682. α-1 : 4-*Glucosans. Part VI.* Further Studies on the Molecular Structures of Glycogens.*

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Twenty-five samples of glycogen, with average chain lengths ranging from 8 to 18 glucose residues, have been degraded by barley β-amylase. The percentage conversions into maltose were 46 ± 7 in all but two instances. *Cardium* glycogen and one sample of rabbit liver glycogen had unusually low β-amylolysis limits of 14 and 25% respectively. These results illustrate the variation in molecular structure which can exist in glycogens.

The highly branched nature of the interior of several glycogens has been confirmed by α-amylolysis.

The use of acetic acid in the purification of glycogen is discussed.

Our earlier studies [1,2] indicated differences in the degree and position of branching among (*a*) glycogens from different biological sources, and (*b*) different glycogen samples from the same source. These observations are extended in the present paper, which reports structural analyses of several additional glycogens by periodate oxidation, and by the use of β- and α-amylase. A preliminary account of part of this work has been published.[3]

Twenty-five samples of glycogen have been analysed. A number of these were kindly provided by Drs. D. J. Bell and G. R. Tristram and the determination of certain chain lengths ($\overline{CL}$) has already been reported.[4] The remainder were isolated by extraction of the tissues with hot water or 30% potassium hydroxide (Pflüger method) and purified by

* Part V, *J.*, 1957, 2205.

[1] For review, see Manners, *Adv. Carbohydrate Chem.*, 1957, **12**, 261.
[2] Bell and Manners, *J.*, 1952, 3641.
[3] Liddle and Manners, *Biochem. J.*, 1955, **61**, xii.
[4] Manners and Archibald, *J.*, 1957, 2205.

precipitation first from 80% acetic acid and then from ethanol.[5] The polysaccharides had glucose contents of 94—97%, specific rotations (in water) of 191° to 201°, and the glycogen–iodine complexes showed maximum absorption at 420—470 mμ. Average chain lengths were determined by measurement of the maximum amount of formic acid liberated by potassium metaperiodate oxidation at room temperature. This method of analysis gives reproducible results which are in agreement with enzymic or methylation analyses of the *same* samples.[2,4]

The glycogens were incubated with barley β-amylase, at pH 4·6 and 35°, and the percentage conversions into maltose determined (see Table 1). The enzyme preparation was free from maltase and α-amylase, but contained Z-enzyme [6] and showed slight hydrolytic activity towards maltotriose. The hydrolytic activities due to Z-enzyme and that towards maltotriose are not significant, since Z-enzyme does not affect the β-amylolysis of glycogens, and maltotriose is not a product of enzyme action.

Peat, Whelan, and Thomas [7] have shown that the side chains in an amylopectin β-limit dextrin contain 2 or 3 glucose residues, whilst Summer and French,[8] from a study of the

TABLE 1. *The action of barley β-amylase on some glycogens.*

Sample no.	Source of glycogen	$\overline{CL}$ [a]	β-Amylolysis limit (%)	Exterior [b] chain length	Interior [c] chain length
1	*Arenicola*	11	43	7—8	2—3
2	*Cardium*	8	14	3—4	3—4
3	Cat liver IV	13[4]	53 [d]	9—10	2—3
4	,, VI	12[4]	52 [d]	8—9	2—3
5	Cock liver	13	39	7—8	4—5
6	Fœtal pig liver	11[4]	49	8	2
7	Fœtal sheep liver (alkali-treated) [e]	13[2]	49	9	3
8	Human muscle II	11	40	7	3
9	*Mytilus edulis* IV	12[4]	51	8—9	2—3
10	,, V	9[4]	40	6	2
11	,, VI	13[4]	46	8—9	3—4
12	,, VII	13	46	8—9	3—4
13	,, VIII	13	45	8—9	3—4
14	,, IX	10	51	7—8	1—2
15	,, X	14	45	8—9	4—5
16	Rabbit liver I	13	25	5—6	6—7
17	,, III	13[4]	51	9	3
18	,, IV	13[4]	45	8—9	3—4
19	,, V	14[4]	51	9—10	3—4
20	,, VI	18[9]	52	12	5
21	,, X	12[4]	49	8—9	2—3
22	,, XII	17	43	9—10	6—7
23	,, XIII	15	46	9—10	4—5
24	Rabbit muscle II	11	39	6—7	3—4
25	Skate liver	13	45	8—9	3—4

