The Cell-Bound α-Amylases of *Streptococcus bovis*

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1. The cell-bound α-amylase of *Streptococcus bovis* has been isolated from other carbohydrases in the cell extract by chromatography on DEAE-cellulose. The enzyme has been compared with the extracellular α-amylase produced by this organism. 2. The two amylases had similar action patterns on amylose, the main product being maltotriose with smaller amounts of maltose and a little glucose. 3. The cell-bound amylase hydrolysed maltopentaose and maltohexaose at a similar rate to the hydrolysis of amylose. Maltotetraose was hydrolysed six times more slowly, and maltotriose 280 times more slowly, than amylose. 4. Studies with end-labelled maltodextrins revealed that the cell-bound α-amylase preferentially hydrolysed the third linkage from the non-reducing end, liberating maltotriose. The linkage at the reducing end of maltotriose was more easily hydrolysed than the other. 5. Egg-white lysozyme and the extracellular enzymes of *Streptomyces albus* lysed the cell walls of *Streptococcus bovis*, releasing amylase into the medium. In the presence of 0·6 M-sucrose 10% of the maximal amylase activity was released by lysozyme. Suspension of the spheroplasts in dilute buffer caused the rupture of the cytoplasmic membrane and the liberation of amylase. 6. A sensitive method for determining the ability of amylases to degrade starch granules is described.

Meyer & Gonon (1951) suggested that the action of α-amylase on amylose could be explained if all the linkages except the terminal ones were hydrolysed at random. Their results with malt and pig-pancreatic α-amylases agreed with this view. So also did those of Whelan & Roberts (1953), who showed that salivary α-amylase acted on amylose to produce maltose and maltotriose in a ratio close to that predicted by Meyer & Gonon's (1951) theory. During the past 10 years the action patterns of several more α-amylases have been elucidated, and it is now clear that a non-random hydrolysis of internal linkages begins to take place as the average size of the molecule becomes smaller. α-Amylases differ from each other in the stage at which non-random hydrolysis begins and in the stage when the rate of hydrolysis decreases. Thus each α-amylase produces a specific group of oligosaccharides.

Robyt & French (1963) have proposed a mechanism for *Bacillus subtilis* α-amylase in which the enzyme is pictured as an endoenzyme that encounters the internal portions of the amylose molecule in a random manner. After one glycosidic bond is broken, one of the two fragments diffuses away, leaving the other fragment still bound to the enzyme, which then continues its reaction by an exo-mechanism with multiple attack.

In the present paper the location of the cell-bound amylases of *Streptococcus bovis* is described, and the properties of the α-amylases obtained from the cell extracts are compared with those of the extracellular α-amylase isolated from the cell-free filtrates. The results, which show that the extracellular α-amylase has a different specificity from those of α-amylases previously investigated, are discussed in relation to the two theories of α-amylase action.

MATERIALS AND METHODS

Carbohydrates. Amylose was prepared from potato starch by the method of Hobson, Pirt, Whelan & Peat (1951). Maltotriose was prepared as described by Peat, Whelan & Kroll (1956). Maltotetraose, maltopentaose and maltohexaose were isolated from the products of the reaction between potato amylose and α-amylase (crystalline) from *B. subtilis*. [^{14}C]Maltodextrins were produced in the reaction between *S. bovis* transglucosylase, potato amylose and [^{14}C]glucose as described by Walker (1965). The label was present only in the glucose residue at the reducing end. Maize-starch granules were prepared by the method of Schoch (1957). Sucrose and melibiose were supplied by British Drug Houses Ltd.

Enzymes. B. subtilis α-amylase was a gift from the Daiwa Kasei Co., Osaka, Japan. Glucose oxidase (pure) and

horse-radish peroxidase were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Lysozyme (crystalline) was a product of California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Crystalline trypsin was a product of Armour Laboratories, Eastbourne, Sussex. The extracellular lytic enzymes of *Streptomyces albus* were prepared according to the method of McCarty (1952) from cultures provided by Dr M. McCarty. The decolorization by adsorption on calcium phosphate was omitted.

Preparation of cell extract and cell-free filtrate. A culture of *S. bovis* obtained from Dr P. N. Hobson was grown in the starch medium described by Hobson & MacPherson (1952). When α-amylase activity in the medium had reached a maximum (44 hr.) the culture was centrifuged lightly to remove calcium carbonate, and the supernatant was centrifuged for 30 min. at 40000 *g*. The supernatant was sterilized by filtration through a Seitz filter pad. The cells were washed twice with 0·9% NaCl and suspended in 0·1 M-phosphate buffer, pH 6·4. The bacteria were disrupted for 15 min. in a Raytheon model DF101 magnetostriction oscillator. The residue was removed by centrifuging for 15 min. at 100000 *g*, and the enzymes in the clear supernatant were dialysed for 16 hr. against 0·01 M-phosphate buffer, pH 6·4.

