SUMMARY

1. Pure E605 has a low *in vitro* activity against cholinesterase.

2. A specimen of E605 previously used (Aldridge, 1950) owed its high activity to the presence of E600.

3. The *S*-phenyl isomer of E605 is a highly active inhibitor and the inhibition follows first-order kinetics. The specimen of *S*-ethyl isomer contains approximately 4% E600.

4. It was observed (Aldridge & Davison, 1952) that in a series of diethyl phenyl phosphates sub-

stituted in the aromatic ring, the more stable to hydrolysis the inhibitor the less effective it is as an inhibitor. This relationship has been extended to include E605, Q2 and the *S*-phenyl isomer of E605.

5. A 'hydrolysis technique' has been developed and kinetic measurements have been utilized for the detection and determination of impurities in organophosphorus inhibitors. These methods are fully discussed.

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Amylases of *Clostridium butyricum* and a *Streptococcus* Isolated from the Rumen of the Sheep

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In the course of an investigation into the digestion of starch by the pig, Baker, Nasr, Morrice & Bruce (1951) isolated a micro-organism responsible for the breakdown of starch in the caecum and identified it as a strain of *Clostridium butyricum*. Later Masson (1951) isolated another strain of *Cl. butyricum* which was the chief agent of starch breakdown in the rumen of a sheep fed on a flaked maize and hay diet. Continuing these investigations into the digestion of starches by the sheep, MacPherson (forthcoming publication) obtained several strains of a *Streptococcus*, probably related to *Strep. bovis*, from the rumen, one being selected for use throughout this work. The extracellular amylase formed when the pig caecum strain of *Cl. butyricum* was grown on a medium containing soluble starch was examined by Whelan & Nasr (1951) and was shown to be of the α -amylase type. Both the sheep rumen *Clostridium* and *Streptococcus* formed an extracellular amylase when grown in a medium containing dissolved starch, and an investigation of these

amylases is the subject of this paper. The amylases would appear to be constitutive and not adaptive enzymes since, when the *Clostridium* was grown in a medium containing maltose as the only carbohydrate, and the *Streptococcus* on a glucose-containing medium, the other constituents being the same as in the starch medium, amylase activity was present in the cell-free filtrates.

There are at present known to be two major groups of starch-hydrolysing enzymes, the α - and β -amylases. In addition, there is the amylase of *Bacillus macerans* which has a synthetic as well as a hydrolytic function, as it catalyses the synthesis of cyclic 'Schardinger dextrins' from starch and starch fractions. Recently, also, various authors have reported the isolation of a maltase from a bacterium and moulds which is also capable of hydrolysing starch entirely to glucose (French & Knapp, 1950; Kerr, Cleveland & Katzbeck, 1951; Philips & Caldwell, 1951*a, b*). The only sugar produced during the hydrolysis of starch-type polysaccharides by β -amylase, which is found in ungerminated cereals, is maltose. Until recently it was thought that amylose, the 'linear' component of starch, was entirely

converted to maltose by this enzyme, but recent work by Peat, Pirt & Whelan (1952) has shown that the purest samples of β -amylase produce only 70% of the theoretical maltose from amylose. The presence of a second enzyme (Z -enzyme), usually present in β -amylase preparations, and which has been shown to be a β -glucosidase (Peat, Thomas & Whelan, 1952), is needed to enable the hydrolytic action of the β -amylase to continue beyond this arrest point. β -Amylase degrades the polysaccharide chain from the non-reducing end group and its action is arrested by the presence of the α -1:6-linkages in amylopectin, the 'branched-chain' component of starch, leading to the formation of a limit dextrin, ' β -dextrin'. The α -amylases on the other hand degrade starch and starch fractions in a more random manner and can hydrolyse the α -1:4-linkages of the inner chains of amylopectin between the α -1:6-branch linkages forming first dextrins and then a mixture of reducing sugars. This property of the α -amylases is generally used for testing for their presence in β -amylase preparations. Pure β -amylase does not attack β -dextrin, but a small concentration of α -amylase will hydrolyse α -1:4-linkages beyond the branch points, thus exposing more non-reducing end groups to the action of the β -amylase and resulting in degradation of the dextrin. The presence of β -amylase in α -amylase preparations is more difficult to detect, but Hobson, Whelan & Peat (1950) have recently introduced a method for characterizing α -amylase by comparing its actions on amylose and β -dextrin and this has been applied to the amylases described in this paper. Quantitative determinations of amylase activity depend on the measurement of either the reducing sugars formed, or the change in iodine stain of starch fractions under standard conditions. It is usual to measure the maltose formed by β -amylase, while, since the reducing power of the initial products of α -amylolysis is small, α -amylase activity is generally determined by observing the time taken for a starch digest to attain a standard iodine colour, or by measuring the change in iodine colour of the digest over a standard time. It should be emphasized, however, that the presence of one amylase as impurity can lead to erroneous results in measuring the activity of the other.

