SUMMARY

1. The oxidation of acetate and its utilization for the synthesis of milk fatty acids in a lactating goat has been investigated with the aid of [*carboxy*-^{14}C] acetate.

2. The half-life of body acetate in the lactating goat is not more than 50 min.

3. Acetate is rapidly utilized for the synthesis of milk fatty acids; the specific activity: time curves of these, after the maxima had been attained 3–4 hr. after the injection, decayed with a half-life of about 4 hr.

4. The plasma fatty acids contained much less ^{14}C than the milk fatty acids and could not have been directly an important source of the latter.

5. The steam-volatile fatty acids had a higher specific activity than the long-chain non-volatile acids indicating their independent synthesis. The hypothesis that the volatile fatty acids of milk originate from the degradation of long-chain acids (particularly of oleic) could not be confirmed. They may represent intermediates in the formation of long-chain acids.

6. Milk cholesterol is synthesized in the udder and not derived from blood cholesterol.

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The Amylase of *Clostridium butyricum*

By W. J. WHELAN and H. NASR
Department of Chemistry, University College of North Wales, Bangor and Rowett Research Institute, Bucksburn, Aberdeenshire

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Baker & Nasr (1947) demonstrated by microscopical observation that the breakdown of raw potato starch in the caecum of the pig is caused by large iodophile sporing rods exercising their action *in situ* upon the surface of the starch granules. The micro-organism was subsequently isolated in pure culture and identified as a strain of *Clostridium butyricum* (Baker, Nasr & Morrice, 1948). The pure culture was able to break down raw potato starch granules *in vitro*, the morphological and histochemical features of the process being similar to those observed in granules present in the caecal contents of pigs fed on potato starch diets. Evidence was presented (Nasr & Baker, 1949) which indicated that the extracellular decomposition of starch and other carbohydrates was accompanied by an intracellular synthesis of iodine-staining polysaccharide operated by a phosphorylating mechanism. The presence of amylase was demonstrated in cell-free culture filtrates when the organism was grown on starch. The isolation of this amylase in a stable solid state and an examination of its properties form the subject

of the work to be described. Some observations have also been made on the secretion of maltase by the organism.

METHODS

Estimation of reducing sugars. The reagent of Shaffer & Hartmann (1921) was used. It was found to be unnecessary to deproteinize solutions before using this reagent, but in later (unreported) experiments with the Somogyi (1945) reagent this step was found to be essential. When acid buffers were contained in the digest a predetermined volume of N-NaOH, sufficient to bring the pH of the digest portion used in the estimation to 7–8, was added to the digest-reagent mixture before heating. Otherwise the procedure was identical with that prescribed by Shaffer & Hartmann.

Measurement of blue value. The blue value of a polysaccharide is defined as the reading on the logarithmic scale of a Spekker photoelectric absorptiometer when the absorption of light (relative to water) by a solution of a polysaccharide-I_2 complex contained in a 4 cm. cell and having the composition, 1 mg. polysaccharide, 2 mg. I_2, 20 mg. KI, 2 drops 6N-HCl/100 ml., is measured, using Ilford gelatin light filters transmitting light of wavelength 680 mμ. (no. 608) (see Bourne, Haworth, Macey & Peat, 1948).

Measurement of absorption value. The expression absorption value (A.V.) describes a measurement made as above in the determination of blue value, but with the difference that the filter wavelength and the polysaccharide concentration are not defined and may vary as, for example, in measurement of the light absorption of a polysaccharide-I_2 complex in the region 430–680 mμ. or in measurements of A.V. using a volume of polysaccharide solution which originally contained the amount required for blue value determination but in which, because of enzymic degradation, the polysaccharide concentration can no longer be specified.

Fractionation of potato starch. Amylose was obtained by the fractionation of unmodified potato starch using the $Al(OH)_3$ method of Bourne, Donnison, Peat & Whelan (1949).

Measurement of α-amylase activity. The method used was devised by Dr P. N. Hobson (Hobson, 1950), to whom we are indebted for permission to publish the details. The amylase solution (3 ml.) was heated to 35° and added to a mixture of soluble (Analar) starch solution (0·6%; 25 ml.) and 0·5M-citrate buffer (3 ml.; pH 6·0), also at 35°. After 30 min. a portion was withdrawn and added to Somogyi (1945) reagent for measurement of reducing power. Provided that the total amount of reducing substance liberated by the enzyme does not exceed the equivalent of 27 mg. of maltose the reducing power is directly proportional to the enzyme concentration.