Purification of the α-amylase in the cell-free filtrate. The clarified filtrate (1 l.) was treated with $(NH_4)_2SO_4$ (507 g.), the pH being maintained between 6 and 6·5. The precipitate that settled overnight was collected on the centrifuge, washed with 40% (w/v) $(NH_4)_2SO_4$ solution and suspended in water (100 ml.). The insoluble material was removed by centrifuging, and the supernatant was treated with maize starch (10 g.) for 30 min. at 4° with occasional stirring. After removal of the starch by centrifuging, the supernatant, which still contained appreciable amylase activity, was treated with a further 10 g. of maize starch. The first batch of starch was eluted by shaking the suspension at 39° for 15 min. with 25 ml. of water, and the second batch with 15 ml. of water. The starch was removed by centrifuging, and the supernatant from the second batch was used to re-extract the first batch of starch granules. The combined extracts were dialysed for 16 hr. against 0·01 M-phosphate buffer, pH 6·4.

Chromatography of the extracellular α-amylase on DEAE-cellulose. A portion (7·5 ml.) of the purified α-amylase was applied to a column (1 cm. × 25 cm.) of DEAE-cellulose (Brown Co., Berlin, N.H., U.S.A.) previously washed with 0·01 M-phosphate buffer, pH 6·4. The column was eluted with increasing concentrations of phosphate buffer, the gradient being linear between 0·01 and 0·30 M. Eighty fractions (7 ml. each) were collected at a flow rate of 50 ml./hr. Tests by the iodine-stain method showed that a single peak of amylase was eluted with 0·10 M-phosphate buffer. The fractions containing the highest activity were combined.

Chromatography of the cell extract on DEAE-cellulose. The dialysed cell extract obtained from 1 l. of culture fluid was fractionated as described above for the purified extracellular α-amylase. The main peak of α-amylase activity was eluted with 0·12 M-phosphate buffer, and a smaller peak with 0·27 M-phosphate buffer.

Paper-chromatographic analyses. The products of α-amylase action on amylose and maltodextrins were desalted with Bio-Deminrolit (The Permutit Co. Ltd.) and separated by chromatography on Whatman no. 3MM paper. The solvent was ethyl acetate–pyridine–water (10:4:3, by vol.). Strips dipped in silver nitrate–NaOH (Trevelyan, Procter & Harrison, 1950) were used as guides and water was used to elute the maltodextrins, which were determined by measuring (Nelson, 1944) the glucose produced on acid hydrolysis. With [^{14}C]maltodextrin substrates the products of amylase action were revealed by radioautography, and after elution determined by the method of Dische, Shettles & Osnos (1949). The radioactivity of the products was measured in an Ekco liquid-scintillation counter N644A used with an N530G scaler (Ekco Electronics Ltd., Southend-on-Sea, Essex).

Determination of carbohydrase activities. (*a*) α-Amylase activity in the fractions eluted from DEAE-cellulose was measured in digests containing 1% soluble starch (1·0 ml.) and enzyme (0·66 ml.). After 4 and 24 hr. at 39° portions (0·25 ml.) were stained with iodine solution (1 ml.) in 25 ml. The iodine solution contained iodine (2 g./l.) and potassium iodide (20 g./l.). The colour was read in the EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with a red (no. 608) filter.

(*b*) α-Galactosidase activity was measured in digests containing melibiose (100 μg. in 0·05 ml.) and enzyme (0·2 ml.). After 2 hr. at 39° the glucose-oxidase reagent of Huggett & Nixon (1957) was added, and the colour developed read at 420 mμ on the Unicam spectrophotometer after 1 hr.

(*c*) Sucrose phosphorylase was determined in digests (0·75 ml.) containing sucrose (10 mg. in 0·1 ml.), enzyme (0·25 ml.) and phosphate buffer (0·2 M). After 20 hr. at 39° the digests were tested for reducing power with the Shaffer & Hartmann (1921) method, and for glucose with the glucose-oxidase method.

(*d*) Starch-granule degradation by fractions with α-amylase activity was determined in digests containing maize-starch granules (5 mg.) and enzyme (0·5 ml.). The tubes were shaken at 39° for 3 hr. and then the granules were removed by centrifuging. A portion (0·1 ml.) of the clear supernatant was tested with glucose-oxidase reagent (0·4 ml.) for 3 hr. at 37°.

Measurement of lysis of Streptococcus bovis *cells.* The fall in the turbidity of the cell suspension was measured in the EEL colorimeter with a red (no. 608) filter. Rhamnose released into the medium was estimated by the method of Dische & Shettles (1948), as modified by Gibbons (1955). The term 'rhamnose release' used below refers to the appearance of soluble fragments containing rhamnose combined with other cell-wall constituents; it is not implied that lytic enzymes release free rhamnose. α-Amylase was determined as described above, with incubation times of 15 and 30 min.