The bacterial amylases so far studied have, with the exception of the *B. macerans* enzyme, been of the α -type. The amylase of *B. subtilis* was investigated by Hopkins & Kulka (1942), and Hopkins, Dolby & Stopher (1942), and by Di Carlo & Redfern (1947*a, b*, 1948). This enzyme has now been obtained in a crystalline form by Meyer, Fuld & Bernfeld (1947) and in its saccharifying and dextrinizing action on starch it appears to be similar to salivary, pancreatic and malt α -amylases, but the nature of the end products of the hydrolysis have not been reported. Hopkins *et al.* (1942), however, showed that the *B. subtilis* enzyme was an α -amylase and that it was similar to malt α -amylase in that it rapidly hydrolysed starch until the sugars liberated corresponded to 40% apparent maltose. Thereafter the rate of hydrolysis was slower until a limiting conversion of 75–80% apparent maltose was found. Rose (1948), investigating the properties of an amylase formed by *B. polymyxa*, found that the reducing power of the products of the hydrolysis of starch by this enzyme did not exceed 80% theoretical maltose. Hockenhull & Herbert (1945) were not able to obtain a maltase-free enzyme from *Cl. acetobutylicum* and, although the rate of hydrolysis decreased when about 70% apparent maltose had been liberated, conversions of starch to considerably greater values than 100% apparent maltose were observed. On the basis of 100% conversion of starch

to glucose by the maltase-containing enzyme it was concluded that the α -amylase hydrolysed starch completely to maltose. However, the recent work of French & Knapp (1950) renders this conclusion invalid as they have shown that the maltase of *Cl. acetobutylicum* alone converts starch and dextrins entirely to glucose. More recently, Whelan & Nasr (1951) described the properties of the amylase of a strain of *Cl. butyricum* isolated from the pig caecum. The enzyme was an α -amylase which rapidly degraded potato starch and amylose until an apparent conversion to maltose of 65–66% was reached. Thereafter the hydrolysis ceased.

METHODS AND MATERIALS

Determination of reducing sugars. The copper reagent of Somogyi (1945*a*) standardized against maltose solution was used. Before being added to the reagent portions of the digests containing the *Cl. butyricum* amylase preparations were neutralized with a predetermined volume of 1*N*-NaOH and deproteinized with $ZnSO_4$ and $Ba(OH)_2$ as described by Somogyi (1945*b*) as the enzyme preparation was, otherwise, found to lower the apparent maltose concentration. Since the streptococcal amylase preparations had only a slight effect on the reagent it was found more convenient to standardize the Somogyi reagent against maltose solutions containing the enzyme preparation in the concentrations of the digests and to determine the reducing power of the digest samples from these standard graphs, after neutralization with a predetermined volume of 1*N*-NaOH. The iodometric technique was used throughout.

Measurement of the iodine stain of polysaccharides.

(1) *Blue value (BV).* The blue value of a polysaccharide is defined as the reading on the logarithmic scale of a Spekker photoelectric absorptiometer (Hilger and Watts, London), when the absorption of light (relative to water) by a solution of a polysaccharide-iodine complex contained in a 4 cm. cell and having the composition, polysaccharide (1 mg.), I_2 (2 mg.), KI (20 mg.)/100 ml. is measured using Ilford gelatin filter 608 transmitting light of wavelength 680 $m\mu$. (see Bourne, Haworth, Macey & Peat, 1948). The blue value is thus a characteristic of the starch fraction. (2) *EEL blue value (EBV).* In this work the light absorption of the polysaccharide-iodine solutions of the concentrations given above contained in a standard $\frac{1}{2}$ in. diameter colorimeter tube was measured in an 'EEL' colorimeter (Evans Electroselenium Ltd., Harlow, Essex) using filter 205 transmitting light of wavelength 680 $m\mu$. A reading of 51.2 on the EEL scale is equivalent to a Spekker reading of 1.40. Thus 1 *EBV* is equivalent to 36.6 *BV*. (3) *Absorption value. (AV).* The absorption value is a measurement of the iodine stain of a polysaccharide solution made in the same way as a blue value except that the wavelength of the light used and the polysaccharide concentration are not standard. The wavelength of light used is stated whenever an absorption value is quoted, but the concentration of polysaccharide may be unknown, as, for example, during an amylolysis. (4) '*EEL*' value (*EV*). This is an absorption value measured on the EEL colorimeter instead of a Spekker absorptiometer.

Fractionation of potato starch. Amylose was obtained from potato starch by the $Al(OH)_3$ -thymol method of Hobson, Pirt, Whelan & Peat (1951). Amylopectin was prepared by fractionation of thymol-amylopectin with methanol as described by these authors. All starch fractions were dried *in vacuo* at 60° over P_2O_5 before use.