RESULTS

Preparation of cell-free filtrates

Cultures were grown at 37° in a medium having the composition shown in Table 1. Subcultures were then made into a similar medium in which sucrose was replaced by 5·0% (w/v) of soluble (Analar) starch, and incubated for 16 days. After a preliminary filtration using filter paper, cell-free filtrates were obtained by passage through a Seitz filter. Sterility of the filtrate was proved by incubation for 7 days in Robertson's meat broth, in which no growth occurred.

Table 1. *Composition of culture medium*

Sucrose	10·0 g.
Peptone	10·0 g.
Precipitated calcium carbonate	6·0 g.
Magnesium sulphate ($MgSO_4.7H_2O$)	0·1 g.
Sodium phosphate ($Na_2HPO_4.12H_2O$)	0·1 g.
Yeast extract, biotin or *p*-aminobenzoic acid	0·1 g.
Tap water	200 ml.

During the development of the above method the following observations were made: (1) The growth of the micro-organisms in pure culture and the amylase activity of the cell-free filtrates were increased by the incorporation in the medium of small quantities of either yeast extract, biotin or *p*-aminobenzoic acid. (2) Other conditions being the same, the filtrates displayed maximum amylase activity when the starch-containing cultures were incubated for 16 days. Thereafter the amylase activity slowly diminished. Activity was assessed qualitatively by observing the rate of change of iodine stain of starch when incubated with the filtrate, excess of iodine being added. The large iodine uptake of the filtrate precluded the quantitative measurement of amylase activity by an iodine-staining or copper-reduction method. (3) The amylase activities of purified freeze-dried enzyme preparations isolated from cultures grown on 0·1 and 5·0% starch were, respectively, in the ratio 1:120 (measured quantitatively by the copper-reduction method).

Preparation of freeze-dried amylase

Preliminary observations. To a portion of cell-free filtrate (50 ml.) was added ammonium sulphate solution (47 g./100 ml.; pH 6·5) with stirring. The solution became turbid at a concentration of 25 g./100 ml. and a precipitate formed and was removed at a concentration of 29 g./100 ml. Further precipitation occurred at higher concentrations of ammonium sulphate and four fractions in all were separated. Each was washed twice with ammonium sulphate solution, of the same concentration as the solution from which the fraction had been precipitated, and then redissolved in water (10 ml.), solution of the first precipitate being accompanied by vigorous frothing. Each of these solutions, as well as the supernatant liquid remaining after removal of the fractions, was tested qualitatively for (*a*) amylase activity by incubation with amylose solution at pH 4·8 and observation of the rate of change of iodine stain, (*b*) phosphorylase activity by incubation with glucose-1-phosphate in citrate buffer (pH 6·0), testing for liberated phosphate with the reagents of Allen (1940), and (*c*) iodine uptake. Results of these tests showed that (*a*) the amylase

activity was largely concentrated in the fraction precipitating at 29 g./100 ml. of ammonium sulphate, (*b*) phosphorylase activity was absent, (*c*) the iodine uptake was minimal in the amylase-containing fraction and was unlikely to interfere with quantitative measurements. Moreover, the yellow-brown colour of the cell-free filtrate was not carried down by the first fraction (containing the amylase), which was white; the latter fractions were heavily pigmented.

Partial separation of the enzyme. To the ice-cold cell-free filtrate was added ice-cold ammonium sulphate solution (pH 6·5) to a concentration of 29 g./100 ml. The precipitate (*A*), after being kept for 1 hr., was separated on the centrifuge. It was washed twice with ammonium sulphate solution (29 g./100 ml.), dissolved in the minimum quantity of water, transferred to a 1 l. flask and freeze-dried at 0·1 mm. pressure using an acetone-solid carbon dioxide mixture in the cold finger. The average yield was 280 mg./100 ml. of cell-free filtrate. A further precipitate (*B*) always settled from the supernatant liquid on being kept for 24 hr. In one experiment this was collected and treated as above. The ammonium sulphate concentration was then raised to 41 g./100 ml. and another precipitate (*C*) was collected and freeze-dried. The total amylase activities of the three precipitates *A*, *B* and *C* were in the ratio 6·55 : 1·0 : 2·5; expressed as activity per unit weight of freeze-dried material they were in the ratio 9·8 : 6·45 : 1·0.

