309. α-1,4-*Glucosans. Part XIII.*[1] *Determination of the Average Chain Length of Glycogens by α-Amylolysis.*

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Twenty-eight samples of amylose, amylopectin, and glycogen containing between 0 and 16% of α-1,6-glucosidic inter-chain linkages have been degraded by equal quantities of salivary α-amylase. A linear relation was found between the apparent percentage conversion into maltose and the percentage of inter-chain linkages. The average chain length of glycogen can therefore be determined by quantitative α-amylolysis.

The method has been applied to the analysis of 10—50 mg. quantities of glycogen from various mammalian sources, including autopsy and biopsy human liver tissue from cases of glycogen-storage disease.

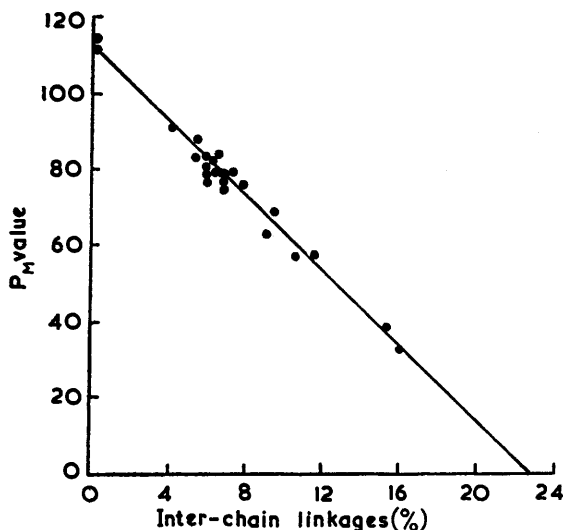
IN our previous studies on glycogens,[2] average chain length ($\overline{CL}$) values were determined by periodate oxidation, in which the proportion of non-reducing end-groups was assessed from the production of formic acid.[1,3] However, the quantity of glycogen required (originally [3] 200—500 mg., and later [1] 70—100 mg.) is greater than that available in many chemical and metabolic studies. We now describe an enzymic method suitable for the determination of $\overline{CL}$ values of 10—50 mg. quantities of glycogen-type polysaccharides.

α-Amylases catalyse an essentially random hydrolysis of the α-1,4-glucosidic linkages in starch and glycogen, the reaction being characterised by a rapid decrease in iodine-staining power and molecular size, and the slow production of reducing sugars.[4] Oligosaccharides with a degree of polymerisation ($\overline{DP}$) > 6 are formed initially, and these are later hydrolysed to give maltose as the major product, together with branched oligosaccharides $\overline{DP}$ > 4. The nature of these α-limit dextrins depends upon the source of α-amylase and the enzyme concentration.[5,6] Low concentrations of salivary α-amylase degrade glycogen or amylopectin to give maltose, maltotriose, and the pentasaccharide 6^3-α-maltosylmaltotriose as the smallest α-limit dextrin [6] (for nomenclature of oligosaccharides, see ref. 7). With higher enzyme concentrations, a second stage of hydrolysis occurs, in which maltotriose is hydrolysed to maltose and glucose and the smallest α-limit dextrin is the tetrasaccharide 6^3-α-glucosylmaltotriose. The oligosaccharides formed from amylopectin during the second stage of hydrolysis by malt α-amylase and *Bacillus subtilis* α-amylase include panose and 6^2-α-maltosylmaltotriose, respectively.[5] It is known that α-amylases have no action on α-1,6-glucosidic inter-chain linkages, but two or three adjacent α-1,4-glucosidic linkages are also resistant to enzymic attack. The degree of α-amylolysis of glycogen should therefore be inversely proportional to the number of resistant linkages and, hence, to the percentage of α-1,6-glucosidic inter-chain linkages. Since randomly branched polymers of high molecular weight contain equal numbers of end-groups and inter-chain linkages, assay of the latter will also enable the $\overline{CL}$ value to be determined.