Preparation of β -dextrin. This was prepared by the hydrolysis of thymol-amyopectin with soya-bean β -amylase as described by Hobson, Whelan & Peat (1950).

Measurement of α -amylase activity. A colorimetric method was used for determination of the amylase activity, as this was found to need less enzyme than the method previously described where the reducing power of an amylase-starch digest was measured after incubation under standard conditions (Hobson, quoted by Whelan & Nasr, 1951). β -Dextrin was used in preliminary experiments, but it was found that the fall in *EV* (680 $m\mu$.) was proportional to the amount of enzyme present only over a small range, so amylose, of blue value 1.2, was substituted for the dextrin. The amylose (approx. 50 mg.) was dissolved in 0.1N-NaOH (10 ml.) by heating in a boiling-water bath for not more than 9 min. After cooling, the solution was made just acid to phenolphthalein with 5N- H_2SO_4 and appropriately diluted. The volume added to the digest mixture was arranged to bring the final concentration of amylose in the digest to 1 mg./ml. The standard digest mixture contained 3.75 ml. amylose, 1.5 ml. of 0.2M-sodium citrate buffer, pH 7.0, and enzyme solution (1.75 ml.), the digest contents being preheated to 35° before mixing. The digest was incubated at 35° for 30 min. when 1 ml. was removed and stained with I_2 (2 mg.), KI (20 mg.)/100 ml. water for measurement of the *EV* (680 $m\mu$.). This *EV* (680 $m\mu$.) was compared with that of a similar portion of a blank digest containing water in place of the enzyme, and, providing the fall in *EV* (680 $m\mu$.) was not more than 24 units on the colorimeter scale it was strictly proportional to the amount of enzyme present. One activity unit is defined as the amount of amylase which will produce a fall in *EV* (680 $m\mu$.) of one colorimeter scale unit under the above conditions.

Standard digests for determination of the action of the enzyme preparations on polysaccharides. Solutions of starch or starch fractions were prepared as above and incorporated in digests containing 7.5 ml. polysaccharide solution (14 mg.), 0.2M-sodium acetate buffer of the appropriate pH value (3.0 ml.), water (1.5 ml.), and enzyme solution (2 ml.; activity 66 units). The digest was incubated at 35° and at intervals portions (usually 3 or 5 ml.) were removed for determination of the reducing power, against portions of a blank digest, containing water in place of the polysaccharide solution, which was incubated in parallel. Where necessary the digest volumes were increased to enable more determinations to be made.

Human salivary α -amylase. Saliva was collected as needed, diluted with an equal volume of water and centrifuged to remove the mucin. A portion of the supernatant liquid was then diluted to give a solution of the required activity.

Preparation of cell-free filtrates. (1) *Cl. butyricum*. Cultures of the *Clostridium* were inoculated into a modified Beijerinck fluid medium (400 ml.) containing 5% soluble starch (Baker *et al.* 1951) and incubated at 40° for 40 hr. After this time all iodine-staining polysaccharide in the medium had been degraded and gas production had almost ceased. The culture was centrifuged for 30 min. at 740 *g* to remove most of the cells, and then passed through a Seitz filter fitted with a Carlson 'EK' sterilizing pad. The volume of sterile filtrate obtained was usually about 300 ml. (2) *Streptococcus*. The medium used for these organisms was a yeast broth (200 ml.; 1% 'Difco' yeast extract, 1% Bacto Tryptone, 0.1% KH_2PO_4 , pH adjusted to 6.8) containing

5% dissolved starch and 3% (w/v) $CaCO_3$. The culture was incubated at 40° for 40 hr. with occasional shaking to suspend the $CaCO_3$. At this time amylase activity was maximal. The culture was then centrifuged until clear (20 min. at 1300 *g*) and sterilized by filtration as above. The volume of filtrate obtained was about 150 ml.

