698. *The Action of Crystalline β-Amylase on Some Glycogens.*

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Glycogens (chain-length *ca.* twelve radicals) on β-amylolysis with the crystalline sweet-potato enzyme lose *ca.* 45% of their exterior chains solely as maltose. Available evidence points to an irregularly branched structure for glycogens; on the average, exterior chains contain *ca.* 7 glucose radicals, and interior chains *ca.* 4. The positions of branching points in the unit chains of amylopectins and 18-radical glycogens are discussed.

By potassium periodate oxidation, chain lengths of thirteen units were found for cat-liver and fœtal-sheep-liver glycogens. Three samples of glycogen from *Mytilus edulis* had different chain lengths (*ca.* 5, 12, and 17). A sample of *Helix pomatia* glycogen had a chain length of *ca.* 7 (cf. Baldwin and Bell, *Biochem. J.*, 1940, **34**, 139). These very short-chain glycogens gave no evidence of being degraded.

The approximate position of the branching points in the constituent unit chains of polysaccharides of the amylopectin–glycogen type can be assessed by studying the action thereon of purified β-amylase. β-Amylase attacks only the exterior chains of 1 : 4-α-linked glucose radicals, and by the stepwise hydrolysis of alternate glucosidic links liberates maltose. Hydrolysis starts at the non-reducing end of the exterior chain and continues until an obstruction is encountered; this appears to be the inter-chain 1 : 6-linkage. The products of the β-amylolysis of these branched polysaccharides are therefore maltose and a dextrin of high molecular weight with outer chain "stubs" consisting of perhaps one or two glucose radicals and still retaining intact the original inter-chain linkages (cf. Bernfeld, *Adv. Enzymology*, 1951, **12**, 379; Hanes, *New Phytologist*, 1937, **36**, 101, 189; Myrbäck and Neumüller, "The Enzymes," New York, 1950, Vol. I, Part 1, p. 653; Peat, *Adv. Enzymology*, 1951, **11**, 339). An accurate determination of the maltose produced by β-amylolysis is therefore a measure of the exterior chain length.

Few studies of the action on glycogens (unit chain length often undetermined) of non-crystalline, and almost certainly mixed, enzyme preparations having β-amylolytic activity have so far been reported. Table 1 emphasises the state of present knowledge, only seven out of 16 specimens having been analysed for chain length.

Table 1. *The β-amylolysis of some glycogens (non-crystalline β-amylases).*

Source of glycogen	Unit chain length * A	Unit chain length * B	Source of β-amylase	Conversion into maltose, %	Ref.
Beef liver (Hoffmann–La Roche)	—	—	Wheat	45	*a*
Mussel (Merck)	—	—	Wheat	45	*a*
Mussel (Merck)	11	—	Wheat	47	*b*
Mussel, fraction I †	—	—	Wheat	43	*c*
,, II	—	—	Wheat	33	*c*
,, III	—	—	Wheat	31	*c*
Hoffmann–La Roche	—	—	Wheat	46	*c*
Hoffmann–La Roche	—	11	Barley	38—46	*d*
Corn (Golden Bantam)	11	—	Wheat	20	*e*
Rabbit liver	—	—	Wheat	45	*e*
Corn (Golden Bantam)	—	10	Wheat	48	*f*
Yeast, fraction I †	—	—	Wheat	47	*g*
,, II	—	—	Wheat	45	*g*
Yeast	—	12	Barley	50	*h*
Rabbit liver	—	12	Barley	43	*h*
Rabbit liver	18	—	Wheat	53	*i*

* A, By methylation. B, By periodate oxidation. † Fractionation by electrodialysis.

[a] Meyer and Press, *Helv. Chim. Acta*, 1941, **24**, 58. [b] Meyer and Fuld, *ibid.*, 1941, **24**, 375. [c] Meyer and Jeanloz, *ibid.*, 1943, **26**, 1784. [d] Carlquist, *Acta Chem. Scand.*, 1948, **2**, 770. [e] Morris, *J. Biol. Chem.*, 1944, **154**, 503. [f] Meyer and Fuld, *Helv. Chim. Acta*, 1949, **32**, 757. [g] Jeanloz, *ibid.*, 1944, **27**, 1501. [h] Northcote, *Biochem. J.*, 1952, in the press. [i] Halsall, Hirst, Hough, and Jones, *J.*, 1949, 3200.