The relation between the extent of α-amylolysis, as measured by the apparent percentage conversion into maltose (P_M), and the percentage of inter-chain linkages in glycogen ($P_{1,6}$) was investigated by incubating equal weights of various polysaccharides of known $\overline{CL}$ with identical amounts of a salivary α-amylase preparation. The digests, which

[1] Part XII, Manners and Wright, *J.*, 1961, 2681.
[2] For a review, see Manners, *Adv. Carbohydrate Chem.*, 1957, **12**, 261.
[3] Bell and Manners, *J.*, 1952, 3641; Manners and Archibald, *J.*, 1957, 2205.
[4] For a review, see Whelan, " Encyclopedia of Plant Physiology," Springer-Verlag, Berlin, 1958, Vol. VI, p. 154.
[5] Whelan, *Stärke*, 1960, **12**, 358.
[6] Walker and Whelan, *Biochem. J.*, 1960, **76**, 257.
[7] Whelan, *Ann. Rev. Biochem.*, 1960, **29**, 105.

contained 0·5 mg. of polysaccharide per ml., 1·9 units of α-amylase per ml. (for assay conditions, see Experimental), and 0·05% sodium chloride solution, were incubated at pH 7 and 37°. The production of reducing sugars was essentially complete within 9 hr., but, for convenience, amylolysis was continued for 24 hr. Paper chromatography showed the presence of glucose, maltose, and higher oligosaccharides, but only a trace of maltotriose, showing that hydrolysis to the second stage [6] had occurred. The P_M values obtained after 24 hours' α-amylolysis for 28 different polysaccharides (the proportion of inter-chain linkages ranged from 0 to 16%) are shown in the Figure. We conclude that there is a significant relation, under these experimental conditions, between the P_M value and the degree of branching. By the method of least squares, these properties are shown to be related by the equation $P_{1,6} = 100/\overline{\mathrm{CL}} = 23 \cdot 3 - 0 \cdot 21(P_M)$. When $P_M = 0$, *i.e.*, the substrate is completely resistant to α-amylase, the calculated $\overline{\mathrm{CL}}$ is 4·3. This figure is significant in view of the production of a tetrasaccharide as the smallest α-limit dextrin, together with higher branched oligosaccharides.[6] The observed P_M values for maize and



Relation between extent of α-amylolysis (P_M value) and the degree of branching of amylopectin-glycogen type polysaccharides.

potato amylose, which do not contain α-1,6-glucosidic linkages, are 114% and 112%, respectively; these results may be compared with the calculated value of 111%, and the P_M value of 115% observed by Walker and Whelan [6] for the second-stage hydrolysis of potato amylose.

The $\overline{\mathrm{CL}}$ value of glycogens can, therefore, be determined from the degree of α-amylolysis under the above experimental conditions. The probable error in $P_{1,6}$ is estimated to be $\pm 1\%$; the corresponding error in $\overline{\mathrm{CL}}$ is equivalent to ± 1 glucose residue when $P_{1,6} = 10\%$, and $\pm 0 \cdot 6$ glucose residue when $P_{1,6} = 12\%$. The method is most accurate for glycogens with $\overline{\mathrm{CL}} \leqslant 14$. The preparation of a graph by using a particular concentration and source of α-amylase will then enable the $\overline{\mathrm{CL}}$ of unknown samples of glycogen to be determined. However, it must be noted that the equation will depend upon (*a*) the enzyme concentration (sufficient α-amylase must be present to ensure that the second-stage of hydrolysis occurs) and (*b*) the amount of maltase present as impurity in the enzyme preparation.

Cori and Larner [8] developed an enzymic method of $\overline{\mathrm{CL}}$ determination based upon the amount of glucose liberated during the digestion of the polysaccharide with muscle phosphorylase and amylo-1,6-glucosidase. The preparation of enzyme and method of analysis are more convenient in the present work.

[8] Cori and Larner, *J. Biol. Chem.*, 1951, **188**, 17.

The accuracy of the α-amylolytic method was verified by analysis of human liver glycogen. Dr. P. W. Kent kindly supplied autopsy samples of liver from a case of glycogen-storage disease (D) in a four-year-old girl, and from a control subject of comparable age (N). Successive extraction of each tissue with cold water (c), followed by hot water (h) gave four glycogen samples. The results of $\overline{CL}$ determinations be periodate oxidation [1] and α-amylolysis are shown in Table 1. β-Amylolysis limits were also determined; they confirm the view that the storage-disease samples have a normal degree of branching.

TABLE 1.
Analysis of human liver glycogens.