RESULTS

Action of the α-amylases in cell extracts on starch solution, maize-starch granules and amylose. Chromatography of cell extracts of *S. bovis* on DEAE-cellulose gave two amylase fractions (Fig. 1). α-Galactosidase and sucrose phosphorylase were eluted between them. Only the fractions from the first peak were able to degrade maize-starch granules. *S. bovis* therefore possessed two cell-bound amylases. The solubilization of starch granules was

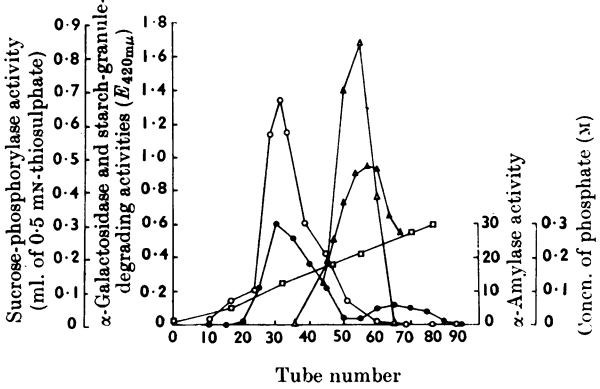

Fig. 1. Separation of cell-bound enzymes of *S. bovis* on DEAE-cellulose. The fractions corresponding to the respective peaks had: amylase activity (●) producing a 50% fall in iodine stain in 4 hr.; starch-granule-degrading activity (○) causing the solution of 61 μg. of dextrin in the activity digest; α-galactosidase (▲) causing 80% hydrolysis of melibiose; sucrose phosphorylase (△) causing the release of 0·27 mg. of monosaccharide. (All conditions were as stated in the Materials and Methods section.) The concentration of phosphate buffer (□) in the fractions was the same in the experiments described in Figs. 1 and 2.

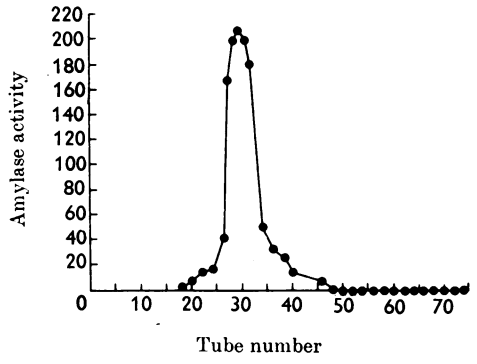

Fig. 2. Chromatography of purified extracellular α-amylase of *S. bovis* on DEAE-cellulose. Amylase activity was measured by the fall in iodine stain that occurred during 2 hr. incubation with starch.

detected by the following method: The amylase partially degrades the granules, liberating dextrins that are rapidly hydrolysed to give mainly maltotriose and maltose. After removal of the granules and addition of glucose oxidase, the hydrolysis continues in the presence of amylase, maltotriase and maltase provided by the glucose-oxidase preparation. This method gave the same results as the reducing-power method described by Walker & Hope (1963) and, being more sensitive, was selected in this case to conserve enzyme.

The purified α-amylase obtained from *S. bovis* culture filtrates was eluted from DEAE-cellulose at a concentration of phosphate buffer similar to that required to elute the major peak of the cell-bound amylase; there was no sign of a second peak (Fig. 2). In the experiments described below the source of cell-bound α-amylase was enzyme from the first and major peak of amylase activity.

Products of the α-amylolysis of amylose. The column eluates that contained maximal amylase activity were combined and dialysed against water. Portions (0·8 ml.) were incubated at 39° with amylose (8 mg.) in digests (2·5 ml.) buffered with tris–maleate at pH 6. When the iodine stain was 75% of the original value the only product detected on the paper chromatogram was maltotriose. Maltotetraose and maltose were first seen in trace amounts when the iodine stain was 55 and 27% respectively of the original. At the stage when the iodine stain was 13% of the original value, maltose and maltotetraose were clearly visible, but maltotriose was by far the most predominant sugar present. The same series of events occurred with the extracellular α-amylase.

When the achroic point was reached, portions were withdrawn for quantitative paper chromatography (Table 1). At a later stage the proportion of maltose and glucose was greater, and that of maltotetraose less, than in samples taken at the achroic point. The results for the cell-bound and extracellular amylases were similar.

Action of cell-bound amylase on maltodextrins. Maltotriose, maltotetraose, maltopentaose and maltohexaose (4 mg.) were incubated with cell-bound amylase (1 ml.) at 39° overnight. Paper chromatography showed that maltotriose had not been hydrolysed, and maltotetraose was partially hydrolysed to give maltotriose as the main product with smaller amounts of maltose and glucose. Maltopentaose had been completely converted into maltose and maltotriose, and the main product from maltohexaose was maltotriose, with smaller amounts of maltotetraose and maltose.

