help to change amylose and dextrin of chain length 50 into red-staining dextrans and to act in opposition to the evidence reported.

SUMMARY

1. The Michaelis constants of soya bean β -amylase with potato amylose and its short-chain fission products are of the same order. The enzyme will therefore exhibit no decided preference for either long or short chains.

2. The early disappearance of the short chains from a mixture of long- and short-chain polysaccharides is demonstrated and must be attributed to a 'multichain' mode of attack by the enzyme, the hydrolysis of short chains being soon completed because they are short.

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The Mechanism of β -Amylase Action

2. 'MULTICHAIN' ACTION ON AMYLOSE FISSION PRODUCTS

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The evidence respectively favouring the 'single chain' and 'multichain' theories of β -amylase action has been summarized in the previous paper (Hopkins & Jelinek, 1954). Particular mention must be made of the work of French, Levine, Pazur & Norberg (1950) who hydrolysed amyloheptaose with soya bean β -amylase and found only maltose and maltotriose among the fission products. Even at the stage of 55% hydrolysis, no pentasaccharide was found, and the 'single chain' hypothesis received support. A preliminary experiment, reported briefly by Hopkins & Jelinek (1949), encouraged investigation of the action of β -amylase on amylose dextrans of chain length 10–30, the progress of which could be followed by changes in the absorption values of the iodine colorations. Such dextrans, prepared from amylose by acid or α -amylolytic fission, are colourless with iodine if shorter than chain length 8 (Swanson, 1948), and

change from red through purple to blue over the range 10–30. The absorption value at 600–680 m μ . (blue value) varies directly with chain length over this range (Bailey, Whelan & Peat, 1950). If an amylose dextrin of chain length 20 is hydrolysed by β -amylase by 'single chain' mechanism the absorption values at all wavelengths should vary inversely with increase in reducing power as percentage from R_m 10, the initial value, to approximately R_m 100 (the R_m value is the reducing power of the theoretically obtainable maltose). If, however, a 'multichain' mechanism operates, when all chains are reduced in length at equal rates, the absorption value will fall to zero by the time that the chain length has been shortened to 8 units (R_m 70) and even a little earlier when measured at 680 m μ . Moreover, the relationship would be linear over this range of chain length.

An additional method of testing for the mechanism by which amylose short-chain fission products are degraded by β -amylase employs chromato-

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graphy. A hexasaccharide undergoing hydrolysis clearly shows the simultaneous presence of maltose, tetrasaccharide and unchanged hexasaccharide.

Although this communication is concerned with short-chain dextrans as substrate, one experiment with amylose is also included in which the presence of short-chain fission products was demonstrated.

MATERIALS AND METHODS

Amylose. This was prepared from potato starch as described by Hopkins & Jelinek (1948).

Amylose dextrans. These were prepared by acting on 0.5% (w/v) amylose with α -amylase (*Bacillus subtilis*) at pH 6, until the iodine reaction was purple, red or achroic, respectively, and boiling to stop further action. In the case of red-staining dextrans (R_1 , R_2), this was followed by evaporation to a thin syrup, then precipitation and reprecipitation with acetone. The product was far from homogeneous at this stage and was repeatedly dialysed through cellophan. This removed all dextrans of shorter chain length and even permitted the slow diffusion of some of the required dextrin itself. No dextrin could be precipitated from the purified product by thymol or *n*-butanol.

Dextrans P_1 and P_2 were prepared by cooling with *n*-butanol at the purple-staining stage to remove the longest chains, then cooling the clear liquid to 3° overnight and collecting the sediment. The latter was redissolved and reprecipitated three times by cooling the aqueous solution to 3° without *n*-butanol. At each successive cooling the absorption values at various wavelengths were read for both the supernatant liquid and the sediment. The pattern of these for the liquid corresponded to dextrans of shorter chain length than that for the sediment, but the difference between liquid and sediment in this respect diminished with successive coolings. An attempt to fractionate the final sediment, P_1 , by once more cooling its solution gave fractions which were indistinguishable in their absorption values at the same concentration. P_2 was obtained from the previous supernatant liquid, purified by dialysis. Further treatment by cooling with *n*-butanol removed nothing from either P_1 or P_2 .