[a] Superscript numbers refer to chain-length determination reported in refs. 2, 4, or 9. [b] No. of glucose residues removed by β-amylase + 2·5. [c] $\overline{CL}$ — exterior chain length — 1. [d] Incubation with crystalline sweet-potato β-amylase gave β-amylolysis limits of 54 and 52% respectively. [e] Before alkali treatment, this glycogen had a β-amylolysis limit of 49%.[2]

action of β-amylase on model substrates, concluded that the exterior chain "stubs" in a glycogen or amylopectin β-limit dextrin "would never be any shorter than 2·5 glucose units." The exterior and interior chain lengths reported in Table 1 have therefore been calculated on the assumption that β-amylase action on glycogen ceased at the second or third glucose residue from the outermost branch points.

[5] Bell and Young, *Biochem. J.*, 1934, **28**, 882.
[6] Peat, Thomas, and Whelan, *J.*, 1952, 722.
[7] Peat, Whelan, and Thomas, *J.*, 1952, 4546.
[8] Summer and French, *J. Biol. Chem.*, 1956, **222**, 469.
[9] Haworth, Hirst, and Isherwood, *J.*, 1937, 577.

It has been suggested [10] that purification of glycogens by precipitation with acetic acid renders them less susceptible to degradation by the enzymes muscle phosphorylase and amylo-1 : 6-glucosidase. After treatment with hot alkali, the glycogens could then be completely degraded by these enzymes. Fœtal sheep liver glycogen, purified by the acetic acid method, has therefore been digested with 0·1N-sodium hydroxide at 100° for 30 min. This treatment did not increase the β-amylolysis limit. Further, four precipitations of rabbit liver or yeast glycogen from 80% acetic acid, followed by an ethanol precipitation, did not decrease the β-amylolysis limits. We conclude that the purification of glycogen with acetic acid does not affect the extent of degradation by β-amylase.

The above results clearly demonstrate the variation in branching characteristics which exist in glycogens, and in particular, the structural differences in samples from the same biological source. Thus, $\overline{CL}$ values for rabbit liver glycogen range from 12 to 18, whilst glycogens (samples 16, 17, 18) with the same chain length differ in position of branching in the constituent chains. Rabbit liver I glycogen is unusual in that both the exterior and interior chains contain *ca.* 6 glucose residues; normally, the exterior chains are roughly twice the length of the interior chains. The presence of relatively short chains in rabbit liver I glycogen has been confirmed, since the extent of degradation by muscle and potato phosphorylase was abnormally low.[11]

Glycogens from *Arenicola* (lug worm), cock liver, and *Cardium* (cockle) do not appear to have been examined previously. The first two samples have normal structures, but the *Cardium* glycogen has unusually short exterior chains (3—4 glucose residues) although the interior chains are of normal length.

The action of purified salivary α-amylase on a number of glycogen-type polysaccharides has also been investigated. Although α-amylases catalyse random hydrolysis of α-1 : 4-glucosidic linkages in these polysaccharides, the extent of degradation should be related

TABLE 2. *The α-amylolysis of some branched α-1 : 4-glucosans.*

Source of polysaccharide	Percentage of α-1 : 6-glucosidic linkages	Apparent % conversion into maltose (P_M) * 2 hr.	6 hr.	24 hr.
Glycogens :				
Arenicola	9	56	71	76
Cardium	13	39	49	54
Cock liver	8	57	72	76
Helix pomatia	14	51	64	67
Mytilus edulis VII	8	60	73	80
,, VIII	8	61	76	80
,, IX	10	62	78	83
,, X	7	61	75	80
Skate liver	8	59	75	80
Amylopectin :				
Waxy maize starch	5	78	93	95
β-Limit dextrins : †				
Helix pomatia glycogen	22	21	26	29
Waxy maize starch	10	48	59	66

* All digests contained 4·6 units of α-amylase per mg. polysaccharide (see p. 3436).

