optimum pH for the extracellular transglucosylase of *S. equinus* is 6.0 at 37 C. This is lower than the optimum pH for the intracellular *S. bovis* transglucosylase which is pH 6.8 at 30 C (Walker, 1965b) and the intracellular *S. mitis* transglucosylase which is pH 7.5 at 46 C (Walker, 1966). It is not surprising that the pH optimum of the transglucosylase
of \textit{S. bovis} is closer to that of \textit{S. equinus} than that of \textit{S. mitis} which is a more distantly related species.

The optimum temperature for activity of \textit{S. equinus} 1091 \(\alpha\)-amylase is 38 C at pH 7.0 while that for \textit{S. bovis} \(\alpha\)-amylase is 39 C at pH 5.8 (Walker and Hope, 1964) or 48 C at pH 7.0 for the enzyme from a Streptococcus probably related to \textit{S. bovis} (Hobson and MacPherson, 1952).

The optimum temperature for the extracellular transglucosylase of \textit{S. equinus} 1091 is 37 C at pH 6.5 while that for the intracellular transglucosylase of \textit{S. bovis} is 30 C at pH 7.5 (Walker, 1965b); for \textit{S. mitis} it is 46 C at pH 6.4 (Walker, 1966).

The activities of various carbohydrates as acceptors for the transglucosylases of \textit{S. equinus} 1091 and \textit{S. bovis} (Walker, 1965b) are compared in Table 12. This comparison shows that these two transglucosylases differ in several respects; however, a more accurate comparison could be made if the same units of activity had been used. The results of the starch agar plate method for testing the relative efficiencies of various carbohydrates as acceptors agreed with the tube test method except for sorbitol. Since sorbitol had no acceptor activity in the tube test, the apparent activity on the starch agar plates might have been due to the oxidation of sorbitol by \textit{S. equinus} 1091 (which can ferment sorbitol) to produce dextrose; the dextrose, in turn, acted as an acceptor. Dextrose,
D-mannose, L-sorbose, maltose, sucrose, and α- trehalose are effective acceptors for D-enzyme, the intracellular transglucosylase of *S. bovis* (Walker, 1965b), and the extracellular transglucosylase of *S. equinus* 1091; however, of these carbohydrates, only dextrose and D-mannose are effective acceptors for the intracellular amylomaltase of *Escherichia coli* (Wiesmeyer and Cohn, 1960).

The transglucosylase of *S. equinus* 1091 should be purified so that its action pattern can be determined and compared to that of *S. bovis* transglucosylase (Walker, 1965b), *S. mitis* transglucosylase (Walker, 1966), amylomaltase (Wiesmeyer and Cohn, 1960), and D-enzyme (Walker and Whelan, 1957).

Erickson\(^1\) found that 1-15 appear in the cell-free liquor of *S. equinus* 1091 when it is grown for 16, 24, and 48 hr at 37 C in TYE medium containing 1.5% maltose; this medium was used in this study for the production of amylase and transglucosylase. If the extracellular transglucosylase of *S. equinus* 1091 synthesizes 1-15 extracellularly in maltose broth, it apparently can use maltose as a donor substrate as well as an acceptor; however, this needs further study for