The molar proportions of the sugars obtained from maltotetraose, maltopentaose and maltohexaose by the action of the amylase were determined by quantitative chromatographic analysis (Table 2). Since maltohexaose gave a product, maltotetraose, that could be further hydrolysed by the enzyme, the maltohexaose digest was analysed at two times, when the conversion of maltohexaose was 64 and 95% respectively. At 64% hydrolysis the molar proportions of maltose, maltotriose and maltotetraose were 1·1 : 4·4 : 1. During the second period of the reaction the proportion of maltotetraose decreased and the final proportions were

Table 1. *Separation and analysis of the main products of the action of* Streptococcus bovis *α-amylase on amylose*

Details of the digests are given in the text. When the amylose was achroic (stage 1) portions of the digests were boiled and deionized, and the products were separated by paper chromatography. The elution of the maltodextrins and their subsequent analysis are described in the Materials and Methods section. The procedure was repeated at a further stage in the hydrolysis, when the apparent conversion into maltose was 87% (stage 2).

Products of enzyme action	Cell-bound amylase				Extracellular amylase			
	Stage 1		Stage 2		Stage 1		Stage 2	
	Wt. (mg.)	Mol. prop.	Wt. (mg.)	Mol. prop.	Wt. (mg.)	Mol. prop.	Wt. (mg.)	Mol. prop.
Glucose	0·06	0·28	0·12	0·46	0·05	0·26	0·17	0·51
Maltose	0·42	1·1	0·51	1	0·39	1·1	0·63	1
Maltotriose	1·17	2	1·23	1·7	1·05	2·1	1·39	1·5
Maltotetraose	0·52	0·67	0·26	0·26	0·68	1	0·11	0·10

Table 2. *Separation and analysis of the products of the action of* Streptococcus bovis *cell-bound amylase on maltodextrins*

The digests contained either maltotetraose, maltopentaose or maltohexaose (4 mg.) and amylase (1 ml.). Portions (0·5 ml.) were withdrawn at the stated times for analysis by paper chromatography.

Products of enzyme action	Maltotetraose Time ... 4 days		Maltopentaose 24 hr.		Maltohexaose 6 hr.		Maltohexaose 24 hr.	
	Wt. (mg.)	Mol. prop.	Wt. (mg.)	Mol. prop.	Wt. (mg.)	Mol. prop.	Wt. (mg.)	Mol. prop.
Glucose	0·16	1·1	0	—	0	—	0	—
Maltose	0·27	1	0·64	1·1	0·12	1·1	0·26	2·0
Maltotriose	0·47	1·2	0·85	1	0·76	4·4	1·0	5·3
Maltotetraose	0·56	—	0	—	0·22	1	0·25	1
Maltohexaose	—	—	—	—	0·64	—	0·08	—

2·0:5·3:1. Maltotetraose was incubated with the amylase for 4 days, and the conversion into products was 60%. The molar proportions of glucose, maltose and maltotriose were 1·1:1:1·2. Thus maltotetraose was the only maltodextrin that yielded glucose on amylolysis. Each of the maltodextrins gave maltotriose as the main product by weight, but it was not possible to decide from these results which linkages in the maltodextrins were broken to release maltotriose.

The rate of hydrolysis of the maltodextrins was compared with that of amylose. The digests (1 ml.) contained substrate (2 mg.) and enzyme (0·6 ml.) buffered at pH 6. Portions were withdrawn at intervals for the determination of reducing power with the Nelson (1944) reagent, and the results are shown in Fig. 3. Maltohexaose and maltopentaose were hydrolysed at a rate comparable with that of amylose, but the hydrolysis of maltotetraose proceeded six times more slowly. No increase in the reducing power of maltotriose could be detected

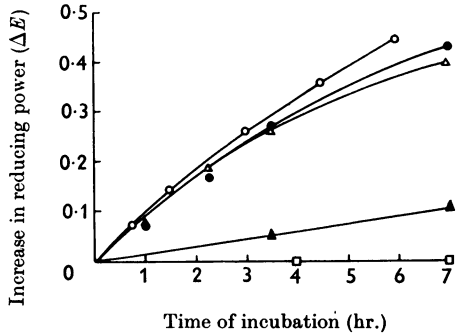

Fig. 3. Rate of hydrolysis of amylose and maltodextrins by cell-bound α-amylase of *S. bovis*. The increase in reducing power that developed when the enzyme was incubated with amylose (○), maltohexaose (●), maltopentaose (△), maltotetraose (▲) and maltotriose (□) was expressed as ΔE ($\Delta E = E_{t_2} - E_{t_0}$). Details of the digests are given in the text. At 7 hr. the reducing power in the maltopentaose digest had doubled, and the reducing power in the maltotetraose digest had increased by 20%.

