LXXII. THE MECHANISM OF DEGRADATION OF STARCH BY AMYLASES.

III. MUTAROTATION OF FISSION PRODUCTS.

By GEORGE GROSVENOR FREEMAN
AND REGINALD HAYDN HOPKINS.

From the Department of Industrial Fermentation, University of Birmingham.

(Received January 31st, 1936.)

KUHN [1925] concluded from measurements of optical rotation that the amylases of pancreas and of *Aspergillus oryzae* apparently yielded α -maltose as a primary fission product of starch, whereas malt amylase produced β -maltose. Subsequently Ohlsson [1930] found by the same method that the two amylases of malt fractionated by his method [1926; 1930] also behaved as α - and β -amylases respectively, and that of ungerminated barley as a β -amylase.

The attempt to accommodate these findings with the conception of starch as being composed of a single type of chemical molecule has led to certain questions. (1) Are these primary fission products actually α - and β -maltose, or are they other bodies? (2) Are the observations of Kuhn to be explained on the basis of changing rotation of the starch itself, *i.e.* that the substrate as distinct from its fission products is mutarotating owing to one of two or more components being selectively hydrolysed? (3) Does dispersed starch consist of two chemically different molecules one containing α -glucosidic linkages only, the other including β - as well as α -glucosidic linkages? In this case the phenomenon observed by Kuhn would be an essential attribute of the substrate and not of the enzyme, it being assumed that α -amylase preferentially attacked the former and β -amylase the latter type of molecule. Van Klinkenberg's [1934] hypothesis includes some such basis as this. There are in any case however powerful *a priori* reasons for rejecting any hypothesis that starch contains two types of molecule so different chemically as those containing only α -linkages and those containing both α - and β -linkages.

Of the above questions, (1) and (2) have been dealt with to some extent. The reducing materials formed in the early stages of hydrolysis are, from the α -enzyme some maltose, but mainly dextrans; from the β -enzyme, maltose only [Freeman and Hopkins, 1936, 1]. α - and β -amylases attack most readily the same 60 % fraction of starch. There is no evidence of substantial selective hydrolysis [Freeman and Hopkins, 1936, 2]. To help to answer question 3 it was necessary to devise experiments to ascertain whether the actions of the amylases give rise to their characteristic mutarotations respectively, irrespective of the physical fraction of starch used as substrate. Kuhn used as substrates amylose and an electro dialysed fraction of starch, whilst Ohlsson used only the latter. Neither of these fractions represents starch as a whole. In the experiments reported here we have allowed forms of α - and β -amylases to act on Lintner soluble starch, amyloamylose, erythroamylose (Samec), glycogen, α -amylo-dextrin (Baker) and α -amylodextrin previously partly degraded by α -amylase. The use of α -amylodextrin was considered particularly important since this is the portion of starch which resists degradation by β -amylase. The mutarotation was observed sometimes directly, as by Kuhn, but usually on a product obtained

by filtration through a specially prepared filter designed to pass only smaller molecules. Dialysis took too long for successful observations. Filtration was adopted, firstly, to remove the enzyme, unchanged substrate and less degraded products, with the idea of observing if the mutarotating substances were among the more degraded reaction products; secondly, because, soon after the commencement of the reaction with α -malt-amylase, a hazy substance forms, which soon prevents polarimetric observation but later flocculates. (This was also observed by Hanes [1935].) On adding to alkali to complete the mutarotation, a flocculum is formed, the removal of which by filtration reduces the optical rotation. Some of the "mutarotation" in the α -sense observed in Exp. 3, and by previous workers, must be due to this removal from solution of optically active material. Filtration before polarimetric observations obviates this.

EXPERIMENTAL.

The substrates used and the enzymes prepared were as described by Freeman and Hopkins [1936, 2]. The particular specimen of soluble starch used had been selected on account of its transparency in solution, and for the sake of uniformity, was used throughout the work recorded previously. All percentages of substrates refer to dry weight. No buffer salts were added when velocities of mutarotation were to be measured; elsewhere a concentration of 0.015 *N* acetate was maintained. The values of $k \times 10^3$ were calculated, in Exp. 1 A, from the 1st and 7th, 2nd and 8th readings *etc.* and in the other experiments in a corresponding manner. The alkali used to complete mutarotation was Na_2CO_3 .

