886. α-1,4-*Glucosans. Part XV.*[1] *Structural Analysis of Glycogens on a Milligram Scale.*

By O. KJØLBERG and D. J. MANNERS.

The average chain length and the relative lengths of the exterior and the interior chains of a *ca.* 5 mg. quantity of a branched α-1,4-glucosan can be determined by α- and β-amylolysis. Interaction of the polysaccharide with iodine and with concanavalin-A can be used to distinguish between glycogen, phosphorylase limit dextrin, and amylopectin-type structures.

These methods have been applied to glycogens from cases of glycogen-storage disease, and to Floridean starches.

SEVERAL distinct types of glycogen storage disease (glycogenosis) have now been recognised;[2-4] these differ in the site of glycogen accumulation, the molecular structure of the deposited polysaccharide, and the relative activity of the various enzymes concerned in glycogen metabolism. In most cases, the accumulation of glycogen is caused by a relative deficiency of one of the enzymes (either glucose 6-phosphatase or amylo-1,6-glucosidase) controlling glycogenolysis, but in rare cases multiple enzyme deficiencies may occur.[5] Present knowledge of the glycogenoses is summarized in Table 1.

TABLE 1.

Types of glycogen-storage disease.

Type	Organs affected	Glycogen structure	Enzyme deficiency
I *	Liver, kidney	Normal	Glucose 6-phosphatase
II	Generalized	Normal	?
III †	Liver, muscle, heart	Limit dextrin	Amylo-1,6-glucosidase
IV	Liver	Amylopectin-type	Branching enzyme
V	Muscle	Normal	?
VIa	Liver	Normal	Liver phosphorylase
b	Muscle	Normal	Muscle phosphorylase

* von Gierke's disease. † Limit dextrinosis; various sub-types may exist (see Manners and Wright, *Biochem. J.*, 1961, **79**, 18P).

Biochemical studies of these diseases involve assay of affected organs for the individual enzymes and structural analysis of the stored polysaccharide. In the latter, three structural types have been encountered, namely, "normal" glycogen, glycogen muscle-phosphorylase limit dextrin (φ-dextrin), and amylopectin, and typical properties are shown in Table 2. The present paper describes methods for the differentiation of these three structural types which can be applied to the analysis of small samples (*ca.* 5 mg.) of polysaccharide isolated from biopsy samples from cases of glycogenosis.

In Part XIII [6] we described an enzymic method for the determination of the average chain length ($\overline{CL}$) of glycogen based on the extent of α-amylolysis under standard conditions. The production of reducing sugars, as apparent maltose, was measured on a 0—3 mg. scale by use of the Somogyi [7] reagent, so that 10—25 mg. quantities of glycogen were required for analysis. However, by applying Park and Johnson's colorimetric method [8] to maltose, 2—14 μg. quantities can be determined so that $\overline{CL}$ values may be measured on

[1] Part XIV, Manners and Wright, preceding paper.
[2] Cori, *Harvey Lectures*, 1952—3, **48**, 145.
[3] di Sant'Agnese, *Ann. New York Acad. Sci.*, 1959, **72**, 439.
[4] Stetten and Stetten, *Physiol. Rev.*, 1960, **40**, 505.
[5] Abrahamov, Mager, and Shafrir, *Bull. Res. Council Israel*, 1961, 9*E*, 83; Steinitz and Reisner, *ibid.*, p. 84.
[6] Manners and Wright, *J.*, 1962, 1597.
[7] Somogyi, *J. Biol. Chem.*, 1952, **195**, 19.
[8] Park and Johnson, *J. Biol. Chem.*, 1949, **181**, 149.

ca. 2 mg. of polysaccharide. The micro-method gives values in good agreement with those obtained by periodate oxidation.[9]

The relative lengths of the exterior and the interior chains in a glycogen-type polysaccharide are usually calculated from the $\overline{CL}$ and β-amylolysis limit.[10] For the latter, 25—50 mg. of glycogen were a convenient quantity when the liberated maltose was measured by using the Somogyi reagent. With Park and Johnson's method, the enzyme digest can be reduced to the analysis of *ca.* 2 mg. of glycogen, and the results with various glycogen samples are in agreement with those determined previously.