Throughout this work fraction *A* has been used. The activity of one such preparation was only slightly diminished on storing in the refrigerator for over a year.

Optimum pH *and temperature of action of the enzyme*

The optimum pH of the enzyme lies between 5·3 and 5·5 (Fig. 1), and its optimum temperature of action under the conditions employed is in the region 46–48° (Fig. 2). As is commonly observed, the apparent optimum temperature varies inversely with the period of action of the enzyme. A pH of 4·8 and a temperature of action of 35·4° were chosen in subsequent experiments.

An A.V. (680 mμ.)/time curve plotted from the data of Fig. 3 reveals that the rate of change of A.V. decreases continuously when the A.V. is less than 40% of its original value. Some of the changes recorded in Figs. 1 and 2 exceeded this value, and therefore the data do not represent with strict accuracy the relative activities of the enzyme at the various values of pH and temperature. Nevertheless, the data do indicate the true pH and temperature optima of action. Proof of this lies in the fact that by means of Fig. 6, in which the relationship between the changes in A.V. (680 mμ.) and reducing power of amylose is depicted, the values of A.V. in Figs. 1 and 2 may be converted into terms of reducing power. The rate of change of reducing power is constant from the outset of the reaction until a point considerably beyond the achroic stage (Fig. 5), and therefore the curves obtained from the new data will accurately represent the relative enzyme activities. Such curves have been plotted, and although they differ to a small extent from Figs. 1 and 2 the same pH and temperature optima are indicated.

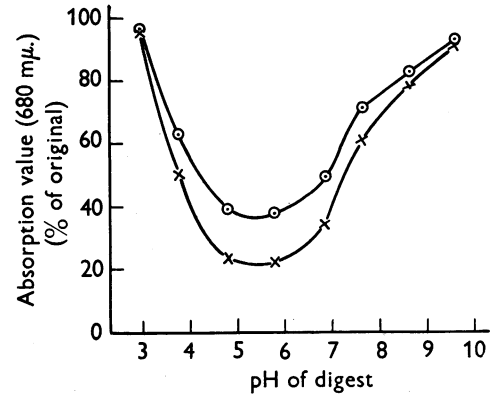

Fig. 1. Effect of pH on amylase activity. Amylose solution (5 ml.; 0·2%, w/v), universal buffer (sodium veronal, sodium acetate, H_2SO_4; 4·5 ml.), enzyme solution (0·5 ml.; 0·1%, w/v). Amylose-buffer mixture heated to 35·4° before adding enzyme. 1 ml. portions (≡1 mg. amylose) removed at 20 (⊙) and 35 min. (×) for determination of absorption value (680 mμ.). (For definition of absorption value, see text.)

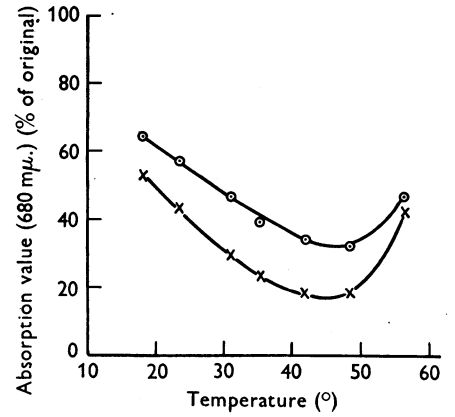

Fig. 2. Effect of temperature of amylase activity. Digest concentrations as in determination of optimum pH; veronal buffer (pH 4·79) used throughout. Amylose-buffer mixture maintained at desired temperature before adding enzyme. Incubation periods: 20 min. (⊙); 35 min. (×).