Preparation of freeze-dried enzymes. Preliminary experiments were carried out to determine the optimum concentrations of $(NH_4)_2SO_4$ for precipitation of the enzymes. All precipitations were carried out as near as possible to +1°. Freeze drying was carried out in 1 l. round-bottomed flasks at 0.1 mm. pressure using an acetone and solid carbon dioxide condenser. (1) *Cl. butyricum*. In a typical preparation an initial precipitation of the enzyme from 275 ml. of culture filtrate was carried out by addition of $(NH_4)_2SO_4$ solution (50% w/v; pH adjusted to 7.0 with NH_4OH) to 40% (w/v) and the precipitate freeze-dried. This precipitate (2.49 g.) was then dissolved in water (100 ml.; solution 1) and $(NH_4)_2SO_4$ solution added slowly to a concentration of 30% (w/v). After storing for 1.5 hr. the precipitate was compacted on the centrifuge in cold pots, washed with cold 30% (w/v) $(NH_4)_2SO_4$ solution, dissolved in 0.02M-sodium citrate buffer (25 ml.; pH 7.0), and freeze-dried to give fraction A. Some $(NH_4)_2SO_4$ was added to the supernatant liquid to bring the concentration to 40% (w/v). The precipitate was removed after 4.5 hr., washed, dissolved in citrate buffer, as above, and freeze-dried to give fraction B. The initial activity of the solution (1) was 19 800 units, the activities of fractions A and B before freeze-drying were 17 000 and 1300 units respectively. After freeze-drying the weight of fraction A was 0.4609 g. and the activity 13 980 units. The weight of freeze-dried fraction B was 1.6296 g., but the activity was not determined. (2) *Streptococcus*. In a typical large-scale preparation $(NH_4)_2SO_4$ was slowly added to the filtrate (152 ml.) to a concentration of 40% (w/v). After standing at +1° for 14.5 hr. the fine precipitate was removed on the centrifuge, washed and dissolved in water (100 ml.). More $(NH_4)_2SO_4$ was then added to a concentration of 35% (w/v) and after storing at +1° for 4.5 hr. a fine suspension formed which was centrifuged off, washed with 35% $(NH_4)_2SO_4$, dissolved in 0.02M-sodium citrate buffer (25 ml.; pH 7.0) and freeze-dried (fraction A). The weight of freeze-dried powder obtained was 0.6923 g. and the total activity 13 140 units compared with 13 200 units before freeze-drying. This was about half the activity of the original filtrate.

Fraction A from each filtrate was used in the following work. These powders kept their activity indefinitely when stored at +1°.

RESULTS

The action of the enzyme preparations on amylose and β -dextrin

Hobson *et al.* (1950) showed that by incubating equal amounts of α -amylase with (1) amylose and (2) β -dextrin and plotting the percentage change in *AV* (680 $m\mu$.) of the amylose-iodine complex against that of the β -dextrin, a curve characteristic of the enzyme and independent of its concentration could be obtained. Two standard digests, both containing the same enzyme preparation, but one containing amylose and the other β -dextrin, were

incubated together, and, at equal periods after addition of the enzyme, portions were removed for determination of the *EV* (680 $m\mu$). The sample from the amylose digest was stained under the standard conditions given above, but that from the β -dextrin digest contained twice the normal concentrations of polysaccharide and was stained with twice the normal concentration of iodine to increase the accuracy of the measurements.

Fig. 1. The comparative action of the enzymes on amylose and β -dextrin. *A*, theoretical curve for β -amylase and Q-enzyme. *B*, curve for R-enzyme. Soya-bean β -amylase (■), clostridial amylase (+), salivary amylase (●), at pH 7.0. Streptococcal amylase (○), at pH 6.0. All digests incubated at 35°.

Q-enzyme and β -amylase attack amylose but not β -dextrin so that a single straight line serves to represent the action of these enzymes. R-enzyme, which catalyses the hydrolysis of the α -1:6-linkages in β -dextrin and amylopectin, acts on β -dextrin with the formation of dextrans of higher blue value (Hobson, Whelan & Peat, 1951), and its action is represented by the curve shown in Fig. 1. α -Amylase such as salivary amylase is the only enzyme which attacks both substrates and the curve depicting its action is shown in Fig. 1 where the results of experiments using salivary α -amylase and the clostridial and streptococcal enzymes are plotted. Mixtures of α -amylase with other enzymes give curves of intermediate shape.

Optimum pH and temperature of action of the enzymes. The optimum pH of action was measured by determining the fall in *EV* (680 $m\mu$) of digests like the standard-activity digests but containing a sodium acetate-sodium veronal buffer the pH of which had been adjusted by the addition of 0.1N- H_2SO_4 (5 ml. 0.143 M-sodium acetate-sodium veronal solu-

tion, x ml. acid, $(18-x)$ ml. water). The constituents were preheated to 35°, mixed, and the *EV* (680 $m\mu$) of portions of the digests were measured after incubation for 15 and 30 min. as in the determination of activity. The pH values of the digests were checked at the end of the incubation.

Fig. 2. The effect of pH on amylase activity at 35°. Incubation periods: clostridial amylase, 15 min. (○), 30 min. (●); streptococcal amylase, 15 min. (+), 30 min. (⊕).

Fig. 3. The effect of temperature on amylase activity. Digests at pH 7.0. Incubation periods: clostridial amylase, 15 min. (○), 30 min. (●); streptococcal amylase, 15 min. (+), 30 min. (⊕).

Both enzymes have a broad pH optimum. That of the streptococcal enzyme under these conditions lies between 5.5 and 6.5 and that of the clostridial enzyme between 4.6 and 6.1 (Fig. 2). This latter value is similar to the optimum pH range of

crystalline *B. subtilis* enzyme (5.3–6.8; Meyer *et al.* 1947), partially purified *B. subtilis* amylase (5–6.5; Di Carlo & Redfern, 1947a) and *B. polymyxa* amylase (6.2–7.5; Rose, 1948).