In our work we used only samples of glycogen prepared by ourselves. Commercially derived preparations may be of uncertain origin, chemically degraded, and even more heterogeneous than average laboratory products (cf. Putzeys and Verhoeven, *Proc. Int. Colloquium Macromol., Amsterdam*, 1949, 267; Kerr, Katzbeck, and Cleveland, *J. Amer. Chem. Soc.*, 1951, **73**, 111). Through the kindness of Professor C. S. Hanes, F.R.S., we were provided with a sample of crystalline sweet-potato β-amylase prepared by Professor A. K. Balls (Balls, Thompson, and Walden, *J. Biol. Chem.*, 1946, **163**, 571; 1948, **173**, 9). The enzyme was free from maltose and α-amylase, and had no Z-enzyme activity (cf. Peat, Pirt, and Whelan, *J.*, 1952, 705) as judged by its inability to hydrolyse soluble laminarin or yeast glucan or to hydrolyse potato amylose to more that 76%. One of us has recently found (Manners, 1952, Thesis, Cambridge) that non-crystalline barley β-amylase preparations, although free from maltase and α-amylase, contain sufficient of a β-glucosidase to attack not only insoluble yeast glucan and soluble laminarin (cf. Peat, Thomas, and Whelan, *J.*, 1952, 722; Dillon and O'Colla, *Nature*, 1950, **166**, 67; *Chem. and Ind.*, 1951, 111), but also to remove the barriers in potato amylose preventing complete β-amylolysis.

We have examined the action of Ball's crystalline β-amylase on liver and muscle glycogens from various mammals, and on certain invertebrate glycogens. Our results, when enzymic action had been carried to completion, are summarised in Table 2 [a preliminary account of these results has recently been given by one of us (Manners, *Biochem. J.*, 1952, **51**, xxx)].

TABLE 2. *The β-amylolysis of some glycogens (crystalline β-amylase).*

Source of glycogen	Unit chain length * A	Unit chain length * B	conversion into maltose (%)	Approx. exterior chain length
Rabbit liver	12[a]	13	43	7
Foetal-sheep liver	—	13	49	8
Cat liver	—	13	48	8
Rabbit muscle	11[a]	13	45	7
Horse muscle	12[b]	11	42	6
Human muscle	—	12	41	6—7
Mytilus edulis, I	—	12	43	6—7
,, II	18[c]	16	47	9—10
,, III	—	*ca.* 5	41	3
Ascaris lumbricoides	13—14[d]	12	49	7
Helix pomatia	—	*ca.* 7	37	4

* See footnote to Table 1.

[a] Bell, *J.*, 1948, 992. [b] Bell, *Biochem. J.*, 1937, **31**, 1683. [c] *Idem, ibid.*, 1936, **30**, 2144. [d] Baldwin and King, *ibid.*, 1942, **36**, 37.

When no further reducing sugar was liberated in each experiment, suitable examination of the digests showed that the β-amylase was still active. In certain experiments, further addition of enzyme failed to increase the β-amylolysis limit. The values in Table 2 therefore represent true β-amylolysis limits. Paper chromatography (Hough, Jones, and Wadman, *J.*, 1950, 1702) showed maltose to be the only sugar present. The absence of D-glucose was confirmed by manometrical treatment of final digests with glucose oxidase (cf. Keilin and Hartree, *Biochem. J.*, 1948, **42**, 230), no oxygen uptake being observed.

We determined the unit chain lengths of the glycogens essentially by potassium periodate oxidation at room temperature (Halsall, Hirst, and Jones, *J.*, 1947, 1399). Halsall *et al.* calculated unit chain lengths from formic acid titrated after 150 hours, this being the time taken for the similar production of 1 mol. of formic acid from β-methylmaltoside (Halsall, Hirst, and Jones, *J.*, 1947, 1429). In our experience, a 150-hour titre gives incorrect values for unit chain lengths; we have therefore allowed oxidation to proceed until a maximum constant formic acid concentration is reached (Table 3). Under these conditions, the final formic acid concentration is *ca.* 0·0025N and is not affected by the excess of periodate (cf. Sarkar, *Nature*, 1951, **168**, 122). We find no evidence of "over-oxidation"; no free iodine appeared in the reaction media. Our results agree closely with those obtained by methylation end-group assay of the *same* samples (Table 2).