Sample	$\overline{CL}$ Periodate oxidn.	$\overline{CL}$ α-Amylolysis	β-Amyloysis limit (%)
N_c	15	16	50
N_h	—	15	47
D_c	15	15	51
D_h	15	14	49

Dr. Kent [9] has examined the enzyme levels in the normal and the abnormal liver and shown that the latter was deficient in glucose 6-phosphatase. When methods described earlier [10] were used, the homogenised diseased tissue hydrolysed glucose 6-phosphate at a rate of $30{\cdot}4 \times 10^{-6}$ mole per hr. per g. of dry wt. (in duplicate experiments) whereas the corresponding value for the normal tissue (taken at autopsy and stored under the same conditions) was 1110. The case thus corresponds to a Type 1 (von Gierke) disease, in the classification of G. T. Cori.[11]

As reported briefly elsewhere,[12] glycogen from a liver biopsy sample (233 mg. of tissue) has been characterised. We are indebted to Dr. A. D. Bain, The Royal Hospital for Sick Children, Edinburgh, for this material. 34 Mg. of glycogen were isolated by the Pflüger technique and 12·7 mg. subjected to α-amylolysis. The P_M value of 48 was equivalent to a $\overline{CL}$ of $7{\cdot}7 \pm 0{\cdot}6$ glucose residues. Since the β-amylolysis limit was only 27% (compare normal values of 40—50%) the glycogen has the structure of a muscle-phosphorylase limit dextrin, and therefore arises from a case of glycogen-storage disease of Type III, or limit dextrinosis.[11]

The α-amylolytic method has also been applied to the analysis of small quantities of mammalian glycogen. For example, Mr. O. Kjølberg recently found a $\overline{CL}$ of 14 for horse eye (levator palpebrae) muscle glycogen by this means.

Since amylopectins contain only 4—5% of inter-chain linkages, an accurate determination of $\overline{CL}$ by α-amylolysis is not possible. However, successive β- and α-amylolysis may give satisfactory results. The polysaccharide (*ca.* 50—90 mg.) is first treated with β-amylase at pH 4·6 for 24 hr.; the enzyme is inactivated by heating, and the β-amylolysis limit determined. The digest is then adjusted to pH 7·0 and incubated with salivary α-amylase under the standard conditions. The total reducing sugar is estimated as maltose, and the P_M value of the β-limit dextrin calculated. From the corresponding $P_{1,6}$ value, and the β-amylolysis limit, the $\overline{CL}$ of the original polysaccharide can be obtained. The results obtained with two amylopectin and five glycogen samples are shown in Table 2, and are in good agreement with those from analysis by periodate oxidation.

The results in Table 2 show a striking similarity in the $\overline{CL}$ of various glycogen β-limit dextrins. This suggests that the degree of branching in the interior of the glycogen molecules is essentially constant, and that the glycogens differ mainly with regard to exterior chain length. The latter would depend upon the metabolic state of the animal at the time of sampling.

9 Kent, personal communication.
10 Çalderbank, Kent, Lorber, Manners, and Wright, *Biochem. J.*, 1960, **74**, 223.
11 Cori, *Harvey Lect.*, 1952—3, **48**, 145.
12 Manners and Wright, *Biochem. J.*, 1961, **79**, 18P.

TABLE 2.

Analysis of polysaccharides by β- and α-amylolysis.

Sample	β-Amylolysis limit (%)	P_M value	$P_{1,6}$ value	$\overline{CL}$ of β-dextrin	$\overline{CL}$ of polysaccharide	$\overline{CL}$ by periodate oxidn.
Glycogens:						
Oyster	39	38	15·4	6·5	11	11
Mytilus edulis XI ...	53	44	14·1	7·1	15	13
Rabbit liver VII	51	42	14·5	6·9	14	14
Rabbit muscle III ...	49	38	15·4	6·5	13	13
Ram liver	46	40	14·8	6·8	13	14
Amylopectins:						
Potato	60	63	10·1	9·9	25	24
Maize	57	60	10·7	9·3	22	19

EXPERIMENTAL

Methods and Materials.—Reducing sugars were estimated as maltose with the Somogyi (1952) reagent.[13] The salivary α-amylase preparation (freeze-dried in 0·2M-citrate buffer of pH 7·0) was that described by Liddle and Manners.[14] The samples of horse and ox muscle glycogen and human liver glycogen have been reported elsewhere.[10,15]