Comparison of the bacterial enzyme with salivary α-amylase

Degradation of amylose by the bacterial amylase. Amylose solution (50 ml.; 0·2 %, w/v), 0·2 M-sodium acetate buffer (30 ml.; pH 4·8) and enzyme solution (20 ml.; 0·4 %, w/v) were together incubated under a layer of toluene at 35·4°, at which temperature the separate constituents had been maintained before mixing, the enzyme being added last. At intervals 1 ml. portions of the digest (≡ 1 mg. amylose) were added to iodine solution for determination of A.V.

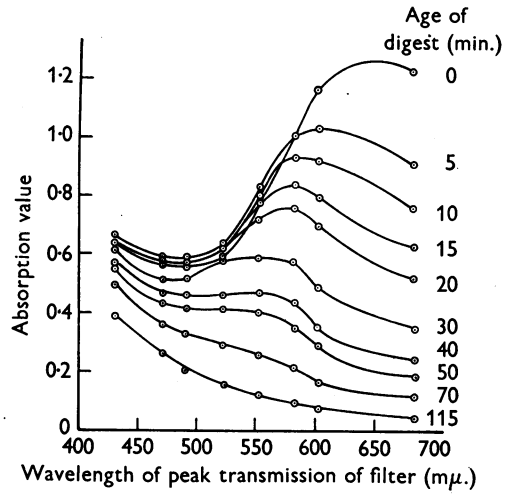

Fig. 3. Hydrolysis of amylose by bacterial amylase. Light absorption curves of iodine-stained degradation products.

(430–680 mμ.) (Fig. 3), and 5 ml. portions were added to Shaffer-Hartmann reagent (5 ml.) for determination of reducing power. Smaller digest portions (x ml. + (5 − x) ml. water) were used when the apparent maltose exceeded 2 mg./5 ml. Both iodine and the copper reagent immediately arrest the enzyme action. Withdrawals for A.V. and reducing power measurements were made alternately at noted times, and from a progress curve of increase in reducing power with time the reducing powers corresponding to the respective absorption values were interpolated and are used in Fig. 6. A correction was made for the small reducing power associated with the enzyme (5 mg. ≡ 0·35 mg. maltose) and all values of reducing power are expressed in terms of the amount of maltose theoretically obtainable. Measurements of A.V. were discontinued when the digest became achroic. After 45 hr. incubation the enzyme was found to be inactive, and a portion of the digest (20 ml.) was transferred to another flask to which was added fresh enzyme solution (5 ml.; 0·4 %). A similar renewal of enzyme was made at 100 hr. from the commencement of the experiment, 9·35 ml. of the second digest being mixed with fresh enzyme solution (5 ml.; 0·4 %) and incubated for a further 62 hr. From 32 to 162 hr. the reducing power remained virtually constant (Fig. 5). No increase in

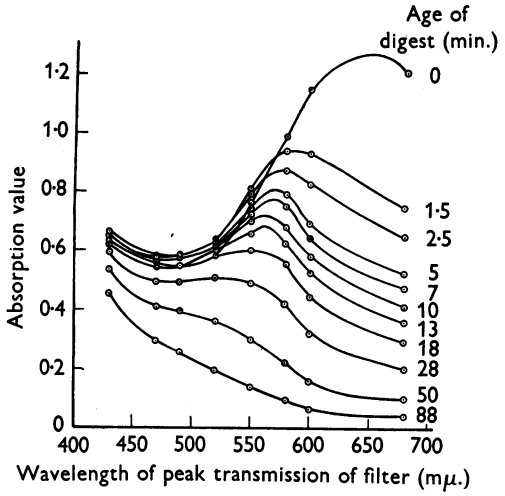

Fig. 4. Hydrolysis of amylose by salivary amylase. Light absorption curves of iodine-stained degradation products.