Achroic dextrin. This, after boiling and cooling, was put directly through a charcoal-Celite column (170 $\times$ 34 mm.) as described by Whistler & Durso (1950) and by Bailey *et al.* (1950). Successive elutions by water, 5, 15, 20, 25 and 30% (all v/v) ethanol yielded glucose, maltose, tri-, tetra-, penta- and hexa-saccharide, respectively. The elution of a given constituent was continued until no further material came off the column. Each eluate was concentrated and precipitated with acetone. The higher saccharides were re-fractionated.

The trisaccharide (maltotriose) had $[\alpha]_D$ (at equilibrium), $+158^\circ \pm 1^\circ$ in water. The hexasaccharide (R_m 32–33) had $[\alpha]_D$ (at equilibrium) $+177^\circ \pm 1^\circ$ in water.

β -Amylase. A sample of β -amylase was prepared from soya beans as described by Newton, Hixon & Naylor (1943). For a specimen of crystalline sweet-potato β -amylase, we are indebted to Dr A. K. Balls.

Absorption values. These were determined as described by Hopkins & Jelinek (1954).

Reducing power. This was measured by the method of Blom & Rosted (1947).

Paper-partition chromatography. This was performed as described previously (Bird & Hopkins, 1954).

RESULTS

Action of β -amylase on the dextrans

Purple-staining dextrans P_1 and P_2 and red-staining dextrans R_1 and R_2 were hydrolysed with β -amylase as summarized in Figs. 1–4, in which the absorption value, expressed as a percentage of the original value at 680 m μ ., is plotted against R_m . The graph in all cases was at first linear, and its initial slope, if maintained, would have intersected the horizontal axis at R_m 50–70. Crystalline β -amylase, acting at pH 3.6 on the dextrin P_1 (purple) in a very rapid reaction and on the dextrin P_2 (reddish purple) was clearly destroying iodine colouring power much faster than it was producing maltose. Changing the conditions to pH 6.8 and 32° did not affect this result. Soya bean β -amylase was more effective in this respect at pH 4.6 in a fast reaction than at pH 5.8 in a much slower reaction. Soya bean β -amylase, preheated to 60° in solution at its natural pH for 1 hr., behaved, in a slow reaction at pH 4.6, just as in Fig. 4 at pH 5.8.

The experiment on which Fig. 2 is based is considered more fully in a later section (Table 2). During the main part of the reaction, the percentage loss in absorption value greatly exceeded the percentage hydrolysis. Blue values (680 m μ .) fell faster than red. When the reaction reached the end of the first linear phase, about 10% of absorption value still remained.

Action of crystalline β -amylase on linear hexasaccharide

A solution of hexasaccharide (1.5%, w/v) was incubated with the enzyme at 21°. Withdrawals of 20 μ l. were placed directly on the paper and the chromatograms were developed.

Figs. 5–7 show that with the progressive disappearance of hexasaccharide, tetrasaccharide concentration increased at first and then remained constant, while maltose concentration steadily increased. Only after the disappearance of hexasaccharide at about R_m 65–70 did the tetrasaccharide concentration diminish. Traces of maltotriose were formed from penta- or hepta-saccharides present in the original substrate. The hexasaccharide used for Fig. 7 was more contaminated with pentasaccharides than the others.

Action of crystalline β -amylase on maltotriose

A solution of maltotriose (0.4%, w/v) was incubated with the enzyme at 26°. After 4 days action at pH 3.6 and at pH 5.8, chromatograms showed that the triose had nearly disappeared, while maltose and glucose had appeared in corresponding amounts. Action went further at pH 5.8 than at 3.6, as expected.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Figs. 1-4. Relationship between percentage loss of absorption value at 680 μ , and increase of R_m during hydrolysis of amylose dextrins by crystalline β -amylase. Fig. 1. Dextrin P_1 . Incubated at pH 3.6 and 21°. Time of half reaction, 2 min. 27 sec. Fig. 2. Dextrin P_2 . Incubated at pH 3.6 and 21°. Time of half reaction, 21 min. Fig. 3. Dextrin R_1 . Incubated at pH 6.8 and 32°. Time of half reaction, 9 min. 30 sec. Fig. 4. Dextrin R_2 . Incubated with soya bean β -amylase. Fast reaction: pH 4.6, 32°. Time of half reaction, 14 min. ($\odot$); slow reaction: pH 5.8, 32°. Time of half reaction, 240 min. ($\triangle$).