† Prepared by the prolonged action of β-amylase on the polysaccharide, removal of maltose by dialysis, and isolation by freeze-drying.

to the proportion of α-1 : 6-glucosidic linkages present, since these linkages and certain adjacent α-1 : 4-glucosidic linkages resist enzymic action.[12] Several polysaccharides were therefore incubated (at pH 7·0 and 35°) with salivary α-amylase, and the apparent percentage conversion into maltose (P_M) determined at intervals (see Table 2). Paper chromatography showed that extensive random hydrolysis had occurred, the products being glucose,

[10] Illingworth, Larner, and Cori, *J. Biol. Chem.*, 1952, **199**, 631.
[11] Liddle, Ph.D. Thesis, Edinburgh, 1956.
[12] Whelan and Roberts, *Nature*, 1952, **170**, 748.

maltose, maltotriose (trace), and oligosaccharides with R_G 0·10, 0·05, and lower chromatographic mobilities.

Since the relative proportion of enzyme and substrate was constant, the variation in P_M values must reflect differences in molecular structure. In general, the extent of α-amylolysis appears to be inversely proportional to the degree of branching in the substrate. The low P_M values for *Helix pomatia* [2] and *Cardium* glycogens are in accord with the earlier evidence for compact and highly branched structures. Conversely, waxy maize starch and its β-limit dextrin have relatively open interior structures, since *ca.* 47 and 33% of the glucosidic linkages in the molecules can be hydrolysed by α-amylase.

Peat, Roberts, and Whelan [13,12] reported that the products of α-amylolysis of a sample of rabbit liver glycogen included *ca.* 5% of maltulose and small quantities of fructose-containing α-dextrins, suggesting that fructose was a minor component of the glycogen. Examination of our α-amylase digests by paper chromatography with the orcinol spray reagent [14] or the acid resorcinol reagent [15] failed to show the presence of ketoses. It is concluded that fructose is not a constituent of the above glycogens.

Experimental

Preparation of Glycogens.—We are indebted to Dr. D. J. Bell for glycogen samples 7, 8, 16, and 17 and the cock liver tissue, and to Dr. G. R. Tristram who provided samples 14 and 15 and many of the animal tissues. The isolation and certain properties of these glycogens are recorded in Table 3. The yield varied from 0·3 to 1·6 g. per 100 g. of wet tissue, and the polysaccharides

Table 3. *The preparation and properties of some glycogens.*

Source of glycogen	Method of isoln.[a]	Method of purifn.[b]	$[\alpha]_D$, H_2O	$\lambda_{max.}$ of iodine complex (mμ) [c]
Arenicola	P	A	+200°	420
Cardium	W	PA	+201	420
Cock liver	W	PA	+191	440
Human muscle	P	A	+195	—
Mytilus edulis VII.....	W	PA	+200	420
,, VIII.....	W	PA	+194	420
,, IX.....	W	PA	+196	—
,, X.....	W	PA	+199	—
Rabbit liver XII.....	W	A	+193	470
,, XIII.....	W	A	+199	470
Rabbit muscle II.....	P	A	+194	—
Skate liver	P	A	+196	420

[a] P = Pflüger method; W = hot-water extraction. [b] A = precipitated with acetic acid and ethanol; PA = deproteinised with picric acid, then as A. [c] Determined as by Peat, Whelan, Hobson, and Thomas (*J.*, 1954, 4440) and corrected for light scattering.

had glucose contents of 94—97% (paper chromatography, and Shaffer–Somogyi estimation [16] after hydrolysis with 1·5N-sulphuric acid for 2 hr. at 100°).

Potassium Metaperiodate Oxidation of Glycogens.—The method was similar to that previously described,[3,4] the formic acid being titrated potentiometrically to pH 5·7 with carbonate-free [17] 0·01N-sodium hydroxide.

β-*Amylolysis of Glycogens.*—Barley β-amylase, from the Wallerstein Laboratories, New York, had an activity of 118 units/mg. by Hobson, Whelan, and Peat's method.[18] The enzyme preparation had a negligible reducing power and showed no maltase activity. It was free from α-amylase since it did not reduce the iodine-staining power of amylopectin β-limit dextrin, or the molecular weight of glycogen β-limit dextrin.[19] On incubation with potato amylose at

[13] Peat, Roberts, and Whelan, *Biochem. J.*, 1952, **51**, xvii.
[14] Bevenue and Williams, *Arch. Biochem. Biophys.*, 1951, **34**, 225.
[15] See Bell, "Modern Methods of Plant Analysis," Vol. II, ed. Paech and Tracey, Springer-Verlag, 1955, p. 21.
[16] Shaffer and Somogyi, *J. Biol. Chem.*, 1933, **100**, 695.
[17] Davies and Nancollas, *Nature*, 1950, **165**, 237.
[18] Hobson, Whelan, and Peat, *J.*, 1950, 3566.
[19] Greenwood, Jones, and Manners, unpublished work.

pH 3·6 and 4·6, 72% and 95% conversion respectively into maltose was observed. This indicates the presence of Z-enzyme in the barley preparation (compare ref. 6). Maltotriose was very slowly hydrolysed by high concentrations of the enzyme (86 units/mg. of maltotriose).