\(^1\)Erickson, A. A., Department of Bacteriology, Iowa State University, Ames, Iowa 50010. Paper chromatographic analysis of the cell-free liquor resulting from the growth of *S. equinus* 1091 in TYE medium with 1.5% maltose. Private communication. 1968.
proof. If this is true, the extracellular transglucosylase of \textit{S. equinus} 1091 is different from the intracellular transglucosylase of \textit{S. bovis} in that the latter cannot use maltose as a donor substrate (Walker, 1965b). Thus, the extracellular transglucosylase of \textit{S. equinus} 1091 may be responsible for the storage of glucose as extracellular oligosaccharides. Oligosaccharides appear in the medium when the cells are grown in the presence of maltose which along with maltotriose is a predominant final product of the hydrolysis of amylose by the \( \alpha \)-amylase of \textit{S. equinus} 1091. As mentioned before, the fact that \textit{S. equinus} 1091 produces a lower pH in dextrose broth than any of the other \textit{S. equinus} or \textit{S. bovis} strains examined, indicates that it is a distinct variety of \textit{S. equinus} that exists in the human intestine. If bacteria resembling \textit{S. equinus} 1091 exist in the human intestine, one of their roles in the ecology of the intestine might be to store as extracellular polysaccharide the maltose as well as the \( G_1 \), \( G_2 \), and \( G_4 \) that are produced by their own \( \alpha \)-amylases and amylases elaborated by other bacteria. If the extracellular transglucosylase of \textit{S. equinus} 1091 is similar in mechanism of action to the intracellular transglucosylase of \textit{S. bovis} (Walker, 1965b), it degrades starch by transferring glucosyl residues (including glucose) from the non-reducing end of the amylose or amylopectin molecule to the non-reducing end of glucose or maltose. Walker (1965b) found that the intracellular
transglucosylase of *S. bovis* could utilize the main product of its α-amylase action, maltotriose, as a substrate for the synthesis of oligosaccharides. Also, Walker (1965b) believed that the production of intracellular iodophilic storage polysaccharide was mediated by this transglucosylase because it was capable of synthesizing from maltotriose maltodextrins of sufficiently long chain length to stain with iodine, provided that glucose was removed by means of a glucose trap.

Dunican and Seeley (1962) found that most of their strains of *S. equinus* synthesized an internal starch-like polysaccharide when the organisms were grown on maltose or on a medium containing starch and a small amount of fermentable monosaccharide. Therefore, *S. equinus* 1091 might also have an intracellular polysaccharide-synthesizing enzyme similar to the amylomaltase of *E. coli* (Monod and Torriani, 1950), *S. pyogenes* (Crowley, 1955) or group D α-hemolytic streptococci (Hobson and Mann, 1955). On the other hand, *S. equinus* 1091 might have an intracellular polysaccharide synthesizing transglucosylase like that of *S. bovis* (Walker, 1965b) or *S. mitis* (Walker, 1966).

Hobson and MacPherson (1952) found that *S. bovis* amylase was not activated by calcium or chloride ions but that dilute solutions were stabilized by small concentrations of calcium ions. It was shown in the present study that *S. equinus* 1091
α-amylase requires both calcium and chloride ions; when CaCl$_2$ is absent the pH optima are 4.75 and 8.0 whereas when CaCl$_2$ is present the pH optimum is 7.0.

Militzer et al. (1946) reported that wheat α-amylase inhibitor inhibits salivary, pancreatic and certain bacterial amylases whereas malt amylase and takadiastase were not inhibited. Evidently _S. equinus_ 1091 α-amylase belongs to the group of bacterial amylases that is inhibited by wheat α-amylase inhibitor. The inhibition obtained in my studies was not great, but probably would have been more pronounced had the enzyme-inhibitor mixture been incubated for a longer time before enzyme assays were performed. This experiment was not repeated, so the results obtained can be considered to be only preliminary. It is interesting that salivary, pancreatic and _S. equinus_ 1091 α-amylases all require chloride ions and all are apparently inhibited by wheat α-amylase inhibitor. According to Militzer et al. (1946) wheat α-amylase inhibitor acts in a non-competitive manner toward salivary α-amylase in the presence of starch, and since the reaction is non-competitive it follows that certain α-amylases have groupings which can combine with the inhibitor while others do not.

