α-1 : 4-*Glucosans. Part I. The Inter-chain Linkages in Glycogens.*

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Four glycogens and two glycogen α-dextrins, subjected to prolonged periodate oxidation (maximal formic acid production) followed by acid hydrolysis, yielded no significant amounts of glucose. It is concluded that these polysaccharides contain only α-1 : 4- and 1 : 6-linkages.

It is often assumed that *all* the inter-chain linkages in *all* glycogens are 1 : 6 (cf., *e.g.*, Myrbäck and Sillén, *Nature*, 1949, **163**, 410; *Acta Chem. Scand.*, 1949, **3**, 190; Cori and Larner, *J. Biol. Chem.*, 1951, **188**, 17), but without conclusive chemical proof apart from one isolated experiment on a single sample (Gibbons and Boissonnas, *Helv. Chim. Acta*, 1950, **33**, 1477). Previous workers have excluded the possibility of 1 : 2-linkages and have shown the presence of some 1 : 6-linkages; certain experiments, however, indicate that glycogens might contain a mixture of inter-chain 1 : 3- and 1 : 6-linkages (Carlquist, *Acta Chem. Scand.*, 1948, **2**, 770; Bell, *J.*, 1948, 992; *Angew. Chem.*, 1948, *A*, **60**, 79). This communication describes experiments which show that over 99% of the inter-chain linkages are 1 : 6 in several samples of glycogen of known biological origin. For a preliminary account see Manners, *Biochem. J.*, 1953, **55**, xx.

First attempts to characterise the inter-chain linkages in branched α-1 : 4-glucosans of the glycogen type were based on methylation studies. Hydrolysis of the trimethyl ether of a 12-unit glycogen should yield tetra-, tri-, and di-*O*-methylglucose in the molecular ratio of 1 : 10 : 1, the last sugar arising from the branch point. In practice, however, the amount of di-*O*-methylglucose always exceeds that of the tetra-*O*-methyl derivative, and the observed ratio of the methyl ethers of glucose approximates to 1 : 9 : 2 (Bell, *Biochem. J.*, 1935, **29**, 2031; 1937, **31**, 1683; *J.*, 1948, 992). Similarly, methylated 18-unit glycogens on hydrolysis give tetra-, tri-, and di-*O*-methylglucose in the ratio 1 : 15 : 2 instead of the expected ratio of 1 : 16 : 1 (Bell, *Biochem. J.*, 1936, **30**, 1612, 2144). In these experiments the observed methoxyl content of the glycogens (43·9—45·5%) indicated that etherification was not complete, despite repeated treatment with the appropriate methylating reagents (tri-*O*-methylglycogen requires OMe 45·6%; cf. Bell, *Ann. Review Biochem.*, 1949, **18**, 87). Part of the di-*O*-methylglucose therefore arose from glucose units which had not been fully etherified and not from the branch points. Furthermore small amounts of di-*O*-methylglucose can arise by demethylation of higher homologues during the acid hydrolysis of methylated glycogen (Bell, *J.*, 1948, 992). Despite these complications, for some time, methylation was the only method for identifying the inter-chain linkages of glycogen. Haworth, Hirst, and Isherwood (*J.*, 1937, 577) showed that a sample of methylated 18-unit rabbit-liver glycogen (OMe 43·3%) yielded a considerable proportion of 2 : 3-di-*O*-methylglucose, and concluded that $C_{(6)}$ was involved in the inter-chain linkages. More recently, Bell (*loc. cit.*) examined the di-*O*-methyl fraction from methylated samples of 12-unit rabbit-liver and 11-unit rabbit-muscle glycogen (OMe 44·7 and 44·2% respectively), and found 2 : 6-di-*O*-methylglucose as the major component in each case, suggesting the possible presence of a number of 1 : 3-linkages. In contrast, Hirst, Hough, and Jones (*J.*, 1949, 928) reported hydrolysis of a methylated rabbit-liver glycogen (OMe 43%) to yield tetra-*O*-methylglucose (1), tri-*O*-methylglucose (8·6), 2 : 3-di-*O*-methylglucose (1·2), 3 : 6-di-*O*-methylglucose (1·5 moles), and mono-*O*-methylglucose (0·3 mole). In general, the methylation studies failed to yield conclusive evidence on the nature of the inter-chain linkages.

An alternative method involves partial depolymerisation with dilute acid. Analysis of the hydrolysate for disaccharides other than maltose will enable the inter-chain linkage of glycogen to be identified, provided that experimental conditions do not allow acid-reversion from glucose to occur. This method has been applied to rabbit-liver glycogen by Wolfrom, Lassettre, and O'Neill (*J. Amer. Chem. Soc.*, 1951, **73**, 595); a partial acid hydrolysate, after acetylation and chromatography, yielded a small amount of β-D-*iso*-maltose octa-acetate, and its isolation was taken as evidence for inter-chain 1 : 6-linkages.