Table 3. *Action pattern of* Streptococcus bovis *amylase on maltodextrins labelled with* ^{14}C *in the glucose residue at the reducing end*

(*a*) Maltotriose. Cell-bound amylase and extracellular amylase were incubated with maltotriose as described in the text. The hydrolysis of maltotriose was 17% in each case.

	Radioactivity (counts/100 sec.)	
Amylase	Glucose	Maltose
Cell-bound	10955	3460
Extracellular	11400	1851

(*b*) Maltotetraose. Cell-bound amylase was incubated with maltotetraose (200 μg.) for 48 hr. The hydrolysis of maltotetraose was 95%.

Products of enzyme action ...	Glucose	Maltose	Maltotriose
Radioactivity (counts/100 sec.)	27180	11430	414
% of total radioactivity	70	29	1
Weight (μg.)	38	66	81
% of total weight	20	36	44

(*c*) Maltopentaose. Cell-bound amylase was incubated with maltopentaose (158 μg.) for 24 hr. The hydrolysis of maltopentaose was 96%.

Products of enzyme action ...	Glucose	Maltose	Maltotriose	Maltotetraose
Radioactivity (counts/100 sec.)	4168	43164	4922	298
% of total radioactivity	8	81	9	1
Weight (μg.)	0	56	83	11
% of total weight	0	36	53	7

(*d*) Maltohexaose. Cell-bound amylase was incubated with maltohexaose (162 μg.) for 24 hr. Hydrolysis was complete and most of the maltotetraose produced was itself hydrolysed.

Products of enzyme action ...	Glucose	Maltose	Maltotriose	Maltotetraose
Radioactivity (counts/100 sec.)	3214	14392	30264	214
% of total radioactivity	7	30	62	0
Weight (μg.)	12	27	108	8
% of total weight	7	18	70	5

under these conditions. When larger digests containing 0·4% of maltotriose were incubated for longer times some hydrolysis was measured, but it proceeded 280 times more slowly than the hydrolysis of starch.

Amylolysis of [^{14}C]*maltodextrins*. The amylase (0·4 ml.) was incubated with maltopentaose and maltohexaose (160 μg.) for 24 hr. at 39°. Similar digests containing maltotriose and maltotetraose were incubated for 6 and 2 days respectively. More enzyme (0·2 ml.) was added to the maltotriose digest at 2-day intervals. Extracellular α-amylase was also incubated with maltotriose in a similar digest. All the digests were desalted, and the products separated by paper chromatography. Radioautographs showed that the labelled products from both maltotriose and maltotetraose were glucose and maltose. Maltopentaose gave labelled maltose, and maltohexaose gave labelled maltotriose. The appropriate areas of each chromatogram were eluted, and the weight and radioactivity of the sugars in the eluates were determined. These results (Table 3) revealed that maltotriose, the major product from each maltodextrin, was released by hydrolysis of the third linkage from the non-reducing end. With maltotriose, the linkage at the reducing end was hydrolysed more easily than the other; 77 and 86% respectively of the total counts were associated with glucose when cell-bound and extracellular amylases were tested.

Location of amylase in the cells. Information on the location of cell-bound enzymes may be obtained by studying enzyme release during the preparation of cell lysates and spheroplasts. Welker & Campbell (1963) used such a method to show that the amylase of *Bacillus stearothermophilus* was an extracellular enzyme. No amylase was released when the cells were treated with lysozyme. Several reports of the formation of osmotically fragile bodies from *Streptococcus faecalis* by lysozyme action have been

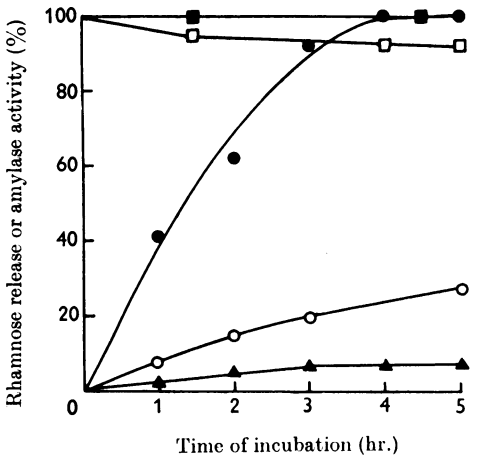

Fig. 4. Effect of lysozyme on *S. bovis*. The fall in turbidity of the cell suspension was measured in the presence (■) and absence (□) of 0·6 M-sucrose. Rhamnose release (○) is shown as the percentage of total rhamnose in the cells. Amylase activity in the medium containing sucrose (▲) is plotted as a percentage of the total activity released after incubation for 5 hr. without sucrose (●).