The action pattern of the α-amylase of _S. equinus_ 1091 was unlike that of any of the _S. bovis_ amylases examined. The products produced by _S. bovis_ 101 α-amylase from amylase, in
order of decreasing quantities, were \( G_3, G_2, \) and \( G_1 \) whereas with \( S. \) equinus 1091 \( \alpha \)-amylase, they were \( G_2, G_3, G_4, \) and \( G_1 \). The \( \alpha \)-amylase action pattern of \( S. \) equinus 1091 indicates that it has a lower degree of multiple attack than is the case for \( S. \) bovis \( \alpha \)-amylases. \( S. \) bovis \( \alpha \)-amylases produce greater quantities of \( G_1-G_4 \) at early hydrolysis times, whereas \( S. \) equinus 1091 \( \alpha \)-amylase produces more higher M.W. oligosaccharides at early hydrolysis times. These \( C_5-C_{12} \) oligosaccharides increase in quantity as hydrolysis proceeds and do not begin to decrease until the point of 55% conversion to apparent maltose.

In Part I of this thesis, it was stated that \( A. \) oryzae produces an \( \alpha \)-amylase (Taka-amylase A) that is similar to that of \( S. \) bovis strains in that the predominant final products on starch include \( G_1-G_4 \), but that there is a difference in that the proportions of each product are not the same. The action pattern of \( S. \) equinus 1091 \( \alpha \)-amylase on amylose is almost identical to that of Taka-amylase A of \( A. \) oryzae on starch (Minoda et al., 1968).

The blue value-reducing curve of the pure \( \alpha \)-amylase of \( S. \) equinus 1091 is almost identical to that of recrystallized, maltase-free Taka-amylase of \( A. \) oryzae (Kung et al., 1953). The curve for \( S. \) equinus 1091 is shifted a very small distance to the left of that for \( A. \) oryzae. Thus both the \( \alpha \)-amylase
action pattern (Minoda et al., 1968) and blue value-reducing value curve (Kung et al., 1953) of A. oryzae are very similar to those of S. equinus 1091.

The blue value-reducing value curves of the α-amylase of A. oryzae and S. equinus 1091 indicate that these enzymes both possess a very low degree of multiple attack (Robyt and French, 1967). These two α-amylases have a lower degree of multiple attack than recrystallized maltase-free pancreatic α-amylase from swine, swine salivary α-amylase, human salivary α-amylase, rat pancreatic and liver α-amylases, recrystallized maltase-free bacterial subtilis α-amylase (Kung et al., 1953), and S. bovis α-amylase (Hobson and MacPherson, 1952). But the α-amylases of S. equinus 1091 and A. oryzae have a higher degree of multiple attack than Clostridium butyricum α-amylase (Hobson and MacPherson, 1952).

S. bovis α-amylase has a degree of multiple attack that is higher than that of human salivary α-amylase (Hobson and MacPherson, 1952), whereas S. equinus 1091 α-amylase has a degree of multiple attack lower than that of human salivary α-amylase. Thus, both the action patterns and degrees of multiple attack for the α-amylases of S. equinus 1091 and S. bovis are different.
SUMMARY

1. Sixty strains of *Streptococcus bovis* were screened for extracellular amylase production. Of these 60 strains only seven produced large zones of hydrolysis on starch plates; three strains produced very small zones. The seven strains that produced large zones of hydrolysis were characterized physiologically and the action patterns of their amylases on starch were determined. The action pattern of one of the strains was also determined using amylose as the substrate.

2. Paper disc assay vs. reducing value curves (saccharogenic/dextrinogenic curves) were obtained for the α-amylase from the seven strains of *S. bovis*. At the higher dilutions, the amylases from different strains had different curves although all of the curves had essentially the same slope.

3. Five general amylase action patterns (Groups 1-5) were observed among the seven *S. bovis* strains. The α-amylases of some of the strains produced considerable quantities of isomaltose and a compound with a $R_f$ value slightly less than that of maltose. Bacterial α-amylases that liberate the branch point of amylopectin as isomaltose have not been previously reported.

4. Physiologically, the seven strains of *S. bovis* exhibited different degrees of relatedness. Also, the action
patterns of these seven strains exhibited correspondingly different degrees of similarity. Those \textit{S. bovis} strains which were closely related physiologically elaborated amylases with similar action patterns. A correlation was found between the degree of pH change produced in dextrose and mannose broths and the action patterns of the amylases. Also a correlation was found between the two main divisions within the \textit{S. bovis} species and the action patterns of the amylases.