Our experiments show that β-amylolysis of a 12-radical glycogen converts *ca.* 45% of the molecule into maltose, leaving unattacked a β-limit-dextrin of chain length *ca.* 6·5. Since

the exterior chain "stubs" of this dextrin contain *ca.* 1·5 glucose radicals (Meyer, *Adv. Enzymology*, 1943, **3**, 109) it follows that (*a*) the glycogen has an exterior chain length of *ca.* 7 glucose radicals, (*b*) the average position of the inter-chain branch point is at the 8th glucose radical from the non-reducing end, and (*c*) the average interior chain length must be *ca.* 4 radicals.

As already noted, β-amylolysis is considered to shorten exterior chains of glycogen by hydrolytic removal of maltose radicals. From the results in Table 2, we observed that, in some instances, β-amylolysis results in the loss of an odd or a fractional number of glucose radicals—wholly, however, in the form of maltose. These numbers must represent the average of the loss of an even number of glucose radicals from individual unit chains, thus indicating a variable exterior chain length, and hence an irregularly branched structure. Other available evidence points to an irregularly branched structure for glycogen (cf. Meyer, *loc. cit.*). Bell and Manners (*Biochem J.*, 1951, **49**, lxxvii) have shown that barley α-amylase will hydrolyse some (but not all) of the interior chains of rabbit-liver glycogen. The residual dextrins so obtained can be hydrolysed further by barley β-amylase. The hydrolysis of an interior chain is assumed to occur at about the mid-point (Myrbäck, *Arkiv Kemi*, 1950, **2**, 417), and since some interior chains thus ruptured are susceptible to further β-amylolysis the fragments must contain at least 3—4 glucose radicals. The original glycogen must therefore contain some interior chains of 6—8 glucose radicals, despite our observed mean interior chain length of *ca.* 4. Swanson and Cori (*J. Biol. Chem.*, 1948, **172**, 815), after a study of the "activation" of phosphorylase by glycogen α-dextrins, suggested that some of the interior chains of glycogen must contain *ca.* 6—8 glucose radicals. Swanson (*J. Biol. Chem.*, **172**, 825), from a study of their iodine complexes, suggested that glycogen β-limit-dextrins, too, contain some interior chains of *ca.* 8 glucose radicals. Meyer and Fuld (*Helv. Chim. Acta*, 1941, **24**, 375) found that mussel glycogen of chain length 11 contained interior chains of an average length of 3 glucose radicals. From Table 2 it can be seen that eight different samples of 12-unit glycogen have an average interior chain length of *ca.* 4 glucose radicals. It therefore seems likely that the interior structure of glycogen is irregular, and that glycogen should be considered as a macromolecule built up of several thousand unit chains, of varied chain length—the average of which is normally 12—which are randomly linked together by means of 1 : 6-glucosidic linkages. We do not propose to comment, at the moment, on the existence of glycogens with unit chains of *ca.* 6 or 18 glucose radicals (cf. Abdel-Akher and Smith, *J. Amer. Chem. Soc.*, 1951, **73**, 994). We have noted, however, that the glycogens from *Helix pomatia* and *Mytilus edulis* III had a negligible reducing power, and that the former gave an opalescent solution, which is indicative of high molecular weight (cf. Harrap and Manners, *Nature*, 1952, in the press. It is concluded that these invertebrate glycogens are the native polysaccharides, and not artefacts produced by degradation during their isolation.

β-Amylolysis of amylopectins has been studied by several groups of workers, *e.g.*, K. H. Meyer *et al.*, Myrbäck *et al.*, and Haworth *et al.*, and the results obtained have been reviewed by Myrbäck (*loc. cit.*), Bell (*Ann. Reports*, 1947, **44**, 217) and Bernfeld (*loc. cit.*). They show that 50—62% conversion of the polysaccharide into maltose takes place, which indicates that the mean situation of the branch point of a 25-radical amylopectin is at *ca.* the 17th glucose radical from the non-reducing end, and that the ratio of the exterior chain length to the interior chain length is 16 : 8. We find that the ratio of the exterior to interior chain lengths of a 12-unit glycogen is also *ca.* 2 : 1. Glycogens therefore differ from amylopectins mainly in the length of their unit chains, and not in the *relative* position of their branch points, even though β-amylolysis removes a relatively greater part of amylopectin (*ca.* 55%) than of glycogen (*ca.* 45%). The latter fact presumably arises from the mode of synthesis of these branched polysaccharides, the P- and Q-enzymes in plants having catalytic properties almost identical with those of the phosphorylase and the branching of animal tissues.