published (Abrams, 1959; Bleiweis & Zimmerman, 1961; Bibb & Straughn, 1962). Chesbro (1961) found that *Streptococcus faecium* was more sensitive to lysozyme than was *S. faecalis*. The action of egg-white lysozyme on *S. bovis* cells was examined by following the decrease in turbidity of the suspension, by observation under the microscope and by measurement of rhamnose release from the cell walls. The cells were harvested after 16 hr. growth and were washed twice with 0·05 M-ammonium acetate buffer, pH 6·5. They were suspended at a cell density of 3·0–4·0 mg. dry wt./ml. of ammonium acetate containing Ca^{2+} (2·5 mM) and Mg^{2+} (8 mM) with lysozyme (0·5 mg./ml.) at 38°. The fall in turbidity was variable from one experiment to another, and the rhamnose determination was a more reliable method for measuring cell-wall dissolution. Occasionally no fall in turbidity could be demonstrated, yet the cell wall was being dissolved, as shown by the release of rhamnose. *S. bovis* cells contained 60 μg. of rhamnose/mg. dry wt., and up to 93% of the total rhamnose was found in soluble components after incubation overnight with lysozyme. Under dark-field illumination the streptococci, which initially had an average chain length of 4, were seen to become separated into single bodies within 3 hr. These were somewhat smaller in size than the original cocci. Most of the amylase release was accomplished in 3–5 hr. (Fig. 4). When the cells were incubated with lysozyme

Table 4. *Release of amylase from* Streptococcus bovis *cells during incubation with lysozyme in various concentrations of sucrose*

At intervals during the incubation with lysozyme portions (1 ml.) of the digests were removed, and the cells were removed by centrifuging. The supernatants (0·1–0·66 ml.) were incubated with 1% soluble starch (1 ml.) in a total volume of 1·66 ml. for 30 min. A portion (0·1 ml.) of the 21 hr. supernatant obtained from the digest incubated without sucrose gave a fall in iodine stain of 21 colorimeter units. This activity is given a value of 100 in the Table, and the amylase activity in all other supernatants is expressed as a percentage of this maximal activity.

Concn. of sucrose (M)	Time with lysozyme (hr.)	Amylase activity in supernatant (%) 1	2	3	21
0		33·2	61·7	66·5	100
0·3		6·8	11·5	13·0	18·7
0·6		3·6	4·7	8·6	12·2
0·9		2·2	4·7	8·6	10·8

in the presence of 0·6 M-sucrose, the amylase activity in the supernatant was less than 10% of the amount released in the absence of sucrose. This suggested that the amylase was situated on or within the cytoplasmic membrane, and that a sucrose concentration of 0·6 M was sufficient to stabilize the spheroplasts. Lysozyme action released a higher percentage of the total amylase activity from cells suspended in 0·3 M-sucrose, but 0·9 M-sucrose gave a result similar to that in 0·6 M-sucrose (Table 4).

After incubation for 4 hr. with lysozyme in 0·6 M-sucrose, the spheroplasts were washed once with 0·6 M-sucrose, then resuspended in 0·05 M-ammonium acetate without sucrose at room temperature for 30 min. The residue was removed by centrifuging, and the amylase in the supernatant was compared with that in a control containing cells that had been treated in the absence of sucrose. Amylase activity was over 8 times that in the control. The supernatants prepared from cell lysates (12·5 ml.) in 0·6 M-sucrose gave an extinction value (260 mμ) of 0·23, whereas the control incubated without sucrose gave a value of 3·2. The extinction of the supernatants obtained after washing the residues and resuspending in 0·05 M-ammonium acetate (5 ml.) was 0·39 for the control and 11·1 for the digest incubated in 0·6 M-sucrose. Thus the results for the determination of ultra-violet-absorbing substances in the supernatants were also consistent with the view that the spheroplasts had been protected during the lysozyme treatment in sucrose, and had burst on suspension in dilute ammonium acetate, allowing the amylase to escape.

Lysis by the extracellular enzymes of Streptomyces albus. The enzyme system of *Streptomyces albus* was also able to lyse the cell wall of *Streptococcus bovis*, releasing rhamnose into the medium. More α-amylase was released from the cells treated in 0·6 M-sucrose than with lysozyme. Since substances that absorbed ultraviolet light were released from cells incubated with *Streptomyces* enzymes, it was probable that the increased amylase activity arose from within the cytoplasmic membrane. When sucrose was omitted little amylase activity could be detected. It was expected that the addition of Ca^{2+} (2·5 mM) to all digests would adequately protect amylases from proteolytic attack, and no explanation is offered for the loss of amylase activity when sucrose was absent.

When the *Streptomyces* enzyme system was added to cells that had been treated for 4 hr. with egg-white lysozyme, the fall in turbidity (sucrose absent) and rhamnose release were greater than expected from the known activities of the two systems. The simultaneous action of lysozyme and *Streptomyces* enzymes resulted in flocculation of the cells. A synergic effect could also be realized with trypsin. Although trypsin alone produced no fall in turbidity and released no soluble fragments containing rhamnose from the cells, an appreciable clearing of the cells occurred within 30 min. of the addition of trypsin to lysozyme-treated cells. The rate of release of soluble components containing rhamnose was greatly increased over that occurring in cells incubated with lysozyme alone, in spite of the fact that lysozyme was removed before trypsin was added. This suggested that the proteolytic component of the *Streptomyces* enzyme system was responsible for the synergic effect with egg-white lysozyme.