Although the yield of *iso*maltose was only 1—2% (a 13-unit glycogen theoretically should give 6·8% of *iso*maltose), none was formed by a similar acid treatment of amylose. These experiments do not, of course, exclude the presence of a small number of 1 : 2- or 1 : 3-linkages.

The method used in the present investigation was that developed by Hirst, Jones, and Roudier (*J.*, 1948, 1779) involving acid hydrolysis of the periodate-oxidised polysaccharide. Each glucose unit, except those linked through $C_{(2)}$ or $C_{(3)}$ will be oxidised, and on hydrolysis will yield a dialdehyde; the presence of glucose in the hydrolysate therefore indicates inter-chain 1 : 2- or 1 : 3-linkages, always assuming that periodate oxidation was complete. This method has been successfully applied to acorn and barley starch (Hirst, Jones, and Roudier, *loc. cit.*; MacWilliam and Percival, *J.*, 1951, 2259) and to certain protozoal polysaccharides (Forsyth, Hirst, and Oxford, *J.*, 1953, 2030; Forsyth and Hirst, *J.*, 1953, 2132). Whilst the present investigation was in progress, the results of Gibbons and Boissonnas (*loc. cit.*) appeared indicating that 97—98% of the inter-chain linkages in a sample of glycogen, of unspecified biological source, were 1 : 6. By a quantitative paper-chromatographic method, they assessed the acid hydrolysate of 6·0 mg. of periodate-oxidised glycogen to contain 0·016 mg. of glucose; *i.e.*, the ratio of the number of inter-chain linkages at $C_{(2)}$ or $C_{(3)}$ to those at $C_{(6)}$ was not greater than 1 : 42. Gibbons and Boissonnas obtained a similar result for potato amylopectin; in both instances, the small amount of glucose found by these workers was possibly due to incomplete oxidation of the interior chains in the polysaccharides since the oxidation period (by sodium metaperiodate) was only 12 days (cf. Experimental section).

Abdel-Akher, Hamilton, Montgomery, and Smith (*J. Amer. Chem. Soc.*, 1952, **74**, 4970) investigated the fine structure of polysaccharides by hydrogenation and subsequent acid hydrolysis of a periodate-oxidised polysaccharide. A sample of glycogen (biological source unspecified) thus yielded 1% of glucose. These workers suggested that the glucose arose from 1 : 3-linkages; the significance of this finding, without additional information, must remain uncertain since the possibility of incomplete oxidation of the glycogen remains.

In the present studies, highly purified glycogens of known biological source and known degree of branching have been used (see Table 1). In an attempt to ensure complete oxidation, glycogens (from cat liver, *Mytilus edulis*, *Helix pomatia*, and *Tetrahymena pyriformis*) were oxidised by saturated aqueous potassium periodate at 15—20° for 40—50 days (cf. Halsall, Hirst, and Jones, *J.*, 1947, 1399; Bell and Manners, *J.*, 1952, 3641); no iodine was liberated, indicating absence of "over-oxidation" (cf. Halsall, Hirst, and Jones, *J.*, 1947, 1427; Greville and Northcote, *J.*, 1952, 1945). Hydrolysates of the oxidised glycogens, neutralised and concentrated, were examined on paper chromatograms and manometrically with glucose oxidase in presence of azide (cf. Bell and Manners, *loc. cit.*). The hydrolysates from cat liver, *Mytilus edulis*, and *Tetrahymena pyriformis* glycogens contained no glucose; that from *Helix pomatia* contained *ca.* 0·1 mg., corresponding to less than 1% of the inter-chain linkages being situated at $C_{(2)}$ or $C_{(3)}$. This glucose may well have originated in incomplete oxidation. Control experiments with glucose showed experimental losses of *ca.* 10%.

In an attempt to facilitate oxidation of the interior chains of glycogens, samples from fœtal sheep liver and *Ascaris lumbricoides* were treated with salivary amylase before periodate oxidation. Salivary amylase hydrolyses α-1 : 4-glucosidic linkages in both the exterior and the interior chains of glycogen, but has no action on the inter-chain linkages (cf. Whelan and Roberts, *Nature*, 1952, **170**, 748). After oxidation of these dextrins no glucose could be detected, by paper chromatography, in their hydrolysates.

The present series of experiments thus shows that glycogens from vertebrate, invertebrate, and protozoan tissues consist of unit-chains of α-1 : 4-linked glucose units which are interlinked solely by 1 : 6-glucosidic linkages. It must be noted that the configuration of the inter-chain linkage is not revealed by these experiments. The results of Wolfrom *et al.* (*loc. cit.*) and Cori and Larner (*loc. cit.*), from chemical and enzymic studies respectively, suggest that the 1 : 6-linkages have an α-configuration. None of the glycogens examined in our study contained fructose (unpublished results; cf. Peat, Roberts, and Whelan, *Biochem. J.*, 1952, **51**, xvii).