It is interesting to compare our results on the β-amylolysis of 12-radical glycogens with that of Halsall, Hirst, Hough, and Jones (*J.*, 1949, 3200) who found that a sample of rabbit-liver glycogen (chain length 18) gave a 53% conversion into maltose.

The exterior chain length of this polysaccharide is therefore *ca.* 11, and the interior chain length *ca.* 6, pointing to a structure intermediate between amylopectin and a typical 12-radical glycogen.

Experimental

Preparation of Glycogens.—The glycogens, isolated from the appropriate tissue by the "Pflüger technique" or by hot aqueous extraction, were purified by precipitation with acetic acid (Bell and Young, *Biochem. J.*, 1934, **28**, 882) and dried at 100° over phosphoric oxide for 3—4 hours in a high vacuum.

Crystalline β-Amylase Solution.—A stock solution (*ca.* 20 mg. in 20 ml. of water) was stored at 0° and diluted as required.

Determination of Reducing Sugar.—Reducing sugar (maltose) was determined by the Shaffer–Somogyi reagent 60 (*J. Biol. Chem.*, 1933, **100**, 695) as modified by Hanes and Cattle (*Proc. Roy. Soc.*, 1938, *B*, **125**, 387). The reagent, calibrated against maltose, would determine 0·5—4·0 mg. of maltose in 5 ml. of aqueous samples, provided the time of heating (15 minutes) was strictly adhered to.

Precautions against Mould Contamination.—Toluene, as used by Halsall *et al.* (*loc. cit.*) with crystalline β-amylase, was found to denature some of the enzyme. A concentration of 0·1—0·2% of propionate (cf. Crook, Carpenter, and Klens, *Science*, 1950, **112**, 656) prevented mould contamination during enzymic digestion.

Preliminary Experiments (*at* 28°).—(a) *Reducing power of the enzyme.* A digest was set up containing β-amylase solution (1 ml.) distilled water (7 ml.), and 0·2M-acetate buffer of pH 4·6 (5 ml.). Aliquot portions (4 ml.) of the digest were analysed for reducing power:

Time of incubation (hrs.)	0	70
Ml. of 0·01N-$Na_2S_2O_3$	0·04	0·02

i.e., the enzyme itself has a negligible reducing power.

(b) *Maltase activity.* A digest was set up containing maltose (15·6 mg.), 0·2M-acetate buffer of pH 4·6 (9 ml.), distilled water (20 ml.), 9% sodium propionate solution (0·5 ml.), and β-amylase solution (0·5 ml.). Aliquot portions (4 ml.) were analysed:

Time of incubation (hrs.)	0	47	200
Ml. of 0·01N-$Na_2S_2O_3$	3·65	3·60	3·57

i.e., the enzyme has no maltase activity.

(c) *α-Amylase activity.* A digest was set up containing 1% starch solution (2 ml.), 0·2M-acetate buffer of pH 4·6 (9 ml.), and distilled water (22 ml.). A sample (4 ml.) was withdrawn for determination of the reducing power of the starch. β-Amylase solution (1 ml.) was added, and aliquot portions (4 ml.) of the digest were analysed at intervals for maltose:

Time of incubation (hrs.)	0·16	0·5	1·67	24	46
Maltose found (mg.)	12·3	12·5	12·5	12·5	12·4

The constancy of the reducing power of the digest shows the absence of α-amylase in the β-amylase preparation.

(d) *Z-Enzyme activity.* Digests were set up containing yeast glucan (37·7 mg.) or soluble laminarin (35·9 mg.), distilled water (6 ml.), 0·2M-acetate buffer of pH 4·6 (5 ml.), and β-amylase solution (1 ml.). Aliquot portions (5 ml.) were analysed:

	Glucan digest	Laminarin digest
Time of incubation (hrs.)	Ml. of 0·01N-$Na_2S_2O_3$	
0	0·41	0·05
70	0·50	0·22

The slight increases in reducing power correspond to a hydrolysis of the glucan of *ca.* 0·14% and of the laminarin of *ca.* 0·3%.