Enzyme digests contained 0·1M-acetate buffer of pH 4·6, and toluene, and were incubated at 35°. Preliminary experiments in which rabbit liver V glycogen was incubated with 40, 60, 80, and 100 units of β-amylase per mg. of glycogen showed that in every case enzyme action was virtually complete within 4 hr. and that on incubation for a further 44 hr. the conversion into maltose did not increase by more than 1%. β-Amylolysis limits were therefore determined, in duplicate, by incubating glycogen (1 mg./ml.) with β-amylase (40—50 units/mg. of glycogen) at pH 4·6 in a total volume of 50 ml. Samples (3 ml.) were analysed at intervals for maltose,[16] and the mean results obtained after 24 and 48 hr. are recorded in Table 1. Duplicate analyses were identical or differed by no more than 1%.

After 48 hours' incubation under the above conditions, the digests contained active β-amylase. Addition of 1 drop of digest to 1% starch solution (2 ml.) caused immediate liberation of reducing sugar. Further, the addition of fresh β-amylase after 48 hr. did not increase the maltose content of the digest.

Portions of the digests were examined by paper chromatography. Ethanol (2 vol.) was added to precipitate protein and polysaccharide, and the supernatant solutions were deionised with IR-4B(OH) and IR-120(H) ion exchange resins, and concentrated. The chromatograms, developed with a silver nitrate–sodium hydroxide reagent,[20] showed the present of maltose and no other sugar.

α-Amylolysis of Glycogens.—α-Amylase was prepared from human saliva by the method of Fischer and Stein [21] except that the final crystallisation stage was omitted, and the enzyme isolated by freeze-drying in 0·2M-citrate buffer (pH 7·0). The enzyme preparation, with an activity of 42 units/mg. by a modification of Fischer and Stein's method,[21] had a negligible reducing power, slowly hydrolysed maltotriose (27 units/mg. of substrate), and contained a trace of maltase activity (37 units/mg. of substrate).

Standard digests containing polysaccharide (*ca.* 50 mg.), 0·5% sodium chloride solution (10 ml.), α-amylase solution (*ca.* 230 units, 1 ml.), and distilled water to 100 ml. were incubated at 35°. Portions (5 ml.) were analysed at intervals, for reducing sugar (see Table 2). The small (*ca.* 2%) increase in reducing power of the digest with waxy maize starch between 6 and 24 hours' incubation showed the virtual absence of maltase activity under the above conditions (4·6 units α-amylase mg. of substrate.)

After 48 hr. a 10 ml. sample was removed from each digest, boiled, concentrated, and examined by paper chromatography. The silver nitrate–sodium hydroxide reagent showed the presence of glucose, maltose (R_G 0·49), maltotriose (traces only), and higher oligosaccharides including sugars with R_G 0·10 and 0·05. When duplicate chromatograms were sprayed with an orcinol reagent,[14] as used by Roberts *et al.*,[12, 13, 22] neither maltulose nor ketose-containing oligosaccharides could be detected. An authentic sample of maltulose had R_G 0·48, and a mixture of glucose and fructose (97 : 3) gave a positive ketose reaction under similar conditions.

Portions (4 ml.) of the digests were also heated with resorcinol (0·45% in water; 2 ml.) and hydrochloric acid–glycerol reagent (5 ml.) at 100° for 15 min. and the solutions examined colorimetrically.[15] If the fructose content of the glycogens was only *ca.* 3% then each aliquot would contain *ca.* 60 μg. of ketose. Under the above conditions, 30 μg. of fructose could be detected, and the intensity of colour with this was greater than that with any of the samples from the α-amylase digests.

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[20] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[21] Fischer and Stein, *Arch. Sci.*, 1954, **7**, 131.
[22] Roberts, Ph.D. Thesis, University College, North Wales (Bangor), 1953.