Glycogen β-dextrins were prepared by incubation of rabbit liver VII glycogen with varying concentrations of barley β-amylase [14] for various periods. Dextrins were isolated after 7, 22, 37, and 48% conversion into maltose, and the $P_{1,6}$ values calculated from these β-amylolysis limits and the $\overline{CL}$ of the original glycogen (13·9).[1]

Activity of α-Amylase.—Fischer and Stein [16] define a unit of α-amylase activity as the quantity of enzyme which liberates 1 mg. of apparent maltose (as measured by the dinitrosalicylic acid procedure) from 2 ml. of 0·5% solution of soluble starch in 3 min. at 20°. These conditions have been modified and reducing sugar was determined with the Somogyi reagent after incubation for 30 min. at 35°. The digest contained 1% soluble starch solution (25 ml.), 0·5% sodium chloride (3 ml.), and α-amylase solution (0·038 mg.; freeze-dried preparation in 2 ml. of water). After 30 min., 12·8 mg. of apparent maltose were liberated, equivalent to an activity of 34 units per mg. of enzyme, based on a 3 minutes' incubation period.

The enzyme (2·75 mg.; freeze-dried preparation in 0·5 ml. of water) was incubated with maltose (25 mg.), 0·5% sodium chloride solution (5 ml.), and water to 50 ml. After 24 and 48 hr., the hydrolysis of maltose was 2% and 3% respectively.

α-Amylolysis of Glycogens.—In preliminary experiments, polysaccharide solution (10 ml.; *ca.* 5 mg./ml.) was incubated with 0·5% sodium chloride (10 ml.), α-amylase solution (5·5 mg.; freeze-dried preparation in 1 ml. of water; 187 units) and water to 100 ml. at 35°. (Since solutions of the polysaccharide were neutral, addition of buffer in excess of that present in the enzyme preparation was unnecessary.) Samples (5 ml.) were removed at intervals for estimation of apparent maltose. α-Amylolysis was essentially complete after 9 hr., and the P_M value increased by only 2—4% during the next 15 hr. Part of this increase is accounted for by the trace of maltase activity.

In later experiments, the digests were reduced to the 25-ml. or 50-ml. scale but the enzyme : substrate ratio remained unaltered. The results are given in Table 3.

Preparation and Analysis of Glycogen from Liver Autopsy Samples.—Boiled liver tissue (69 g.) from the glycogen-storage disease case was homogenised with cold water and centrifuged. Glycogen was precipitated from the extract with ethanol, after deproteinisation with 4% trichloroacetic acid solution at 0°. After purification by further precipitation with ethanol, the yield of glycogen (D_c) was 1·36 g. The residue was extracted twice with boiling water (500 ml.) for 30 min. in an atmosphere of nitrogen. After deproteinisation, precipitation with ethanol, and purification, 2·03 g. of glycogen (D_h) were obtained. The total yield (D_c and D_h) was 4·9% of the tissue weight.

The control sample of normal liver tissue (51 g.) was extracted similarly, the yields of

[13] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[14] Liddle and Manners, *J.*, 1957, 3432.
[15] Lawrie, Manners, and Wright, *Biochem. J.*, 1959, **73**, 485.
[16] Fischer and Stein, *Arch. Sci.*, 1954, **7**, 131.

TABLE 3.
P_M values of polysaccharides.

	$P_{1,6}$ [a]	P_M (24 hr.)	$P_{1,6}$ (calc.) [b]
Glycogens and glycogen β-dextrins			
Human liver (S.K.)	15·9	33	16·4
Rabbit liver VII 48% dextrin	15·2 [c]	38	15·3
Rabbit liver VII 37% dextrin	11·6 [c]	57	11·3
Rabbit liver VII 22% dextrin	9·4	62	10·3
Oyster	9·5	68	9·0
Human liver (A.K.)	6·9	75	7·8
Human kidney (A.K.)	7·0	76	7·4
Horse psoas muscle (post-rigor)	5·9	76	7·4
Rabbit liver VII 7% dextrin	7·9	76	7·4
Horse l. dorsi muscle (pre-rigor)	6·0	78	6·9
Horse heart muscle (post-rigor)	6·1	78	6·9
Horse psoas muscle (pre-rigor)	6·2	79	6·7
Pig liver (normal)	6·6	79	6·7
Ox sternocephalicus muscle (post-rigor)	6·7	79	6·7
Ox psoas muscle (post-rigor)	6·8	79	6·7
Rabbit liver VII	7·2	79	6·7
Horse diaphragm muscle (pre-rigor)	6·0	80	6·5
Horse diaphragm muscle (post-rigor)	6·0	80	6·5
Pig l. dorsi muscle	6·5	81	6·3
Ox psoas muscle (pre-rigor)	6·1	82	6·1
Horse l. dorsi muscle (post-rigor)	6·0	83	5·9
Ox sternocephalicus muscle (pre-rigor)	5·4	83	5·9
Pig liver (sugar fed)	6·5	84	5·7
Starch components			
Potato amylopectin	4·2	91	4·2
Potato amylopectin β-dextrin	10·5 [c]	56	11·6
Maize amylopectin	5·3	88	4·8
Potato amylose	0	112	—
Maize amylose	0	114	—