The optimum temperature was determined by incubating the standard activity digests at different temperatures and determining the fall in *EV* (680 m μ .) after 15 and 30 min. in the usual manner.

The results in Fig. 3 show that both enzymes have the same optimum temperature of $48 \pm 1^\circ$ under the digest conditions employed. For subsequent experiments a temperature of action of 35° was chosen for both enzymes.

Fig. 4. The relationship between *EV* (680 m μ .) and percentage apparent conversion to maltose at 35° for amylose degraded by the clostridial amylase at digest pH values of 4.8 and 7.0 (+); streptococcal amylase at pH 6.0 (○); salivary amylase at pH 7.0 (○). Typical β -amylase curve (■).

The action of the enzymes on amylose

The relationship between absorption value and percentage apparent conversion to maltose. An amylose of blue value 1.21 was used in these experiments to facilitate comparison of the results with those obtained by Whelan & Nasr (1951), as it was found that slightly different curves were obtained when amylose fractions of different blue values were used. Digests of the standard proportions, but with the total volume increased to 42 ml., were employed. A buffer of pH 7.0 was used for salivary α -amylase and two, of pH 7.0 and 4.8, for the clostridial amylase, the same curve being obtained at both pH values. For the streptococcal enzyme a buffer of pH 6.0 was used. Withdrawals for measurement of *EV* (680 m μ .) (see Methods section), and for measurement of reducing power, as maltose, were made alternately at noted intervals and from the progress curve of fall in *EV* with time the values corresponding to the respective reducing powers were found.

The results are shown in Fig. 4, a typical curve obtained with β -amylase being included for comparison.

Tests for the presence in the preparations of enzymes other than α -amylase

β -Amylase, Q- and R-enzymes. The results obtained from the comparison of the action of the enzyme preparations on amylose and β -dextrin obviate the presence of more than traces of β -amylase, Q- and R-enzymes.

Maltase. The polysaccharide solution in the standard digests was replaced by a maltose solution of the same concentration. The action of the clostridial enzyme on maltose was tested at pH values of 4.8 and 7.0 and that of the streptococcal enzyme at pH 6.0. Portions of the digests were removed for determination of the reducing powers, as maltose, at approximately 10 hr. intervals up to 48 hr.

No change in reducing power was found and the enzyme preparations were therefore free from maltase. Quantitative determinations, using portions of the digests as enzyme source, showed that the enzymes in the digests containing maltose retained their initial activity after incubation for 48 hr.

Phosphorylase. Phosphorylase activity was determined using the method of Green & Stumpf (1942) except that since the phosphorylase activity of the enzyme preparations was small, incubation was carried on for 1 hr. before the deactivation of the digests by addition of trichloroacetic acid. For determination of the initial concentration of inorganic phosphate a digest containing all the constituents but the enzyme was incubated in parallel, then trichloroacetic acid was added, and, immediately after, the enzyme preparation. Both digests were centrifuged and the inorganic phosphate in a suitable sample determined by the method of Allen (1940).

Experiments using the enzyme fractions obtained at 30 and 40% (w/v) concentrations of ammonium sulphate in the preliminary fractionation of the clostridial amylase showed that the fraction precipitated by 30% w/v $(\text{NH}_4)_2\text{SO}_4$ had a very slight phosphorylase activity, but that the fraction precipitated by 40% w/v $(\text{NH}_4)_2\text{SO}_4$ had three times as much. For the determination of the phosphorylase activity of the freeze-dried preparations the enzyme was used in a concentration of 220 α -amylase units/digest. The phosphorylase activity of the streptococcal amylase preparation was of the order of 1 unit/13 000 α -amylase units, and of the clostridial amylase 1 unit/10 000 α -amylase units.

Phosphatase. The composition of digests and the experimental conditions were the same as for the determination of phosphorylase activity except that 0.1M-sodium α -glycerophosphate replaced 0.1M-glucose-1-phosphate. Neither enzyme preparation liberated inorganic phosphate from the glycerophosphate. α -Glycerophosphatase activity is therefore absent under these conditions.

Invertase. The polysaccharide in the standard digests was replaced by a sucrose solution of the

same concentration. The buffers used were of pH values 4.8 and 7.0 for the clostridial amylase and 6.0 for the streptococcal enzyme. After incubation for 20 hr. none of the digests showed any reducing power thereby indicating the absence of invertase.

The effect of chloride and calcium ions on the activity of the enzymes

Chloride ions. Two digests were prepared as for the activity determinations except that one contained sufficient NaCl to bring the final concentration to 0.14 M. The digests were incubated at 35° for 30 min. when portions (1 ml.) were removed for determination of *EV* (680 m μ) as in the determination of activity.