The action of β-amylase on potato amylose was also studied as follows: the digest contained amylose solution (2·21 mg./ml.; 10 ml.), 0·2M-acetate buffer of pH 4·6 (5 ml.), distilled water (19 ml.), 9% sodium propionate solution (0·5 ml.), and β-amylase solution (0·5 ml.). The

amylose concentration was determined by Pirt and Whelan's method (*J. Sci. Food Agric.*, 1951, **5**, 224). The course of hydrolysis was as follows :

Time of incubation (hrs.)	17	41	335
% Conversion to maltose	69·5	71·2	75·6

In the presence of Z-enzyme, β-amylase hydrolysis of amylose was almost complete (Peat, Thomas, and Whelan, *J.*, 1952, 722).

Main Experiments.—Enzymic digests were set up containing glycogen (30—60 mg.), 0·2M-acetate buffer of pH 4·6 (5—1 ml.), distilled water (10—25 ml.), 9% sodium propionate solution (0·5—1·0 ml.). A sample (usually 3 ml.) was removed for the determination of the reducing power of the glycogen. β-Amylase solution (1 ml.) was then added. Samples were analysed, at intervals, for maltose, after incubation at 28°. The final β-amylolysis limits so obtained are recorded in Table 2 (mean of duplicate experiments which agreed within ±1%). When a constant maximum titre has been obtained, one drop of each digest was added to 2 ml. of 1% starch solution. The presence of active enzyme was shown by the formation of reducing sugar, controls being carried out with 1 drop of each digest in 1 ml. of water. In some experiments a second portion of β-amylase solution was added, after β-amylolysis has proceeded for 22 hours, but without increase in the β-amylolysis limit.

Examination of the β-Amylolytic Digests for D-*Glucose.*—(a) *Paper chromatography.* Samples of the digests were examined on paper chromatograms (Hough, Jones, and Wadman, *J.*, 1950, 1702), aniline phthalate being used as the developing agent. In all instances, maltose alone was detected.

(b) *Glucose oxidase.* Aliquots (3 ml.) of the digests were treated with glucose oxidase (cf. Keilin and Hartree, *Biochem. J.*, 1948, **42**, 230) in the presence of azide (Monod, personal communication). No oxygen uptake was observed. A control experiment, using 1·58 mg. of glucose, gave an oxygen uptake of 195 μl., equivalent to 123 μl./mg. of glucose (theory, 124·4 μl./mg. of glucose). Thus the only reducing sugar present in the β-amylolytic digests was maltose, and hence, the determination of the increase in reducing power (as maltose) of an aliquot portion of the digest was a true measure of the extent of β-amylolysis.

Potassium Periodate Oxidation of Glycogens.—The oxidations (Halsall, Hirst, and Jones, *J.*, 1947, 1399) were carried out at 15—20° in brown bottles (cf. Head, *Nature*, 1950, **165**, 236), on rotating rollers. Aliquots, after addition of ethylene glycol, were titrated against 0·01M-sodium hydroxide, in a stream of carbon dioxide-free air (methyl-red).

Preliminary experiments on muscle glycogens from horse, man, and rabbit showed that production of formic acid continued after 150 hours (Table 3). The unit chain lengths, in glucose radicals, calculated from the 150-hr. titre were: horse muscle glycogen, 16; rabbit muscle glycogen, 17; and human muscle glycogen, 15.

TABLE 3. *Oxidation of muscle glycogens by saturated potassium periodate solution.*

	Formic acid production (mg.)				Formic acid production (mg.)		
Time (hrs.)	Human (414·8 mg.)	Rabbit (411·7 mg.)	Horse (684·4 mg.)	Time (hrs.)	Human (414·8 mg.)	Rabbit (411·7 mg.)	Horse (684·4 mg.)
64	4·1	3·0	6·8	166	8·4	6·8	13·4
93	6·0	4·9	9·4	208	9·6	8·1	15·1
138	7·4	5·5	11·8	256	10·5	8·5	15·9
150	7·9	6·6	12·4	332	10·5	8·6	16·0

The method of Halsall *et al.* (*loc. cit.*) was therefore modified in the present study, in that the unit chain length was calculated from the maximum formic acid titre (after *ca.* 300 hours). The results so obtained are summarised in Table 2.

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