[a] Percentage of α-1,6-glucosidic linkages, calculated from $P_{1,6} = 100/\overline{CL}$, where $\overline{CL}$ was determined by periodate oxidation (refs. 1 and 3). [b] Percentage of α-1,6-glucosidic linkages calculated from $P_{1,6} = 23{\cdot}3 - 0{\cdot}21(P_M)$. [c] $\overline{CL}$ calculated from original $\overline{CL}$ and β-amylolysis limit.

glycogen (N_c) and (N_h) being 0·40 g. and 0·15 g., respectively, equivalent to a total yield of 1·1% of the tissue weight.

Further extraction of the aqueous-extracted residues with 30% potassium hydroxide solution at 100° gave no glycogen.

Three of the glycogens (D_c, 97·4 mg.; D_h, 91·0 mg.; N_c, 60·4 mg.) were oxidised with 0·1M-sodium metaperiodate at 20°, as described previously.[1] The production of formic acid was measured after 48, 92, 122, and 147 hr., and the formic acid concentration extrapolated to zero time indicated $\overline{CL}$ values of 14·7, 15·1, and 15·2 glucose residues, respectively.

The P_M values obtained on α-amylolysis (for 26 hr. in this experiment) were: D_c, 80; D_h, 77; N_c, 81; and N_h, 79; corresponding to $\overline{CL}$ values of 15·4, 14·1, 15·9, and 15·0 glucose residues, respectively. In a control experiment, another human liver glycogen (sample A.K.) [10] with $\overline{CL}$ 14·5 by periodate oxidation had a P_M value of 75.

Preparation and Analysis of Liver Biopsy Sample.—The tissue sample (233 mg.) was digested with 30% potassium hydroxide (1·5 ml.) at 98° for 20 min. The resulting pale brown solution was centrifuged, saturated sodium sulphate (0·2 ml.) added to the supernatant solution, and the glycogen precipitated by the addition of ethanol (2 ml.). It was purified by seven further precipitations with ethanol, the whole procedure being carried out in a single graduated centrifuge tube. The glycogen was dissolved in water (6·4 ml.); acid hydrolysis of an aliquot part indicated the presence of 34 mg. of polysaccharide, equivalent to 14·6% of the tissue weight.

Biopsy glycogen (12·7 mg.) was incubated at 37° with α-amylase. Liver glycogens [10] A.K. (15·4 mg.) and S.K. (14·2 mg.) were also analysed. The following P_M values were obtained:

Time of incubation (hr.)	1·5	7	9	46
Biopsy glycogen	41	48	48	51
A.K. Liver glycogen	60	76	76	80
S.K. Liver glycogen	26	34	35	38

$P_{1,6}$ values were calculated from the P_M after 9 hr. In previous analyses, P_M values (24 hr.) of 75 and 33 had been noted for the A.K. and S.K. samples.

On incubation of the biopsy glycogen (15·8 mg.) with β-amylase (50 units/mg.) at pH 4·6 and 35°, the percentage conversion into maltose was 19, 23, and 27 after 5, 20, and 45 hr., respectively.

α-Amylolysis of β-Limit Dextrins.—Polysaccharide (50—90 mg.) was incubated with barley β-amylase (25 units/mg.) at pH 4·6 in a total volume of 30 ml. After 24 hr. at 35°, the enzyme was inactivated by heating and the maltose content measured. A 20 ml. sample was removed, adjusted to pH 7·0, and reincubated at 37° with α-amylase, and 0·5% sodium chloride (5 ml.) in a total volume of 50 ml. Enzyme action was complete after 9 hr. and the P_M value then determined.

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