The technique of filtration through a ferric phosphate gel will be described in a later communication.

Exp. 1. Enzyme, pancreatic (α) amylase; substrate, soluble starch. 200 ml. 2.68% starch + A, 15 ml., B, 30 ml. of 5% pancreatin. Temp. 20°, p_{H} 6.8, filtrations at A, 28–36 min., B, 17–26 min.; 0.1 ml. of 5% HgCl_2 added to 50 ml. of filtrate to arrest enzyme action.

Table I. *Optical rotation of filtrates (2 dm.) and velocity constant of mutarotation.*

Time from commence- ment of readings min.	A			B		
	Rotation (angular degrees)	Mutarotation (angular degrees)	$k \times 10^3$	Rotation (angular degrees)	Mutarotation (angular degrees)	$k \times 10^3$
0	9.44	-0.82	6.32	7.16	-0.52	6.25
5	9.38	-0.76	6.23	7.13	-0.49	6.87
10	9.33	-0.71	5.96	7.08	-0.44	6.03
15	9.27	-0.65	5.70	7.05	-0.41	5.65
20	9.23	-0.61	5.88	7.03	-0.39	—
25	9.18	-0.56	(5.48)	—	—	—
30	9.15	-0.53	—	6.97	-0.33	—
40	—	—	—	6.93	-0.29	—
50	9.08	-0.46	Mean 6.02	6.90	-0.26	Mean 6.20
60	8.98	-0.36	—	—	—	—
70	8.93	-0.31	—	—	—	—
80	8.90	-0.28	—	—	—	—
∞	8.62	—	—	6.64	—	—
0	8.65 (alkali added)			6.62 (alkali added)		
<i>R</i> of filtrate, hydrolysis %		63.4				45
<i>p</i> _H of filtrate		6.72				6.84
Original reaction mixture, hydrolysis %:						
(1) At time of filtration		64				58
(2) Final (24 hours)		70				71.3

Exp. 2. Enzyme barley (β) amylase; substrate soluble starch. A, 200 ml. of 3.5% starch + 40 ml. of amylase ($\equiv$ 80 g. barley). B, 200 ml. of 2.68% starch + 30 ml. amylase ($\equiv$ 60 g. barley). Temp. 20°, p_H 4.6. Filtered at 10–16 min., then 0.1 ml. of 0.85% $AgNO_3$ added to arrest enzyme action. Read from 20 min. onwards.

Table II. *Optical rotation of filtrates (2 dm.) and velocity constant of mutarotation.*

Time from commencement of readings min.	A			B		
	Rotation (angular degrees)	Mutarotation	$k \times 10^3$	Rotation (angular degrees)	Mutarotation	$k \times 10^3$
0	8.28	+0.52	6.25	5.84	+0.37	6.05
5	8.31	+0.49	5.84	5.87	+0.34	3.35
10	8.34	+0.46	4.90	5.90	+0.31	5.64
20	8.41	+0.39	—	5.93	+0.28	—
30	8.45	+0.35	Mean 5.66	5.96	+0.25	Mean 5.68
40	—	—	—	6.00	+0.21	—
45	8.49	+0.31	—	—	—	—
∞	8.80	—	—	6.21	—	—
0	8.80 (alkali added)	—	—	6.25 (alkali added)	—	—
50	8.80 (alkali added)	—	—	6.25 (alkali added)	—	—
R of filtrate, hydrolysis %	41.6	—	—	37.4	—	—
p_H of filtrate	4.88	—	—	4.52	—	—
Original reaction mixture, hydrolysis %:						
(1) At time of filtration	47	—	—	47.4	—	—
(2) Final (24 hours)	55.2	—	—	59.1	—	—

Exp. 3. 75 ml. of 2.68% starch + 50 ml. of α - or β -malt amylase ($\equiv$ 100 g. malt). Temp. 20°, p_H 4.6.