Figs. 5-7. Chromatograms showing progress of action of crystalline β -amylase on linear hexasaccharide at 21°. S = substrate. C = control mixture of maltose and maltotriose.

Chromatogram no.	R_m		
	Fig. 5	Fig. 6	Fig. 7
S	34	—	—
1	54	48	51
2	70	57	58
3	93	64	63
4	95	70	78
5	96	—	89
6	98	—	93
7	—	—	—
8	—	—	97
pH	3.6	3.6	5.0
Time of half reaction (min.)	2	10	10

Fig. 5.

Fig. 6.

Fig. 7.

Figs. 5-7. For legends see foot of p. 142.

Table 1. *Fractionation of β -amylase/amylose hydrolysates*
 (Absorption value (A.V.) ratio = A.V. read at 680 m μ /A.V. read at 490 m μ .)

Enzyme	pH		Maltose	Flocculum	Supernatant solution	Original amylose
Soya bean	5.8	Yield (%)	80	16.6	3.4	—
		A.V. ratio	—	—	1.62	—
Soya bean	4.6	Yield (%)	70.3	27.2	2.5	—
		A.V. ratio	—	—	1.2	3.0
		Chain length	—	260	—	480
Soya bean	4.6	Yield (%)	88	9.7	2.3	—
		A.V. ratio	—	—	0.76	—
		Chain length	—	—	20	—
Crystalline (sweet potato)	5.8	Yield (%)	62.5	34.4	3.1	—
		A.V. ratio	—	—	0.94	3.0
		Chain length	—	425	—	590

Fractionation of amylose undergoing β -amylolysis

Amylose (0.2%) was hydrolysed at 32° with β -amylase and the reaction stopped by boiling. The reaction mixture was frozen overnight and then allowed to thaw at room temperature; the flocculated sediment was separated by centrifuging and the supernatant solution was analysed. The conditions of the various hydrolyses and results of the fractionation are summarized in Table 1. Absorption values were read over the range 680–470 m μ , and the spectrograms indicated that short-chain material was under investigation. The ratio of the absorption values at 680 m μ . and at 490 m μ . is given in Table 1. For amylose this ratio is about 3.0.

When β -amylolysis of amylose was interrupted at stages earlier than R_m 50, no evidence was obtained of the presence in the reaction mixture of measurable quantities of short-chain fission products other than maltose. But at about R_m 60 and at subsequent stages, about 3% of products, the chain length of which ranged from 20 upwards as judged by their iodine stain, could be separated by freezing and thawing the reaction mixture. When a fraction of mean chain length 20–35 was obtained, its approximate chain length could be judged from the pattern of its absorption values by comparing this pattern with those of dextrans such as P_1 , P_2 and others prepared for this and other work. The concentration of the dextrin could also be estimated by this means. Determination by weighing, after complete removal of maltose by repeated dialysis, was unsatisfactory since some red-staining dextrans slowly diffused through cellophan. The quantities stated in Table 1 were therefore calculated by difference.

For determination of chain length by periodate oxidation the supernatant fraction was exhaustively dialysed before analysis. In the case of the sediment, freedom from adherent maltose was ensured by reprecipitation until the supernatant liquid had

no reducing power. The results showed that about 3% of the fission products were recovered as short-chain material as indicated by the iodine spectrogram. More could be extracted from the sediment at higher temperatures, but it was admixed with longer-chain material. The chain length of the residual amylose at 60–70% hydrolysis was much less than that of the original.

A control 'fractionation' of the original amylose yielded no carbohydrate in the supernatant fraction.

DISCUSSION

In considering the action of β -amylase on amylose-type dextrans of chain length 10 and upwards and giving iodine coloration, it is convenient to disregard the possibility that some of the molecules may contain barriers to the enzyme of the type responsible for the limited action on amylose reported by Peat, Pirt & Whelan (1952). Only a negligible portion of short-chain dextrans is produced in this way.

If the 'single chain' mechanism operates, the enzyme would hydrolyse a single chain completely before attacking another, and iodine coloration would diminish linearly with formation of maltose, finally disappearing at R_m 100. In Figs. 1–4 the extra diagonal line represents the theoretical progress under the 'single chain' mechanism.