Experimental

Periodate Oxidation of Glycogens.—Glycogens [cat liver (434 mg.), *Helix pomatia* (450 mg.), *Mytilus edulis* (78 mg.), and *Tetrahymena pyriformis* (106 mg.)] were each oxidised by saturated solutions of potassium periodate (80 ml.) at room temperature and in diffuse daylight for 40—50 days. A pilot experiment (unreported) with rabbit-liver glycogen had shown that under these conditions, the oxidation of interior chains was incomplete after 18 days. Ethylene glycol (5 ml.) was added, solid was filtered off, and the filtrate was dialysed until free from iodate. 2N-Sulphuric acid (33 ml.) was added to the dialysed solutions (~100 ml.) and the mixture was kept at 95° for 8 hr. After careful neutralisation with hot aqueous barium hydroxide, and removal of barium sulphate by centrifugation, the hydrolysate (pH 5) was concentrated at 45° to *ca.* 2 ml. Samples (0·02 ml.) were examined on paper chromatograms (Hough, Jones, and Wadman, *J.*, 1950, 1702); the results are recorded in Table 1. The remainder of the concentrates were transferred to the main compartment of Warburg manometer cups, together with 0·1M-phosphate buffer (pH 7, 1·1 ml.) and 0·1M-sodium azide (0·1 ml.). Glucose oxidase solution (0·1 ml.) was placed in the side arm of the manometer cups. The observed oxygen uptakes are given in Table 1. Under these conditions, 1 mg. of glucose gave an oxygen uptake of 123 μl. (Bell and Manners, *loc. cit.*). Suitable examination of the enzyme digests showed that the glucose oxidase was not inhibited by the other hydrolysis products of the oxidised glycogens.

Table 1.

Analysis of acid-hydrolysate of periodate-oxidised glycogen

Source of glycogen	$10^{-6}M$ *	Chain length †	Paper chromatogram	Possible oxygen uptake (μl.) ‡	Observed oxygen uptake (μl.)
Cat liver	10·0	13	—	46	0
Mytilus edulis	—	5	—	21	0
Helix pomatia	—	7	Trace glucose	88	10
Tetrahymena pyriformis	9·8	13	—	11	0
Fœtal sheep liver	14·9	13	—	—	—
Ascaris lumbricoides ...	8·7	12	—	—	—

* Mol. wt. determined by light-scattering data (Harrap and Manners, *Nature*, 1952, **170**, 419).
† Potassium periodate oxidation assay (Bell and Manners, *loc. cit.*; Manners and Ryley, *Biochem. J.*, 1952, **52**, 480). ‡ Possible oxygen uptake, assuming 1% of the inter-chain linkages are at $C_{(2)}$ or $C_{(3)}$.

Control Experiment.—Glucose (54 mg.), dissolved in 0·5N-sulphuric acid (133 ml.), was kept at 95° for 8 hr. After neutralisation and concentration, as previously described, the concentrate was diluted to 50 ml. with distilled water. Aliquot portions (1 ml.) were treated with glucose oxidase; the oxygen consumed was 118 ± 1 μl. (89% of the expected 134 μl.).

Periodate Oxidation of Glycogen α-Dextrins.—Samples of fœtal sheep-liver glycogen (517·8 mg.) and *Ascaris lumbricoides* (750·7 mg.) were dissolved in water (25 ml.). Sodium chloride solution (0·5 ml.; 3% w/v), 0·1M-phosphate buffer (pH 7; 2·0 ml.) and diluted saliva (10 ml.) were added. After incubation at room temperature for 10 min., the enzyme was inactivated by boiling. At this stage, the solutions were achroic to iodine and had lost their opalescence, and the hydrolysis (as " maltose ") had reached 47% and 36% (Schaffer–Somogyi modified reagent 60; Bell and Manners, *loc. cit.*) with the mammalian and invertebrate glycogen respectively. To the α-dextrin solutions, 8% (w/v) sodium metaperiodate (25 ml.) was added, and the mixtures kept in the dark at room temperature for 14 days. Excess of periodate was neutralised by ethylene glycol (5 ml.), and the mixtures were then extracted with successive portions of chloroform to remove iodine liberated during oxidation of the reducing groups of the α-dextrins. Iodate was removed by Ag^+, and excess of Ag^+ by hydrogen sulphide. The solutions were concentrated to 50 ml. and then hydrolysed (0·5N-sulphuric acid) as previously described. The neutralised concentrate of each α-dextrin hydrolysate was examined on a paper chromatogram; neither hydrolysate contained any reducing sugars. A control experiment, with 10 mg. of glucose and 2·0 g. of potassium iodate in water (50 ml.), when examined on a chromatogram, gave an intense brown spot at the same R_G as authentic glucose.

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