423. α-1 : 4-*Glucosans. Part V.* End-group Assay of Glycogens by Periodate Oxidation, and the Oxidation of Maltose by Sodium Metaperiodate.*

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Fifteen samples of glycogen have been assayed by potassium metaperiodate oxidation, and with only two exceptions, average chain lengths of 10—14 glucose residues were obtained.

Oxidation of glycogen and of maltose, dissolved in sodium chloride, by sodium metaperiodate for 25 hr. at 2° (Potter and Hassid [1]) has been studied. Under these conditions, oxidation is incomplete.

PERIODATE oxidations have been widely used in structural investigations of polysaccharides, in particular, of starches, glycogens, and dextrans. By estimating the formic acid produced during oxidation, the proportion of triol groups in the polysaccharide can be assessed and, in starches and glycogens, the ratio of non-terminal to non-reducing terminal glucose residues (*i.e.*, average chain length, $\overline{\text{CL}}$) determined.[2] Further, examination of an acid hydrolysate of a periodate-oxidised glucosan enables 1 : 2- or 1 : 3-glucosidic linkages to be detected.[3] In the present investigation, the average chain lengths of several samples of glycogen have been determined by (*a*) potassium metaperiodate oxidation at room temperature, and (*b*) sodium metaperiodate oxidation at 2°. A preliminary account of part of this work has been published.[4]

Assay of glycogen by oxidation with potassium periodate was first made by Halsall, Hirst, and Jones,[5] $\overline{\text{CL}}$ values being calculated from the production of formic acid after 150 hours' oxidation. Later, Bell and Manners [6] found that samples of mammalian-muscle glycogen ($\overline{\text{CL}}$ 12, by methylation) had apparent chain lengths of 15—16 after 150 hours' oxidation. However, when oxidation was continued to maximum production of formic acid (after 300—400 hours' oxidation), $\overline{\text{CL}}$ values of 12 ± 1 were obtained. This apparent discrepancy was ascribed to variation in the oxidation rate with room

* Part IV, *J.*, 1956, 2831.

1 Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488.
2 Brown, Dunstan, Halsall, Hirst, and Jones, *Nature*, 1945, **156**, 785.
3 Hirst, Jones, and Roudier, *J.*, 1948, 1779.
4 Manners, *Biochem. J.*, 1953, **55**, xx.
5 Halsall, Hirst, and Jones, *J.*, 1947, 1399.
6 Bell and Manners, *J.*, 1952, 3641.

temperature.[7] A similar effect was also noted by Carlquist,[8] who found that in 144 hr. at 15° or 21° a glycogen sample gave 8·4 or 9·5 moles of formic acid per 100 glucose residues. Accordingly, end-group assays have been carried out on 15 different samples of glycogen, and $\overline{CL}$ values, evaluated from the final constant concentrations of formic acid, are recorded in Table 1, together with the specific rotations and methods of preparation of the glycogens.

TABLE 1. *End-group assay of glycogens by oxidation with potassium periodate.*

Source of glycogen [a]	Method of isoln.[b]	Method of purifn.[c]	$[\alpha]_D$ (in H_2O)	$\overline{CL}$	Source of glycogen [a]	Method of isoln.[b]	Method of purifn.[c]	$[\alpha]_D$ (in H_2O)	$\overline{CL}$
Cat liver IV	P	A	—	13	Rabbit liver III...	W	A	+196°	13
Cat liver VI	P	A	—	12	Rabbit liver IV	W	A	—	13
Fœtal pig liver ...	W	A	+191°	11	Rabbit liver V ...	W	A	+196	12
Helix pomatia I ...	P	A	+192	10	Rabbit liver X ...	C	E	+193	12
Helix pomatia II	P	A	+182	7	*Tetrahymena pyriformis* II	P	A	+195	14
Mytilus edulis IV	W	PA	+196	12	*Trichomonas gallinae* II	P	A	+200	13
Mytilus edulis V...	W	A	—	9					
Mytilus edulis VI	C	A	+195	13					
Rabbit liver II ...	P	A	+198	12					

[a] Roman numerals refer to different samples from the same biological source. [b] P = Pflüger method; W = hot-water extraction; C = commercial preparations. [c] A = acetic acid precipitation (Bell and Young, *Biochem. J.*, 1934, **28**, 282); PA = deproteinisation with picric acid; E = electrodialysis.