Table III. *Optical rotations and mutarotations of reaction mixtures and filtrates.*

Time min. sec.		Rotation (1 dm.)	Rotation after alkali addition (angular degrees)	Muta- rotation	Iodine reaction	Conversion (iodimetric) % maltose
α -Amylase						
0	0	(2.94)*	(2.94)*	—	Blue	0
1	0	3.04	—	—	—	—
2	0	3.09	—	—	—	—
2	10	3.06	2.75	- 0.31	Red	25.8
7	40	—	2.66	—	Yellow	32.1
18	45	—	2.63	—	Colourless	37.9
42	30	—	2.58	—	„	42.7
1440	0	—	2.18	—	„	83.6
22	0	filtrate 2.21	1.96	- 0.25	„	22.9
26	0					
β -Amylase						
0	0	(2.97)*	(2.97)*	—	Blue	0
3	0	2.71	2.77	+ 0.06	—	26.7
7	30	2.40	2.60	+ 0.20	Blue	40.3
17	30	2.35	2.46	+ 0.11	Blue-violet	54.7
40	30	2.35	2.38	+ 0.03	„	57.5

* Calculated.

Exp. 4. A, 100 ml. of 3.00% α -amylodextrin + 20 ml. α -amylase ($\equiv$ 40 g. malt). B, 4.00% α -amylodextrin, hydrolysed by α -malt-amylase to 20.7%

hydrolysis, the enzyme destroyed and 100 ml. of the product equivalent to 2.84 % of undegraded α -amylodextrin + 20 ml. barley amylase ($\equiv$ 40 g. barley). C, 100 ml. of 3.00 % α -amylodextrin + 20 ml. α -malt-amylase ($\equiv$ 40 g. malt) + 20 ml. barley amylase ($\equiv$ 40 g. barley). Temp. 20°, p_H 4.6. Results in Table IV (4 A, 4 B, 4 C). Percentage hydrolysis in the reaction mixtures is indicated in col. 6, the figures in brackets referring to the filtrates, and all in terms of the substrate concentration in the original reaction mixtures.

Exp. 5. 100 ml. of 3.0 % glycogen + 20 ml. A, α -malt-amylase; B, barley amylase. Temp. 23°, p_H 4.6. Results in Table IV (5 A, 5 B).

Exp. 6. 60 ml. of 1.95 % amyloamylose + 10 ml. A, α -malt-, B, barley amylase. Temp. 20°, p_H 4.6. Results in Table IV (6 A, 6 B).

Exp. 7. 60 ml. of 1.95 % erythroamylose + 10 ml. A, α -malt-, B, barley amylase. Temp. 20°, p_H 4.6. Results in Table IV (7 A, 7 B).

Table IV. *Progress of reactions and mutarotations of filtrates.*

	Time of filtration min.	Time of reading min.	Rotation (1 dm.)	Rotation after alkali addi- tion (1 dm.) (angular degrees; corrected)	Muta- rotation (1 dm.)	Conversion (iodimetric) maltose %
α -Amylodextrin						
4 A	—	5	—	—	—	16.2
	—	12	—	—	—	20.2
	8–15	17	2.92	2.78	–0.14	(11.5)
	—	35	—	—	—	25.3
	33–38	40	2.92	2.79	–0.13	(15.8)
	—	8 (hr.)	—	—	—	50.1
4 B	—	7	—	—	—	10.6
	10–12	14	3.85	3.95	+0.10	(6.6)
	—	30	—	—	—	12.6
	30–32	34	3.74	3.81	+0.07	(7.5)
	—	90	—	—	—	14.3
4 C	—	5	—	—	—	27.0
	—	12	—	—	—	33.0
	8–13	15	2.77	2.71	–0.06	(22.2)
	—	35	—	—	—	37.8
	33–38	40	2.61	2.55	–0.06	(24.7)
	—	4½ (hr.)	—	—	—	49.9
Glycogen						
5 A	10–20	22	3.93	3.80	–0.13	(20.5)
	32–38	40	4.51	4.43	–0.08	(26)
5 B	10–14	16	4.11	4.21	+0.10	(20)
	30–34	36	3.88	3.97	+0.09	(25)
Amyloamylose						
6 A	10–12	14	1.01	0.96	–0.05	—
6 B	10–12	14	1.11	1.17	+0.06	—
Erythroamylose						
7 A	10–12	14	2.19	2.04	–0.15	—
	30–32	34	2.02	1.89	–0.13	—
7 B	10–12	14	1.94	2.01	+0.07	—
	30–32	34	1.73	1.86	+0.13	—

DISCUSSION.