Calcium ions. Two digests were prepared each containing amylose solution (7 mg.; 3.75 ml.), 0.2 M-sodium acetate solution the pH of which was adjusted to 7.0 (1.5 ml.) and enzyme solution (0.75 ml.). In addition, one digest contained CaCl₂ solution (1 ml.) to give a final concentration of 0.07 M, and the other NaCl solution (1 ml.) such that the final concentration was 0.14 M. The digests were incubated at 35° and at 30 min. the *EV* (680 m μ) of portions (1 ml.) of each were measured.

The results are shown in Table 1. The presence of calcium ions causes the slightly higher *EV* of the samples from digests containing calcium chloride. A similar effect is found with starch-iodine solutions containing calcium nitrate. Neither enzyme is activated by calcium or chloride ions under the conditions employed, but in some (unreported) experiments it was found that dilute solutions of the enzymes were stabilized by the presence of small concentrations of calcium ions.

The hydrolysis of starch and starch fractions by the enzymes

Solutions of the amylose, and amylopectin from potato starch were prepared and incorporated in digests of the standard proportions containing buffers of pH values 4.8 and 6.0 for the clostridial amylase and 6.0 for the streptococcal amylase. The digests were incubated at 35°, and, at intervals, the reducing sugars present were determined and expressed as maltose. The activities of the enzymes in the digests were determined at intervals.

After 27 hr. the streptococcal enzyme retained its initial activity in the digests (4.7 units/1 ml.), but at 72 hr. the activity had fallen to 2.9 units/1 ml., and

at 118 hr. it was 0.8 unit/1 ml. The clostridial amylase retained its full activity at 24 hr., and the activity at 118 hr. was 1 unit/1 ml. At 72 hr. fresh enzyme was added to portions of each digest containing amylose and, to the digests containing the clostridial amylase, starch and amylopectin to bring the activity to the initial value; the extents of conversion to reducing sugars did not, however, markedly increase above those of the original digests. The apparent conversions to maltose of the different substrates by the two enzyme preparations are shown in Figs. 5 and 6; in each case the values found for starch are almost exactly those calculated from the extent of the hydrolysis of the two constituents, amylose and amylopectin.

Identification of the main products from the hydrolysis of amylose by the streptococcal-enzyme preparation

A standard digest containing 28 mg. of amylose was incubated at 35°, the reducing power being determined at 21 and 23.5 hr. (90.7% apparent maltose). At 27 hr. 20 ml. of the digest were shaken alternately with cation- and anion-exchange resins (Amberlite 1 R 100/8 and 1 R 4 B; Rohm and Hass Co., Philadelphia, U.S.A.) to remove the buffer and the solution evaporated to dryness under reduced pressure at 45°. The resulting residue was dissolved in water (0.5 ml.) and spots of this solution, to the total volumes given below, were layered on to the starting line of a strip of Whatman no. 1 filter paper. The first chromatogram had spots consisting of approximately 0.01 ml. of amylolysis products, and 0.01 ml. of amylolysis products mixed with 0.005 ml. of 1% glucose solution, together with reference spots of 0.002 ml. of 1% glucose, 0.002 ml. of 1% maltose and 0.002 ml. of 1% maltotriose. The chromatogram was developed with a solvent consisting of A.R. amyl alcohol (British Drug Houses Ltd.), pyridine and water in the proportions by volume of 7:7:6 (Jeanes, Wise & Dimler, 1951) for 20.75 hr. at 20°; the solvent, which had run 32 cm., was then dried off, and the chromatogram replaced and developed for a further 19.5 hr., the solvent running the same distance. After drying, the paper was coloured by spraying with a benzidine reagent (Bacon & Edelman, 1951).

Three spots corresponding to glucose, maltose and maltotriose were found in the α -amylolysis products. A similar chromatogram was developed with the top layer of a benzene, *n*-butanol, pyridine, water mixture (in the proportions by volume 10:50:30:30)

Table 1. *The effect of calcium and chloride ions on the activity of the enzymes*

(Digests 1 contained amylose (7 mg.), 0.2 M-sodium citrate (1.5 ml., pH 7.0), and enzyme in a total volume of 7 ml. In digests 2 the sodium citrate was replaced by sodium acetate, and the enzyme activity differed from digests 1. The *EV* gives a measure of the enzyme activity; see text. Initial *EV* of amylose, 42.0.)