During isolation of glycogen by the Pflüger method (digestion of the tissues with hot 30% potassium hydroxide), there is no appreciable alkaline-degradation of the polysaccharide.[9] By methylation, the glycogens from rabbit liver III and X and *Helix pomatia* I had chain lengths of 12.[10-12] The latter type of glycogen therefore differs significantly in degree of branching from *Helix pomatia* II glycogen isolated in 1949; the presence of 7-unit chains in this has been confirmed. Fœtal pig liver glycogen, which has not previously been studied, resembles the majority of mammalian glycogens in degree of branching.

An alternative periodate method used by Potter and Hassid [1] involves oxidation at 2° of a solution of the polysaccharide in 1·5% sodium chloride with 1·5 mols. of sodium metaperiodate and determination of the formic acid produced after 25 hr. Chain lengths of 22—27 were reported for various amylopectins, although these results do not appear to have been confirmed by methylation assay of the *same* samples. Other end-group assays of glycogens and amylopectins by this method have been reported; [13-15] the $\overline{CL}$ values were greater than those obtained by other methods of assay of the same samples. In particular, the results differed from enzymic assays (using phosphorylase and amylo-1 : 6-glucosidase), as shown in Table 2.

It appears that with Potter and Hassid's procedure periodate oxidation and, hence, production of formic acid, are incomplete after 25 hr.; it has therefore been applied to glycogens already assayed by potassium periodate. Six samples of glycogen (and one of amylopectin), in sodium chloride solution, were oxidised with sodium metaperiodate at 2°, and the concentration of formic acid was determined after 25 hr. (Table 3). Since the $\overline{CL}$ values from the titres after 25 hr. were greater than those from potassium periodate assay, production of formic acid was incomplete. Six further quantitative experiments

[7] Manners, Ph.D. Thesis, Cambridge, 1952.
[8] Carlquist, *Acta Chem. Scand.*, 1948, **2**, 770.
[9] Greenwood and Manners, *Proc. Chem. Soc.*, 1957, 26.
[10] Bell, *Biochem. J.*, 1935, **29**, 2031.
[11] Haworth and Percival, *J.*, 1932, 2277.
[12] Baldwin and Bell, *Biochem. J.*, 1940, **34**, 139.
[13] Cori and Larner, *J. Biol. Chem.*, 1951, **188**, 17.
[14] Schlamowitz, *ibid.*, p. 145.
[15] Polglase, Smith, and Tyler, *ibid.*, 1952, **199**, 97.
[16] Illingworth, Larner, and Cori, *ibid.*, p. 631; Schlamowitz, personal communication.

showed that only 80—90% of the theoretical periodate was reduced after 25 hr. Schlamowitz's report [14] that formic acid was completely liberated within 20—25 hr. could not be confirmed (see p. 2209).

The period of 25 hr. for oxidation was chosen by Potter and Hassid [1] on the grounds that their "model" saccharide, maltose, yielded the theoretical 3 mols. of formic acid in this time. Their method is therefore based on the unproved assumption that oxidation of a disaccharide occurs at the same rate as that of a polysaccharide of molecular weight

TABLE 2. *Chain lengths of glycogens and amylopectins determined by periodate oxidation and enzymic methods.*

Sample	Sodium periodate oxidn.[a]	Enzymic assay	Ref.	Sample	Sodium periodate oxidn.[a]	Enzymic assay	Ref.
Rabbit liver glycogen	18	14·7	13	Potato amylopectin	27	21·8	13
Rabbit liver glycogen	22	15·9	16	Sago amylopectin ...	22	17	13
Rabbit liver glycogen	23	17·2	16	Wheat amylopectin...	23	18·5	13
Corn amylopectin ...	26	21·2	13				

[a] Procedure of Potter and Hassid.[1]

TABLE 3. *End-group assays of glycogens by periodate oxidation.*

Sample	Potassium periodate oxidn.*	Sodium periodate oxidn.† *A*	Sodium periodate oxidn.† *B*	Sample	Potassium periodate oxidn.*	Sodium periodate oxidn.† *A*	Sodium periodate oxidn.† *B*
Ascaris lumbricoides	12	15—16	11	*Mytilus edulis* VI...	13	—	14
Cat liver VI	12	—	12	Rabbit liver I	13	—	13
Commercial ‡	—	—	12	Rabbit liver II ...	12	14	—
Helix pomatia II ...	7	9	—	Rabbit liver V ...	12	—	12
Human liver	6	8	—	Rabbit liver X......	12	—	12
Human muscle ...	12	—	11	*Trichomonas fœtus*	15	18	15
Mytilus edulis V ...	9	13	—				

* See Table 1 and ref. 6. † *A*, Potter and Hassid's procedure; *B*, modified procedure.