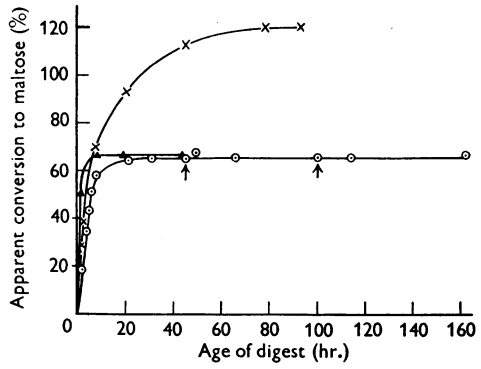

Fig. 5. Reducing power/time curves for bacterial amylase acting on potato amylose (-⊙-) and potato starch (-▲-), and for salivary amylase acting on potato amylose (- × -). Fresh enzyme added (↑). Because of the small scale of the graph, measurements of reducing power taken before 100 min. of incubation have been omitted. These values are, however, incorporated in Fig. 6.

reducing power occurred over a period of 24 hr. when enzyme solution (1 ml.; 0·4 %) was incubated with maltose solution (10 ml.; 0·2 %, w/v) at 35·4°. The enzyme preparation was therefore free from maltase.

Degradation of amylose by salivary amylase. Early morning saliva was collected and diluted with an equal volume of water and the precipitated mucins were removed on the centrifuge. Of this solution 8 ml. were diluted to 60 ml. with water and heated

to 35·4°, 30 ml. then being added to a mixture of amylose solution (40 ml.; 0·25 %) and 0·2 M-sodium acetate buffer (30 ml.; pH 4·8) maintained at the same temperature (final amylose concentration, 1 mg./ml.). The progress of the reaction was followed exactly as in the previous paragraph; the results are recorded in Figs. 4–6. A correction was again applied

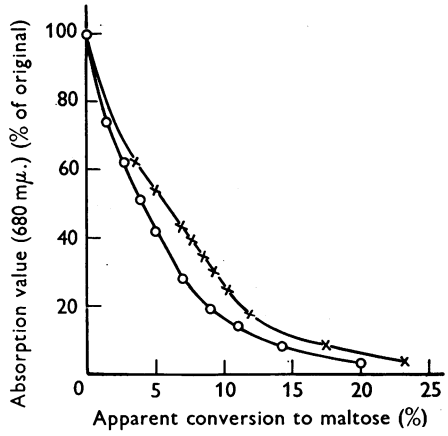

Fig. 6. Relationship between absorption value (680 mμ.) (expressed as percentage of the original) and percentage apparent conversion to maltose for amylose (blue value 1·21) degraded by bacterial (○) and salivary (×) amylases.

for the reducing power of the diluted (60 ml.) amylase solution (5 ml. ≡ 0·45 mg. maltose). Maltase was found to be present when the usual test was applied. The reducing power of the test solution increased to 106 % of its initial value in 24 hr.

Maltase secretion

When the organism was cultured on a starch-containing medium amylase, but not maltase, was secreted. In an attempt to discover whether the organism could secrete maltase it was cultured as above in two media containing (*a*) 5 % starch + 5 % maltose and (*b*) 5 % maltose respectively. From the cell-free filtrates precipitates were isolated by addition of ammonium sulphate solution to a concentration of 40 g./100 ml. The precipitates were washed with ammonium sulphate solution and freeze-dried as above. The two preparations were found to contain maltase in approximately equal amounts. The amylase activity of the precipitate from (*a*) was comparable with that of the standard preparation, but only a trace of amylase was present in the precipitate from (*b*); on incubation with starch the iodine stain persisted for several days. Under similar conditions the preparation from the starch-containing medium rendered the starch achroic within an hour.

DISCUSSION

Previous workers have studied two types of bacterial amylase. The first, the amylase of *Bacillus macerans*, is possessed of a synthetic as well as a degradative function in that it produces cyclic six- and seven-membered rings of glucose units (Schardinger dextrins) from starch, the amylose component giving the greater yield (cf. Kerr, 1942). The second type of bacterial amylase has been described by Hopkins & Kulka (1942) and Hopkins, Dolby & Stopher (1942) from *B. subtilis*, and by Hockenhull & Herbert (1945) from *Clostridium acetobutylicum*. The former enzyme has been crystallized by Meyer, Fuld & Bernfeld (1947). In its saccharifying and dextrinizing action on starch it appears to be similar to pancreatic, salivary and malt α-amylases (Bernfeld & Fuld, 1948), but the nature of the end products produced by the crystalline enzyme has not been reported. Hopkins and co-workers showed that the *B. subtilis* enzyme was an α-amylase and was similar to malt α-amylase in particular, in that both enzymes rapidly hydrolysed starch until 40 % apparent maltose was liberated. Thereafter the rate of hydrolysis was very much slower, the malt enzymes reaching a higher conversion limit (93 %) than the bacterial enzyme (75–80 %). Both enzymes were free from maltase. Hockenhull & Herbert (1945) were not able to obtain a maltase-free enzyme preparation, but did show that the *Cl. acetobutylicum* amylase was of the α-type and that the purified enzyme hydrolysed starch rapidly at first, the rate decreasing when 70 % apparent maltose had been liberated. The upper limit of hydrolysis was conditioned by the maltase present, conversions of considerably greater than 100 % apparent maltose being observed. On the basis of a 100 % conversion of starch to glucose by the maltase-containing enzyme these workers concluded that the amylase was 'of the apparently rather rare type which converts starch completely to maltose, with no other stable by-products'. To ascribe this function to the enzyme renders it unique rather than rare. There is no other amylase which behaves in this way.