Salts added to digests Source of the enzyme	<i>EV</i> (680 m μ) of amylose after incubation for 30 min. at 35°			
	1		2	
	0.14 M-NaCl	No salts	0.14 M-NaCl	0.07 M-CaCl ₂
A rumen <i>Streptococcus</i>	35.0	35.0	32.6	34.1
<i>Clostridium butyricum</i>	34.5	34.5	33.8	35.2

for 44.5 hr. at 20°, the solvent being allowed to drip off the bottom of the paper, and after drying was coloured with the benzidine reagent. Again spots corresponding to glucose, maltose and maltotriose were found in the α -amylolysis products, the separation of the sugars being greater in this solvent. No material of chain length greater than maltotriose was detectable on these chromatograms. The most abundant products of the hydrolysis are thus glucose, maltose and maltotriose, the intensity of the spots suggesting that the maltotriose was present in large amount, and the glucose in comparatively small amount.

The action of the enzyme preparations on maltotriose and maltotetraose

The maltotriose and maltotetraose used were isolated from the end products of hydrolysis of amylose by the clostridial amylase by chromatography on charcoal in the manner of Whistler & Durso (1950) and Bailey, Whelan & Peat (1950). The maltotriose had $[\alpha]_D + 168^\circ$ (in water) and an

Table 2. *The hydrolysis of maltotetraose by the bacterial amylases*

(The digests were incubated at 35° at pH values of 4.8 for the clostridial enzyme and 6.0 for the streptococcal enzyme, and contained maltotetraose (14 mg.), 0.2M-sodium acetate (3 ml.), enzyme (66 units), water to 14 ml.)

Source of enzyme	Time of incubation	Apparent conversion of tetraose to maltose (%)
<i>Clostridium butyricum</i>	2 min.	37.1
	4.25 hr.	59.3
	20 hr.	81.5
A rumen <i>Streptococcus</i>	2 min.	46.5
	21 hr.	80.3
	45.5 hr.	91.9
	94.5 hr.	95.6

apparent chain length (from the copper-reducing power measured as maltose) of 2.82 glucose units and gave the theoretical amount of glucose on acid hydrolysis. The maltotetraose had $[\alpha]_D + 176^\circ$ and an apparent chain length of 4.2 and was hydrolysed to the theoretical amount of glucose. No carbohydrate impurities could be detected by chromatography and a graph of $\log(1/R_p - 1)$ per chain length of these sugars with glucose and maltose gave a straight line. The digests were of the same proportions as the standard digests, but containing a known amount (approx. 14 mg.) of the sugar. A buffer of pH 4.8 was used for the clostridial enzyme and of pH 6.0 for the streptococcal enzyme. The reducing power of digests containing maltotriose was measured up to 22 hr. in the case of the clostridial enzyme, and a further reading was taken at

51 hr. in the case of the streptococcal amylase. No increase in reducing power occurred. The results for the digests containing maltotetraose are shown in Table 2. The maltotetraose is slowly hydrolysed. The standard tests showed that the enzyme in all the digests was active after the last determinations of reducing power had been made.

DISCUSSION

The simultaneous action on amylose and β -dextrin of the two enzymes described in this paper (Fig. 1), together with the results obtained by a comparison of the change in iodine stain and the apparent conversion to maltose of amylose, indicate that the enzymes are of the α -amylase type. With both enzymes the iodine stain of the polysaccharide changes from blue, through purple and red and then disappears during the hydrolysis of amylose or starch, a result which is given by α - and not by β -amylases. The two enzymes differ in the extent of conversion of amylose to sugars, during the initial stages of the hydrolysis. The enzyme of the rumen strain of *Cl. butyricum* shows a similarity to that of the enzyme from the pig caecum strain examined by Whelan & Nasr (1951), but differs from the streptococcal enzyme, which is similar to that of salivary α -amylase. This difference between the enzymes could be explained by assuming that the streptococcal and salivary α -amylases make a more end-wise attack on the amylose chain splitting off low molecular weight sugars with a high reducing power and leaving dextrans of long chain length and high blue value. The clostridial enzymes, on the other hand, rapidly degrade amylose in a more random manner forming dextrans of short chain length and low blue value. Thus at any given apparent conversion to maltose the clostridial amylase digests have a lower *EV* (680 m μ .) than the salivary and streptococcal amylase digests. Further work to identify some of these intermediate products is in progress. This difference between the enzymes is maintained up to the end of the hydrolysis, the clostridial enzymes giving lower conversion limits than the salivary and streptococcal enzymes. On comparison of the extents of conversion of amylose to reducing sugars there is also, however, a difference between the amylases of the two strains of *Cl. butyricum*. The amylase from the pig caecum strain of organism hydrolysed starch and amylose to 65–66% apparent maltose, a figure which was not increased on prolonged incubation or addition of more enzyme. The amylase from the sheep rumen strain of *Cl. butyricum* rapidly hydrolyses amylose to about 80% apparent maltose and this extent of conversion slowly increases on prolonged incubation (Fig. 5). Starch and amylopectin are hydrolysed to smaller extents. The streptococcal

enzyme, however, again appears to be similar to salivary α -amylase in the final apparent conversion to maltose. Roberts & Whelan (1951) have recently reported a final conversion of amylose to 90% apparent maltose by salivary α -amylase. With the streptococcal enzyme also starch and amylopectin have lower extents of conversion to sugars than amylose. This is most likely due to the presence of α -1:6-linkages in amylopectin which are not hydrolysed by the α -amylases and which act as obstructions to the enzymes and lead to the formation of dextrans of molecular weight greater than maltose and maltotriose; these dextrans are then slowly hydrolysed. This is in accordance with the results of other workers (Meyer & Gonon, 1951; Myrbäck, 1950) who concluded that α -amylases do not hydrolyse α -1:6-linkages.