‡ Purchased from British Drug Houses Ltd. [waxy maize starch had $\overline{CL}$ values of 18 and 22 by potassium and sodium periodate oxidation (method *A*) respectively].

$\sim 10^7$. We have noted, however, that different samples of glycogen are oxidised, under identical conditions, at slightly differing rates. We have re-examined the periodate oxidation of maltose in sodium chloride. In one experiment, after 25 hr. 2·3 mols. of formic acid were produced, and 4·7 mols. of periodate reduced. In additional experiments, maltose, in water or in 3% sodium chloride, was oxidised with varying amounts of sodium metaperiodate. After 25 hr. 1·7—2·6 mols. of formic acid were present, and after *ca.* 120 hr. 2·4—3·1 mols. Although strictly reproducible results could not be obtained in the presence of sodium chloride (see p. 2208), release of formic acid and uptake of periodate were never theoretical after 25 hours' oxidation at 2°.

The formation and subsequent hydrolysis of a formyl ester were incidentally indicated, since production of the third mol. of formic acid continues in the *absence* of periodate. A mixture of maltose in sodium chloride and sodium metaperiodate was divided after 48 hr. at 2° when it contained 2·6 mols. of formic acid. One half was stored at 2° for 24 hr.: the formic acid content increased to 2·9 mols. To the other half ethylene glycol was added, to reduce the remaining periodate, and the formic acid determined immediately and at intervals thereafter. During 24 hr. the formic acid content increased slowly from 2·6 to 2·9 mols. In a similar oxidation by aqueous sodium metaperiodate, we found 2·4 mols. of formic acid released after 48 hours' oxidation, 2·7 mols. after neutralisation of periodate and storage at 2° for 24 hr., and 2·6 mols. after 72 hours' total oxidation. Aliphatic formyl esters are also slowly hydrolysed in presence of sodium metaperiodate at 2° without consumption of periodate. It is apparent that the periodate oxidation of maltose resembles that of lactose [17] and cellobiose,[18] and involves (*a*) an initial oxidation

[17] Meyer and Rathgeb, *Helv. Chim. Acta*, 1948, **31**, 1540.
[18] Head and Hughes, *J.*, 1954, 603.

in which 4 mols. of periodate are reduced and 2 mols. of formic acid produced, and (*b*) a slower stage involving the release of a third mol. of formic acid by hydrolysis of a formyl ester.

The above results on the rate of production of formic acid have been confirmed by others. Morrison and his co-workers [19] found that maltose oxidised by Potter and Hassid's procedure gave 2·5 mols. of formic acid, whilst Potter and his collaborators [20] observed that at 3° the expected 3 mols. of formic acid were not produced after 9 days.

For the end-group assay of glycogens, Potter and Hassid's method has therefore been modified: oxidation in *aqueous* solution is continued for 7—10 days, and the maximum concentration of formic acid determined. With "model" compounds consisting of glycogens already assayed by potassium periodate, the oxidation (determined by periodate-uptake and formic acid production) was normally complete within 7 days. Typical results are reported in Table 3. We noted, however, that with certain mammalian liver glycogens formic acid is produced unexpectedly slowly; [21] the reasons for this are being investigated.

The present study provides further evidence that the *average* length of the chains in glycogens is normally *ca.* 12 glucose residues. We have now assayed some 30 samples of glycogen and, of these, 23 had $\overline{CL}$ values of 10—14. These results agree with those of Abdel-Akher and Smith [22] who for another 37 individual glycogens found average chain lengths of 10—14. This suggests that in most animal tissues the *relative* activity of phosphorylase and branching enzyme during glycogen synthesis is very similar. However, the activity of this enzyme system in different specimens of *Mytilus edulis* appears to vary since glycogens with $\overline{CL}$ values of *ca.* 5, 9, 12, 13, and 17 have been isolated (see Table 1 and ref. 6).

Experimental

Glycogen Samples.—We are indebted to Dr. J. S. D. Bacon for *Mytilus edulis* V glycogen, to Dr. G. D. Greville for the cat liver glycogens, to Dr. E. E. Percival for rabbit liver X glycogen and to Dr. J. F. Ryley for the protozoal glycogens (cf. ref. 23). *Helix pomatia* I glycogen was prepared by deacetylation of the corresponding acetate which was kindly provided by Dr. D. J. Bell. *Mytilus edulis* VI glycogen was purchased from L. Light and Co. Ltd. The remaining samples were isolated and purified as indicated in Table 1. The rotations of 0·2—0·4% glycogen solutions were measured in 2 dm. polarimeter tubes.