The filtered solutions contained very little enzyme, in Exp. 1 A none. Mercuric chloride (Exp. 1 B) and silver nitrate (Exps. 2 A and 2 B) were added to prevent further enzyme action. Traces of chlorides in the pancreatic amylase

prevented the use of silver in the case of Exp. 1 B. Control tests showed that these salts in the concentrations used effectively checked enzyme action but had no perceptible effect on the rate of mutarotation of maltose.

α -Amylase and β -amylase, with all the substrates used, gave filtrates which exhibited α - and β -mutarotations respectively, the unimolecular velocity constants of which approximated to those of β -maltose under the same conditions. The initial fission products of α -amylase are reducing and mutarotating "dextrins" and some α -maltose, the later products, *e.g.* at 60–70 % hydrolysis as in Exp. 1, mainly α -maltose to which the observed velocity constants refer. The velocity constants of mutarotation of pure β -maltose to equilibrium have been determined by us over a wide range of temperature and p_H in the absence of salts, the relevant results being summarised in Table V.

Table V. *Unimolecular velocity constants of mutarotation at 20° (unbuffered).*

p_H	$k \times 10^3$			
	4.52	4.88	6.72	6.84
β -maltose to equilibrium	5.00	5.00	—	5.48
α -amylase fission products	—	—	6.02	6.20
β -amylase fission products	5.68	5.66	—	—

In the presence of acetate buffers, 0.04*N*, the values of the constant for β -maltose were substantially higher, *e.g.* 5.8 instead of 5.0 at p_H 4.66, and 11.16 instead of 5.4 at p_H 6.81. The ions, Na^+ and Cl^- , which were present in small concentrations in the reaction mixtures have no catalytic effect on mutarotation, but unavoidable traces of other ions may account for the values of $k \times 10^3$ being high.

SUMMARY.

Experiments recorded, taken in conjunction with those in Parts I and II, indicate that the α - and β -types of amylase, as typified by those of the pancreas, malt and ungerminated barley, respectively attack starch in different ways. Whilst each enzyme degrades most easily the same 50–60 % of the starch, including the whole of the amyloamylose portion, the α -enzyme alone can attack the α -amylodextrin portion. Comparatively little of the fission products arises from degradation of the latter during the first 50–60 % of the hydrolysis of starch by the α -enzyme.

Further differences in the mode of action are evident. In the initial stages of hydrolysis, α -amylase produces reducing dextrins and a little maltose, giving α -mutarotation, whilst β -amylase produces β -maltose as almost the sole reducing product. Later the reducing products resulting from the action of the α -enzyme include increasing proportions of α -maltose, as was indicated by the observations of Kuhn and confirmed with certain precautions in this work. The velocities of mutarotation to equilibrium of the filtered reaction products of advanced hydrolyses with both enzymes agree reasonably well with those observed for β -maltose under similar conditions. The possibility that the observations of Kuhn and of Ohlsson were due, in the cases of one or both enzymes, to mutarotation of unattacked substrate, one component of which had been selectively hydrolysed, is excluded.

The sense of mutarotation of the reaction products is a characteristic of the enzyme and is independent of the substrate employed, whether it be soluble

starch, one of its components or glycogen. In short α - and β -amylases, attacking the same material, produce respectively α -dextrins (and later α -maltose) and β -maltose.

One of us (G. G. F.) is indebted to the Department of Scientific and Industrial Research for a grant.

REFERENCES.

- Freeman and Hopkins (1936, 1). *Biochem. J.* **30**, 442.
—— — (1936, 2). *Biochem. J.* **30**, 446.
Hanes (1935). *Can. J. Res. B*, **13**, 185.
Kuhn (1925). *Liebig's Ann.* **443**, 1.
Ohlsson (1926). *Compt. Rend. Trav. Lab. Carlsberg*, **16**, No. 7.
—— (1930). *Z. physiol. Chem.* **189**, 17.
Van Klinkenberg (1934). *Ergebn. Enzymforsch.* **3**, 73.