5. The action pattern of \textit{S. bovis} H12 indicated that an extracellular transglucosylase was produced along with \(\alpha\)-amylase. Extracellular bacterial transglucosylase had not been previously reported.

6. The action patterns of the amylase of \textit{S. bovis} were compared with those of several bacteria and fungi. The suggestion was made that \textit{S. bovis} \(\alpha\)-amylase might have commercial value because of their unique action patterns and because \textit{S. bovis} has relatively simple nutritional requirements. In addition, \textit{S. bovis} does not require oxygen, which is an expensive item in industrial fermentations, for growth.

7. Seven \textit{S. equinus} strains were obtained, screened for extracellular amylase production and thoroughly characterized physiologically. \textit{S. equinus} 1090 and 1091 were retained for further study. These two strains were very different from each other physiologically. It was concluded that \textit{S. equinus}
1091 belongs to that distinct variety of *S. equinus* that exists in the human intestine and has a high fermentative power in dextrose broth. *S. equinus* 1091 fermented six more carbohydrates and produced a much greater quantity of acid in dextrose and D-mannose than *S. equinus* 1090. *S. equinus* 1091 synthesized $G_3-G_{15}$ from maltose in TYE broth, presumably by means of its extracellular transglucosylase.

8. *S. equinus* 1091 produced a much greater quantity of extracellular transglucosylase but only half as much amylase as strain 1090. However, strain 1091 produced as much amylase as the other five strains of *S. equinus* examined. *S. equinus* 1090 was the only strain of the seven examined that was not repressed in amylase production by 1.5% or 2.0% dextrose on a tryptone yeast extract agar plate.

9. Calcium carbonate (3.0%) did not inhibit the production of amylase by *S. bovis* strains but it did retard the production of amylase and transglucosylase by *S. equinus* strains.

10. For *S. equinus* 1091, preliminary experiments on agar plates were used to determine the effect of media and dextrose concentration on amylase and transglucosylase production; to determine the temperature optimum for transglucosylase production; to determine the effect of the brand of soluble starch on transglucosylase production; to determine the effect of concentration of dextrose or maltose on the size of the
amylolytic zone; to determine the kinetics of transglucosylase production; and to determine the relative efficiencies of various carbohydrates as acceptors for transglucosylase.

11. The following factors affecting production of amylase and transglucosylase in broth by *S. equinus* 1091 were studied: effect of calcium carbonate, medium, age of inoculum, concentration of maltose, and length of fermentation.

12. The transglucosylase of *S. equinus* 1091 was separated from its α-amylase by means of DEAE-cellulose chromatography.

13. The amylase and transglucosylase of *S. equinus* 1091 were purified by concentration by ultrafiltration and acetone precipitation. After a second acetone precipitation, the α-amylase had been purified 48-fold and the transglucosylase 28-fold. With another preparation, the specific activity of *S. equinus* transglucosylase was increased 25-fold simply by washing five times in an ultrafilter with a 50,000 M.W. membrane.

14. The α-amylase of *S. equinus* 1091 required *Ca^{++}* and *Cl*⁻ ions; had an optimum temperature of 38°C at pH 7.0 and optimum pH of 7.0 at 37°C in the presence of *CaCl₂*. Two pH optima of 4.75 and 8.0 at 37°C were observed in the absence of added *CaCl₂*; and had a lower degree of multiple attack than that of *S. bovis* and a degree of multiple attack and
amylase action pattern very similar to that of *Aspergillus oryzae*.

15. The transglucosylase of *S. equinus* 1091 did not require CaCl₂, had a temperature optimum of 37°C at pH 6.5 and optimum pH of 6.0 at 37°C. The enzyme could use dextrose, D-mannose, L-sorbose, maltose, sucrose and αα-trehalose as glucosyl acceptors.
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