Potassium Periodate Oxidation of Glycogens.—The method previously described [6] was used.

Sodium metaperiodate oxidations.

Analytical Methods.—Formic acid was determined, after neutralisation of periodate with ethylene glycol, by titration with 0·01N-sodium hydroxide in a stream of carbon dioxide-free air with (*a*) methyl-red as indicator, when repeating Potter and Hassid's experiments,[1] or (*b*) a glass electrode and pH meter to an end-point at pH 5·8. Periodate uptake was estimated by the methods of Barnebey [24] or Fleury and Lange.[25] Results are expressed as moles per mole of maltose or, for glycogens, moles per mole of anhydroglucose residue.

Oxidations in Presence of Sodium Chloride.—During oxidations of glycogen or maltose in 1·5% sodium chloride solution with sodium metaperiodate, reagent blanks were also analysed. On storage at 2°, these became acid (pH *ca.* 3), and periodate was precipitated. Production of formic acid is therefore calculated from sodium hydroxide titres after correction for the *initial* acidity of the reagent blanks. Further, the results are approximate since the sodium hydroxide titrations include not only release of formic acid by periodate oxidation, but also, and to an unknown extent, acidity due to the interaction of sodium chloride and metaperiodate. The

[19] Morrison, Kuyper, and Orten, *J. Amer. Chem. Soc.*, 1953, **75**, 1502.
[20] Potter, Silveira, McCready, and Owens, *ibid.*, p. 1335; see also Wolff, Hofreiter, Watson, Deatherage, and MacMasters, *ibid.*, 1955, **77**, 1656.
[21] Cf. Perlin, *ibid.*, 1954, **76**, 4101.
[22] Abdel-Akher and Smith, *ibid.*, 1951, **73**, 994.
[23] Manners and Ryley, *Biochem. J.*, 1952, **52**, 480; 1955, **59**, 369.
[24] Barnebey, *J. Amer. Chem. Soc.*, 1916, **38**, 330.
[25] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107.

precipitation of periodate, which occurred within 24—96 hr., prevented accurate measurement of periodate reduction.

(a) *Glycogens.* Glycogen (30—280 mg.) in 3% sodium chloride (5 ml.) was oxidised with 0·27—0·40M-sodium metaperiodate (5 ml.) at 2° for 25 hr. Ethylene glycol (neutral; 3 ml.) was added, and the mixture kept for 1 hr. at room temperature in the dark, before titration. A control of sodium chloride and metaperiodate was similarly analysed. The results are given in Table 3.

The rate of formic acid production was studied by treating rabbit liver IV glycogen (189·8 mg.) and *Mytilus edulis* I glycogen (110·0 mg.) in 3% sodium chloride (10 ml.) with 0·37M-sodium metaperiodate (10 ml.) at 2° :

Time of oxidn. (hr.)	Apparent chain length (glucose residues)	
	Rabbit liver IV glycogen	*Mytilus edulis* I glycogen
51	16·2	16·2
100	15·0	15·2
166	14·6	14·2
291	12·6	13·2

For measurement of periodate uptake, 50 mg. of glycogen were oxidised for 25 hr. The results were glycogen from *Ascaris lumbricoides*, 0·98 mole per anhydroglucose residue; cat liver IV, 0·89; cat liver VI, 0·94; horse muscle, 0·92; fœtal sheep liver, 0·95, and rabbit liver IV, 0·94. On complete oxidation, these glycogens reduce 1·08—1·09 mols. of periodate.

(b) *Maltose.* Maltose hydrate (223·4 mg.), dissolved in 3% sodium chloride (10 ml.), was oxidised with 0·37M-sodium metaperiodate (10 ml.; 6 mols.) at 2°. The results were :

Time of oxidn. (hr.)	2	4	25	48	72
Formic acid prodn. (mols.)	1·5	1·7	2·3	2·7	3·1

In a second experiment, anhydrous maltose (162·8 mg.) in 3% sodium chloride was treated with 0·27M-sodium metaperiodate (10 ml.; 6 mols.) at 2° :

Time of oxidn. (hr.)	0·5	1	2	22	25	96	145
Periodate uptake (mols.)	3·2	3·9	4·0	4·6	4·7	—	—
Formic acid prodn. (mols.)	1·1	1·2	1·4	2·3	2·3	2·7	3·3

Maltose was also oxidised with varying amounts of sodium metaperiodate (4·4—34·0 mols.); after 25 hr. 1·7—2·5 mols. of formic acid were released, and after 95 hr. 2·5—2·9 mols.