It is generally agreed that the action of α-amylases on starch is to hydrolyse the molecules to short dextrins containing about six to nine glucose units, which are then attacked, in some cases much more slowly, yielding maltose and dextrins containing an isomaltose residue in which two glucose units are joined by an α-1:6-glucosidic link; these dextrins arise from the amylopectin component of starch. Because of the random mode of action of the α-amylase this residue is not situated in the same position in every dextrin molecule, with the result that a whole range of these short, branched dextrins is found. Myrbäck (1948), in a review of amylase action, has discussed the nature of these branched

dextrins. It is certain that α-amylase does not hydrolyse the α-1:6-linkage (Myrbäck, 1948; Bourne *et al.* 1948). Therefore the amylase preparation of Hockenhull & Herbert (1945) must, in addition to maltase, have contained an enzyme capable of hydrolysing the branch link in order that 100% conversion of starch to glucose should be observed. Such an enzyme has recently been isolated from the broad bean and the potato by Hobson, Whelan & Peat (1950).

It was felt that the study of the action of the *Cl. butyricum* enzyme would be simplified by the use of amylose instead of starch as substrate. The number of 1:6-links would then be minimal. The actions of the *Cl. butyricum* enzyme and salivary α-amylase on pure potato amylose were compared. The patterns of the light absorption curves of the iodine-stained degradation products liberated by the two enzymes are closely similar (Figs. 3 and 4). The changes in colour of the iodine stain in both cases were from blue through purple and red to the achroic stage. There is no doubt that in this respect the bacterial amylase is of the α-type. The patterns are closely similar to that of acid hydrolysis of amylose (Swanson, 1948) and are quite distinct from the pattern of β-amylase action during which, when the experiment is performed in a similar manner, the wavelength of peak absorption remains unaltered (Swanson, 1948; see, however, Bourne & Whelan, 1950). When, however, the reducing powers developed by the salivary and bacterial amylases are compared (Fig. 5), a marked difference is apparent. That of the salivary enzyme was complicated by the presence of maltase, the apparent conversion to maltose being at least 120%. Maltase-free saliva brings about an almost quantitative conversion of amylose to maltose (unpublished observations). The final reducing power developed by the bacterial enzyme was 65·5% apparent maltose, and this value was maintained over a considerable period, despite the addition of further quantities of enzyme. The end product of the action of *Clostridium* amylase on amylose is not therefore the same as that of salivary amylase. That the difference in action of the two enzymes is apparent from the outset of the reaction can be seen from the relationships between A.V. (680 mμ.) of the iodine-stained degradation products and the reducing power developed (Fig. 6). At equal percentage decreases of A.V. (680 mμ.) the reducing power developed by the bacterial enzyme is lower than that developed by salivary α-amylase. The maltase present in the latter preparations cannot account for this difference. Of the several possibilities that present themselves the most logical explanation of these differences would appear to be that the initial products of the action of the bacterial amylase, although achroic, are of higher molecular weight than the dextrins produced by salivary amylase. The end products produced by the *Clostridium* enzyme must also be of greater average chain length than maltose, the end product of salivary amylase action. Thus the reducing power developed by the former enzyme will be relatively smaller throughout the reaction. In a later publication it is hoped to define the nature of these unknown end products. They have been isolated on a relatively large scale and have partially crystallized. Preliminary observations indicate that the major constituents are maltose and maltotriose in the molar ratio 2:1. There is no glucose present. Crystalline β-amylase attacks the mixture very slowly. It is completely fermented by baker's yeast.