More amylase was released when trypsin or the *Streptomyces* enzymes were added to lysozyme-treated cells suspended in 0·6 M-sucrose. This increased activity could be accounted for by an action of these enzymes on the cytoplasmic membrane, allowing some of the α-amylase to escape. Ultraviolet-absorbing material was also released. When sucrose was absent, the amylase that had already been released by lysozyme was rapidly inactivated after the addition of *Streptomyces* enzymes.

Similarly, no further release of enzyme could be detected when trypsin was added to lysozyme-treated cells in the absence of sucrose.

A small amount of amylase was released from the cells by trypsin. This was about 10% of the total amylase liberated by lysozyme in the absence of sucrose. Amylase release caused by trypsin was similar in digests incubated with or without sucrose. Since the proportion of amylase released by trypsin was equivalent to that released when lysozyme acted on the cells in 0·6 M-sucrose, it was probable that both these enzymes were liberating amylase that was located externally to the cytoplasmic membrane, possibly on the surface of the cell wall.

All the results described in this section are summarized in Table 5.

Table 5. *Action of trypsin and the extracellular lytic enzymes of* Streptomyces albus *on* Streptococcus bovis *cells pretreated with lysozyme*

The incubation mixtures contained 30 mg. dry wt. of cells in 10 ml. or a similar suspension of cells that had been treated with lysozyme (5 mg. per 10 ml.) for 4 hr. at 38°. Portions (9 ml.) of the cell suspensions were incubated either with the *Streptomyces* enzyme system (1 ml.) or 0·05 M-ammonium acetate buffer, pH 6·5 (1 ml.), or with trypsin (0·25 mg. in 1 ml.). The lysozyme-treated cells were centrifuged and resuspended in 0·05 M-ammonium acetate buffer, pH 7·8 (9 ml.), before the incubation with trypsin. All treatments were carried out in the presence and absence of 0·6 M-sucrose.

(*a*) The turbidity of the digests was measured in the EEL colorimeter and the results are shown in colorimeter units. The values refer to digests containing no sucrose. The results in parentheses were obtained when sucrose was present.

	Turbidity					
	Control cells			Cells pretreated with lysozyme		
Time (hr.)	Trypsin	*Streptomyces* enzymes	Lysozyme	Trypsin	*Streptomyces* enzymes	Lysozyme
0	75	75 (61)	75 (65)	53 (57)	58 (65)	57 (64)
0·5	—	—	—	43	43	54
1	—	—	—	40	39	55
2	75	72 (60)	66 (64)	37 (55)	35 (62)	—
4	75	68 (61)	60 (64)	34 (53)	32	52 (65)

Table 5—*continued.*

(*b*) The removal of rhamnose from the cell walls into the surrounding medium was measured in the digests incubated without sucrose.

	Rhamnose release (%)				
	Control cells			Cells pretreated with lysozyme	
Time (hr.)	Trypsin	*Streptomyces* enzymes	Lysozyme	Trypsin	*Streptomyces* enzymes
0	0	0	0	29	29
2	—	7	23	57	79
4	1	13	29	77	96
8	2	18	43	—	—
17	—	—	—	97	97
21	3	50	91	—	—

(*c*) Amylase release was measured by the iodine-stain method, and results are given as fall in iodine stain (EEL colorimeter units) of starch that occurred when portions (0·5 ml.) of the supernatants were incubated in standard starch digests for 15 min. at pH 6·5. No amylase was released from cells incubated without lytic enzymes.

	Amylase activity									
	Control cells						Cells pretreated with lysozyme			
	Trypsin		*Streptomyces* enzymes		Lysozyme		Trypsin		*Streptomyces* enzymes	
Concn. of sucrose (M) ...	0·6	0	0·6	0	0·6	0	0·6	0	0·6	0
Time (hr.)										
0	—	—	—	—	—	—	0	0	5	30
2	2	2	6	2	3	20	7	0	13	1
4	3	3	12	2	5	33	7	1·5	21	1
8	—	—	14	3	5	45	—	—	—	—
17	—	—	—	—	—	—	9	—	5	1·5
21	5	6	8	2	6	60	—	—	—	—

(*d*) The release of materials absorbing ultraviolet light was measured at 260 mμ in 1 cm. cells in the Unicam spectrophotometer. The extinction values are corrected for values given by the enzymes, and by cell suspensions incubated without enzymes. Incubation was for 4 hr.