In the above experiments, free iodine was present after *ca.* 90 hr. showing that over-oxidation had occurred.

Oxidations in Absence of Sodium Chloride.—In these oxidations, the reagent control of aqueous sodium metaperiodate was stable on storage at 2°.

(a) *Maltose.* Maltose hydrate (220·4 mg.) in water (10 ml.) was oxidised with 0·37M-sodium metaperiodate (10 ml.; 6 mols.) at 2° with results as follows :

Time of oxidn. (hr.)	2	4	25	48	72
Periodate uptake (mols.)	3·6	—	4·2	4·2	4·4
Formic acid prodn. (mols.)	1·4	1·6	2·2	2·5	2·8

On repetition, 2·1 mols. of formic acid were produced, and 4·2 mols. of periodate consumed after 25 hr. Oxidation of maltose with 4·0 or 11·6 mols. of periodate gave the following results :

Time of oxidn. (hr.)	2	4	25	48	72
Formic acid prodn. (mols.) :					
(*a*) 4·0 mols. of oxidant	1·3	1·4	2·0	2·1	2·3
(*b*) 11·6 mols. of oxidant	1·8	1·9	2·6	2·9	3·0

In the above experiments, the " formic acid " production is slightly lower (*ca.* 0·2 mol. of apparent formic acid) than in those in presence of sodium chloride. The difference is attributed to the acidity developed during the interaction of sodium chloride and metaperiodate.

(b) *Glycogens. Trichomonas fœtus* glycogen [23] (106·4 mg.) in water (25 ml.) was oxidised with 0·37M-sodium metaperiodate (5 ml.) at 2°. Portions (5 ml.) were removed at intervals, ethylene glycol (1 ml.) was added, and the formic acid titrated, with the following results :

Time of oxidn. (days)	2	4	6	10
Formic acid prodn. (mg.)	1·66	1·81	1·93	2·00
Apparent chain length (glucose residues)	18·0	16·4	15·4	15·0

Under similar conditions, *Ascaris lumbricoides* glycogen (100 mg.) gave 2·26 mg. of formic acid after 3 days, and 2·35 mg. after 5 days; 0·99 and 1·05 mols. of periodate were reduced within 2 and 3 days, respectively.

For end-group assays, glycogen (*ca.* 100 mg.) in water (23 ml.) was oxidised with 0·4M-sodium metaperiodate (2 ml.) at 2°. Portions (5 ml.) were removed at intervals for determination of formic acid. $\overline{CL}$ values, calculated from the final formic acid concentration, are given in Table 3; 1·06—1·09 mols. of periodate were reduced during these oxidations.

Ascaris lumbricoides glycogen (102·5 mg.) and *Mytilus edulis* VI glycogen (95·8 mg.) were also oxidised at room temperature (15—17°). The final production of formic acid (after 8 days) was 2·54 and 2·13 mg. respectively, corresponding to average chain lengths of 11 and 13 glucose residues. During the oxidation of glycogens with a *limited* excess of periodate (*ca.* 30%), appreciable " over-oxidation " does not therefore occur.

Hydrolysis of Formyl Esters.—Mixtures of maltose (*a*) in sodium chloride and (*b*) in water with sodium metaperiodate were divided after 48 hours' oxidation at 2°. One-half of each solution was stored at 2° for a further 24 hr. Ethylene glycol was added to the remaining solutions, which were then titrated with sodium hydroxide (methyl-red). The neutralised solutions were stored at 2° for 24 hr., alkali being added at intervals to maintain the pH. Results were :

Time after redn. of periodate (hr.)	0	2	3	24
Formic acid prodn. (mols.) (*a*)	2·6	2·7	2·8	2·9
(*b*)	2·4	2·5	2·6	2·7

The normal oxidations resulted in the production of (*a*) 2·9 and (*b*) 2·6 mols. of formic acid after 24 hr.

At 2°, and in presence of 3% sodium chloride and 0·37M-sodium metaperiodate, ethyl and *n*-propyl formate were slowly hydrolysed. No periodate was consumed.

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