In order directly to compare the *Cl. butyricum* amylase with the bacterial enzymes of other workers who had used starch as the substrate, the former enzyme was incubated with whole potato starch. The hydrolysis was rapid and the reducing power reached a constant value of 66·5% apparent maltose (Fig. 5). This value was closely similar to that found when amylose was the substrate, i.e. 65·5%. The enzyme is therefore distinct from the *B. subtilis* amylase of Hopkins *et al.* (1942); the rate of hydrolysis of starch by this enzyme became exceedingly slow after approx. 40% apparent maltose had been liberated. There would appear, however, to be some similarity between our enzyme and that from *Cl. acetobutylicum* (Hockenhull & Herbert, 1945), which, when purified, hydrolysed starch to 70% apparent maltose, and thereafter more slowly. From the fact that maltase appeared to hydrolyse completely the product of amylase action it cannot be argued that maltose, and not some higher oligosaccharide, was the only product, because Sugihara & Wolfrom (1949) have shown that maltase will attack the trisaccharide maltotriose.

Despite this apparent similarity between the amylases of *Cl. acetobutylicum* and *Cl. butyricum* there is a curious difference between the maltase secretion of the two organisms. When the former organism was grown on starch both amylase and maltase were secreted; grown on maltose, only maltase appeared (Hockenhull & Herbert, 1945). When *Cl. butyricum* is grown on starch only amylase is secreted; there is no maltase. Maltase does, however, appear together with amylase when the culture medium contains both starch and maltose and in similarity to the *Cl. acetobutylicum* organism, maltase with (in our experiments) a small trace of amylase is found when *Cl. butyricum* is grown on maltose. The maltase may be isolated and preserved as a stable freeze-dried solid.

SUMMARY

1. From cell-free culture filtrates of *Clostridium butyricum* a starch-degrading enzyme preparation has been isolated in the form of a stable

freeze-dried powder. Its properties have been examined.

2. The enzyme belongs to the group of α-amylases, but differs from either salivary or malt α-amylase in that its action on potato amylose and starch ceases abruptly when 65·5 and 66·5% apparent maltose respectively have been liberated.

3. Some observations on the maltase secretion of the organism have been made.

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The Relationship Between the Constitution and the Effect of Chemical Compounds on Plant Growth

3. CHLORINATED BENZALDEHYDES AND BENZOIC ACIDS

By R. L. JONES, T. P. METCALFE and W. A. SEXTON
Imperial Chemical Industries Limited, Dyestuffs Division, Research Laboratories, Blackley, Manchester

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In the course of a systematic investigation of the phytotoxic properties of different chemical types, we have turned our attention to aromatic aldehydes and ketones and some of the carboxylic acids corresponding thereto. Interest in aldehydes and ketones was stimulated by the finding of Gray & Bonner (1948*a*, *b*) that leaves of the shrub *Encelia farinosa* contained 2-methoxy-5-acetylbenzaldehyde and that this is toxic to tomato seedlings at concentrations as low as 50 p.p.m. This compound, containing both aldehyde and ketone groups, was synthesized by Mr N. Greenhalgh in these laboratories and tested against germinating rape and wheat using our standard technique. It was very toxic to both species at 50 p.p.m., less so at 10 p.p.m. and showed no selective effect. The examination of a number of simple substituted benzaldehydes and acetophenones suggested that the former grouping was perhaps of greater interest, and since the aldehydes are more readily accessible than the ketones, our investigation has been concentrated upon the aldehydes. At the same time the corresponding carboxylic acids were examined, since although auxin-like activity is generally considered to demand some type of bridge linking the carboxylic acid group to the aromatic nucleus, several substituted benzoic acids show marked formative effects when applied to plants (Zimmerman & Hitchcock, 1941).

METHODS

Preparation of compounds. Nearly all the compounds used in this investigation are known ones, and many were already available in these laboratories. Attention is called, however, to the compounds substituted in the 2:3:4- and 2:3:6-positions (aldehydes, and acids) since the literature contains serious errors. The preparation and characterization of these