With the 'multichain' mechanism, on the other hand, all iodine coloration would disappear by the time that all chains have been shortened to chain length 8 (and the blue value would disappear even earlier), i.e. before R_m 100. The most striking difference between the results to be expected from the two mechanisms should be obtained if the dextrin employed is composed of the shortest possible chains which give measurable colour with iodine. With 'multichain' hydrolysis, a dextrin of chain length 14 would lose all of its blue value on being shortened to 10 units, represented by a rise in R_m from 14.3 to 43. However, the production of

such a dextrin by fission of amylose entails difficulties in freeing it from dextrans of different chain length, a most important precaution. If, for instance, the product consisted of a mixture of dextrans of chain length 16 and 12, the removal of 2 units from all chains would be accompanied by fall in iodine colour values, as expected. Thereafter, the fission of dextrans of chain length 10 would contribute to reducing power without corresponding diminution of blue value. The graph, relating absorption value at 680 m μ . to R_m , would become less steep than hitherto. Some such effect is to be seen in Figs. 1-4. This source of error becomes more serious when relatively short-chain dextrans are used in this type of experiment. Furthermore, any contamination of the original material with achroic dextrin would result in the graph being from the outset less steep than it should have been, and the shorter the chain length of the dextrin employed, the more difficult is it to avoid such contamination. On the other hand, the greater the mean chain length, the nearer to 100 the value of R_m will be when the material becomes achroic. In fact dextrans of chain length 50 and upwards become almost indistinguishable from amylose in these experiments. Ordinary dialysis is not sufficiently selective if dextrans of chain length over 20 are to be purified. Bacterial α -amylase was used to hydrolyse the amylose, as, at the red-staining and subsequent stages, greater uniformity of chain length was expected than if acid was used. The methods adopted after some trials are given in the Experimental section.

The conclusion drawn from Figs. 1 to 4 is that the 'multichain' mechanism functions under the experimental conditions. The chain lengths of the dextrans ranged from over 30 in Fig. 1 to about 16 in Fig. 4, declining throughout the series. Corresponding to these, the initial linear parts of the graphs, extended to intersect the horizontal axis, did so at R_m 65, 65, 58 and 47, respectively. As expected, more decisive results were obtained with the dextrans R_1 and R_2 of shorter chain length. However, it also appears that the rate of the reaction exercised an influence. Fig. 4 shows results for a fast and a slow reaction with the same dextrin, R_2 , the

faster reaction giving the steeper curve. Another experiment, not reported in detail, in which soya bean β -amylase, which had been preheated in solution to 60° for 1 hr., gave results very similar to those of the slow reaction of Fig. 4. In these cases, however, it was noticed that the hydrolysis stopped at an unusually early stage. If the weak enzyme were for some reason to experience special difficulty in hydrolysing dextrans of chain length 6 or less, the 'single chain' mechanism could also account for the result since, in the case of either mechanism, the reaction would finish at about 75% R_m and the liquid would be achroic. This is the only way in which any of the results reported here can be reconciled with the 'single chain' hypothesis.

There are other distinctions between the manifestations of 'single' and 'multichain' mechanisms. With the former, the absorption value should fall linearly with increase in R_m , as already seen, and it should diminish at the same rate at all wavelengths. Table 2 shows that the blue values diminish more rapidly than the red and that both diminish much faster than would be expected on the 'single chain' hypothesis. On the 'single-chain' hypothesis also, one would expect the rate of maltose formation to fall during the reaction with diminishing molar concentration of substrate. However, it remained uniform (implying a zero-order reaction), scarcely diminishing by R_m 80. After this a marked retardation occurred. Allowing for iodine-staining dextrans still present, the residual achroic dextrans present at this stage would possess a mean chain length of 4.

The residual iodine-staining material at this stage includes some which cannot be hydrolysed by the pure β -enzyme. Indeed, it is of interest to note that a little red-staining residue (2-5%) was always present when β -amylolysis of these dextrans had reached its limit. The limit of such action on potato amylose is R_m 70 (Peat *et al.* 1952), and the obstruction to further action which occurs there evidently persists in some of the molecules of these iodine-staining dextrans. This was the case irrespective of whether the latter had been prepared by α -amylolytic or acid fission.