Fig. 5. The hydrolysis of starch fractions by the clostridial amylase at 35°. Digests at pH 4.8; amylose (○), starch (●), amylopectin (●). Digest at pH 6.0; Amylose (+). Addition of enzyme to bring the activity to the initial value (↑).

In a later publication it is hoped to describe the isolation of the end products of the hydrolysis of amylose and starch by these enzymes, and a preliminary fractionation has shown that the clostridial enzyme produces from amylose a mixture of sugars of which maltose and maltotriose are the main constituents, together with a little glucose and maltotetraose. The end products of the action of the streptococcal enzyme on amylose were shown by paper chromatography to contain glucose, maltose and maltotriose. As mentioned above, neither enzyme hydrolyses maltose or maltotriose, so the slow increase in reducing power on prolonged incubation of the digests containing starch and starch fractions is most likely due to the degradation of remaining traces of maltotetraose, which is hydrolysed by the enzymes (Table 2), and other dextrans of slightly longer chain length. Whelan & Nasr (1951) reported that a preliminary investigation of the products of hydrolysis of amylose by the pig caecum clostridial amylase showed the presence of maltose and maltotriose, and later Roberts & Whelan (1951) isolated

the end products of hydrolysis of amylose by salivary α -amylase and showed these, again, to be maltose and maltotriose. It would thus appear that maltotriose is a main product of the degradation of amylose by all these amylases. The difference in the final reducing powers of the digests containing amylose would then be mainly due to differences in the proportions of maltose, maltotriose and glucose present. In the products of hydrolysis of amylose these enzymes differ from the malt α -amylase and pig pancreatic amylase studied by Meyer & Gonon (1951) which are stated to give maltose and glucose as the end products of hydrolysis of amylose, maltotriose being a comparatively slowly hydrolysed intermediate product.

In further experiments it was found that the two organisms grew on media containing glucose,

Fig. 6. The hydrolysis of starch fractions by the streptococcal amylase at 35°. Digests at pH 6.0; amylose (○), starch (●), amylopectin (●). Addition of enzyme to bring the activity to the initial value (↑).

maltose or maltotriose as the only carbohydrates and that acids were produced by fermentation of these sugars. Thus these organisms can use as energy sources the main products of the hydrolysis of starch by their isolated extracellular amylases.

SUMMARY

1. Starch-degrading enzymes have been isolated in the form of stable freeze-dried powders from cell-free filtrates of cultures of sheep rumen strains of *Clostridium butyricum* and a *Streptococcus* grown in media containing dissolved starch. Their properties have been examined.

2. The enzymes belong to the group of α -amylases. The hydrolysis of starch and starch fractions by the enzymes has been investigated and the two enzymes have been shown to differ in the extents of conversion of these substrates to reducing sugars. The branch linkages in amylopectin act as obstructions to both enzymes.

3. The presence in the enzyme preparations of β -amylase, maltase, invertase, phosphatase or Q-

and R-enzymes could not be detected, but small traces of phosphorylase were present.

4. Maltose and maltotriose with some glucose are the main end products of the hydrolysis of amylose by these enzymes. Neither amylase hydrolyses these sugars, but maltotetraose is degraded by both enzymes.

5. A simple method for determination of α -amylase activity is described.

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The Free Amino-acids Occurring in the Body Tissues and Blood of the Rat and of the Cow

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Previous to the work reported here, few papers have been published showing the distribution of free amino-acids among the tissues of any one animal. Awapara, Landau & Fuerst (1950) have reported on the free amino-acids in rat tissues, while Gordon (1949) has investigated the intracellular fluid of calf embryo muscle and compared its free amino-acid composition with that of adult ox muscle and adult ox serum.

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EXPERIMENTAL

Total N. Micro-Kjeldahl estimations were carried out on the fresh tissue.

Preparation of protein-free filtrates. These were prepared from rat and cow tissues, blood and urine. The rats used in the experiments were of no specified age, but were mature males of approx. 400 g. body wt. They were slaughtered by dislocation of the neck and the organs removed as quickly as possible. Cow tissues were obtained from the slaughterhouse and were removed from the body as soon as possible after the death of the animal. They were then packed in ice for transport to the laboratory where they were immediately