	$E_{260m\mu}$			
	Control cells		Cells pretreated with lysozyme	
Concn. of sucrose (M) ...	0·6	0	0·6	0
Lysozyme	0	3·3	0	3·3
Streptomyces enzymes	0·98	3·4	1·9	3·4
Trypsin	0	0·4	1·6	2·8

DISCUSSION

Bacterial enzymes may be cell-bound or may be liberated into the medium around the cells. Pollock (1962) has further divided cell-bound enzymes into surface-bound and intracellular enzymes. The term intracellular is reserved for enzymes that are within the cytoplasmic membrane, whereas surface-bound enzymes may be fixed to the cell wall or to structures underlying it, and are outside the cytoplasmic membrane. In the present work the amylases that were released from the cells by ultrasonic oscillation have been termed cell-bound, and the amylase appearing in the culture medium during growth was

termed extracellular. The results have indicated the similarity between one of the cell-bound amylases and the extracellular α-amylase. These amylases were eluted from DEAE-cellulose with the same concentration of phosphate buffer, they had identical action patterns on amylose and both could degrade starch granules. The second amylase in the cell-bound extract has not been studied beyond the finding that starch granules were not degraded. The possibility that this amylase was also liberated into the medium has not been investigated. The starch-adsorption step used in the purification of the extracellular α-amylase might have removed the second amylase if it were present, as some amylases that do not degrade maize-starch granules are not adsorbed on the granules (Walker & Hope, 1963).

The experiments with lysozyme showed that little amylase was released from the cells under conditions that protected the cytoplasmic membrane. This raised the question whether the α-amylase in the medium arose from autolysis of the cells during the 48 hr. period of growth. *S. bovis* transglucosylase (Walker, 1965) is a cell-bound enzyme and has not been detected in cell-free filtrates. It was released from the cells by ultrasonic disruption and by the action of lysozyme. The absence of transglucosylase in cell-free filtrates suggested that α-amylase in the medium around the cells was liberated without cell lysis.

The action pattern of the cell-bound amylase of *S. bovis* on maltodextrins and on amylose indicated that it has a different specificity from the α-amylases previously described. The major product from all the substrates was maltotriose and the experiments with labelled maltodextrins revealed that this arose from the hydrolysis of the third α-(1→4)-glucosidic linkage from the non-reducing end of each dextrin. This linkage was hydrolysed preferentially even when it coincided with the linkage at the reducing end, as in maltotetraose. The linkage at the non-reducing end of maltotetraose was not attacked, as no radioactive maltotriose was produced during the α-amylolysis of end-labelled maltotetraose. In maltohexaose, as in maltotetraose, the second linkage most susceptible to hydrolysis proved to be the second from the reducing end. With maltopentaose, this coincided with the third linkage from the non-reducing end, causing 80% of the total hydrolysis to take place at this linkage.

The amylase of *B. subtilis* also produced appreciable amounts of maltotriose from amylose, but maltohexaose was the main product of the reaction (Robyt & French, 1963). No maltotriose was produced in the slow reaction with maltohexaose or with maltoheptaose.

The hypothesis of Meyer & Gonon (1951) stated that α-amylase could act in a random fashion with equal ease on all the α-(1→4)-linkages except the terminal ones in an amylose chain. For an α-amylase that had no action on maltotriose this would lead to the production of maltose and maltotriose in the molar ratio 2·35:1 (Whelan & Roberts, 1953). For the amylase of *S. bovis* this ratio was 0·63:1. The theory predicted that the products of α-amylase action on maltohexaose would be maltose and maltotriose in the molar ratio 3:1 (cf. Whelan & Roberts, 1953), but *S. bovis* α-amylase gave a 1:2·7 molar ratio. Clearly the theory of Meyer & Gonon (1951) could not explain the distribution of the products of *S. bovis* α-amylase action on either amylose or maltodextrins.

The appearance of maltotriose at an early stage in the hydrolysis of amylose suggested the occurrence of multiple attack. Random fragmentation of long chains of maltodextrins should have resulted in significant amounts of maltotetraose and maltose at the stage when maltotriose first became apparent during the hydrolysis of amylose. The production of maltotriose alone could be explained if, after random attachment of the α-amylase to the polysaccharide chain, the enzyme continued to react several times with the same chain, releasing maltotriose. This is the mechanism that Robyt & French (1963) have postulated for the α-amylase of *B. subtilis*.

The action of *S. bovis* amylase on maltotriose was extremely slow. Both the linkages were attacked, but the linkage at the reducing end was the more susceptible to hydrolysis. Maltotriose is generally stable to the action of both α- and β-amylases unless massive quantities of enzyme are added. Pazur & Budovich (1955) investigated the hydrolysis of end-labelled maltotriose with salivary α-amylase and found a greater rate of hydrolysis of the glucosidic bond nearest the reducing end.

Since maltotriose was a major product of the action of *S. bovis* amylase on amylose, the fate of this trisaccharide in the cell is of interest. The further hydrolysis by α-amylase is unlikely to be the means whereby maltotriose is utilized.

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