Table 2. Loss in absorption values during β -amylolysis of dextrin P_2

(The reaction mixture, containing dextrin P_2 (0.09%) and crystalline β -amylase, was incubated at pH 3.6 and 21°. The reaction was of zero order to R_m 50, and almost so to R_m 81.6. A slower reaction continued, reaching R_m 94 after 3 hr. Hydrolysis in column 2 is expressed as % of the range leading to R_m 81.6, i.e. 100% hydrolysis is attained at R_m 81.6.)

Time (min.)	Hydrolysis (%)	Loss in absorption value (%) at: (m μ)			
		680	600	490	470
2	5.4	17	13	10	7
5	13.5	28	26	20	19
10	26.6	45	42	40	40
20	52.2	72	71	68	67
42	100	89	89	87	87

The chromatograms (Figs. 5-7), in which hexasaccharide was substrate, show the presence during hydrolysis of tetrasaccharide, the amount of which did not diminish until all signs of hexasaccharide had disappeared. If the 'multichain' mechanism functions, we should expect hexasaccharide concentration to decrease while tetrasaccharide and maltose concentrations increase. Meanwhile, R_m will rise from 33 to 66, but shortly before this hexasaccharide should have disappeared, since below a certain concentration, it would not show on the chromatogram. From R_m 66 onwards tetrasaccharide concentration should decrease while maltose concentration should continue to increase. The three chromatograms show these changes. The spots corresponding to maltotriose are due to the presence of relatively small quantities of this product derived from corresponding quantities of penta- and/or hepta-saccharide in the original substrates. The very rapid reaction (Fig. 5) shows the slow fission of maltotriose, which did not start to diminish until all tetrasaccharide had disappeared.

These results are in marked contrast to those of French *et al.* (1950) who worked with amyloheptaose. As it remained a possibility that the 'multichain' mechanism may have been due to the high rates of reaction, some reactions which were several times faster than others under otherwise similar conditions have been included in both sets of experiments; for example, the rate in Fig. 1 was 9 times as fast as in Fig. 2, while the rate in Fig. 5 was 5 times as fast as in Fig. 6. It was possible for the 'multichain' mechanism to function and yet, by increasing the quantity of enzyme without altering other conditions, to increase the rate several times.

Finally, the β -amylolysis of amylose itself, summarized in Table 1, indicates that quantities of short-chain fission products were formed during the reaction. Such would not pass a cellophan membrane in dialysis (except perhaps in the case of the supernatant fraction which contained 88% of the theoretical yield of maltose) and may well be missed when sought by such experimental methods. Furthermore, such short-chain products would certainly be precipitated by *n*-butanol together with the longer-chain residue still present.

Under conditions which preclude all but the faintest activity of α -amylase or Z-enzyme, such as the use of crystalline β -amylase, the reaction becomes exceedingly slow at R_m 70 (Peat *et al.* 1952). The appearance of short-chain products as this stage is approached suggests that some chains are hydrolysed completely by the time that the reaction slows down, and that they are hydrolysed by the 'multichain' mechanism.

The presence of short-chain products in our

reaction mixtures at 80 and 88% hydrolysis by soya bean β -amylase could easily be attributed to the presence of traces of α -amylase. The Z-enzyme of Peat *et al.* (1952) could account for action proceeding beyond 70% but not for short-chain fission products unless the 'multichain' mechanism is assumed. The hydrolysis with crystalline β -amylase to 62.5%, still some way short of the arrest point with the crystalline enzyme, indicated some shortening of chain length (from 590 to 425) in the long-chain residue. It is not yet possible to state the nature of the barrier to action of the crystalline enzyme which occurs at 70-72% with potato amylose (Peat *et al.* 1952) and is confirmed with our materials, but its bearing on the results of experiments such as this is evidently important.

SUMMARY

1. The hydrolysis of amylose dextrins of chain length 16-30 by soya bean and crystalline sweet-potato β -amylases has been followed by measurement of absorption value of iodine coloration and reducing power.
2. The relatively rapid disappearance of the iodine colour is consistent with a 'multichain' mechanism in which all chains are liable to be attacked.
3. Paper chromatographic analyses of the fission products of hexasaccharide by crystalline β -amylase indicate that the intermediate product, tetrasaccharide, accumulated until the hexasaccharide disappeared. Maltotriose was slowly hydrolysed.
4. Diminution in chain length of the residue and the presence of short-chain fission products during hydrolysis of amylose by β -amylase were observed and indicate a 'multichain' mechanism.

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