The intensity of iodine-staining of a glycogen-type polysaccharide [11] and the turbidity produced by the addition of concanavalin-A [1] are both related to the degree of branching. These procedures, described elsewhere,[1,11] require only 1 mg. of glycogen, and enable a clear distinction to be made between the three structural types. A combination of these methods and α- and β-amylolysis therefore enables the structure of a branched α-1,4-glucosan to be established.

TABLE 2.

Properties of glycogen-type polysaccharides.

	Glycogen	φ-Dextrin	Amylopectin
Average chain length *	10—14	6—8	18—24
β-Amylolysis limit (%)	40—50	14—28	50—60
Exterior chain length *	6—10	2—4	9—16
Interior chain length *	2—4	2—4	6—8
α-Amylolysis limit, %	75—85	33—48	85—92
Absorption spectra of iodine complex:			
$\lambda_{max.}$ (mμ) ($E_{max.}$)	420—500 (0·1—0·4)	—	530—550 (0·9—1·3)
Glycogen value †	0·8—1·3	>1·3	0

* Expressed as glucose residues. † Turbidity reaction with concanavalin-A (see Part XIV [1]).

TABLE 3.

Analysis of glycogens from muscle biopsy samples.

Case	G. R.	J. W.	J. W.	J. W.
Muscle	Gastrocnemius	Gastrocnemius	Soleus	Pectoralis major
Glycogen content (%) *	4·1	11·3	7·0	3·7
Weight of sample (mg.)	950	530	170	200
Yield of glycogen (mg.) †	39	58	4·0	1·2
α-Amylolysis limit (%)	73	70	70	69
1,6-Inter-chain linkages (%)	9·0	9·3	9·2	9·6
$\overline{CL}$	11	10·8	10·9	10·4
β-Amylolysis limit (%)	49	35	32	—
$\overline{ECL}$	8	6—7	6	—
$\overline{ICL}$	2	3—4	4	—
Glycogen value	1·12	1·23	1·18	—
Iodine absorption spectra:				
$\lambda_{max.}$ (mμ)	445	445	—	—
$E_{max.}$	0·30	0·27	—	—

* Determined on samples dried on filter paper, by Dr. Thomson, using the method of Good, Kramer, and Somogyi, *J. Biol. Chem.*, 1933, **100**, 485. † Based on glucose content of an aliquot part after acid hydrolysis.

The methods were first applied to the analysis of muscle glycogen from biopsy samples from two cases of glycogenosis. The samples were kindly supplied by Dr. W. H. S. Thomson, The Western Infirmary, Glasgow, who has reported elsewhere [12] the results of clinical and biochemical investigation on these cases.

[9] Manners and Archibald, *J.*, 1957, 2205; Manners and Wright, *J.*, 1961, 2681.
[10] Bell and Manners, *J.*, 1952, 3641; Liddle and Manners, *J.*, 1957, 3432.
[11] Archibald, Fleming, Liddle, Manners, Mercer, and Wright, *J.*, 1961, 1183.
[12] Thomson, Maclaurin, and Prineas, *J. Neurol. Neurosurg. Psychiatry*, in the press.

Patient G. R. (48 yr., male) was a case of muscle glycogenosis characterized by a deficiency of phosphorylase, corresponding to Type VIb (see Table 1). Glycogen was isolated from a gastrocnemius muscle sample and found to have a normal glycogen structure (see Table 3). This finding is in agreement with the biochemical investigations.

Only two other cases of Type VIb have so far been reported. In one of these,[13] a biopsy sample of thigh muscle contained 4% of glycogen (normal value *ca.* 1%) which had a $\overline{CL}$ of 14 and a phosphorolysis limit of 41%, showing that the degree of branching was within the normal range. In this, and one other case [14] in which structural analysis of the glycogen was not reported, evidence for the presence of UDPG-glycogen transferase and the virtual absence of phosphorylase was obtained.

In the second case (patient J. W.; 4 yr.; male) glycogen was obtained from three muscle biopsy samples (other tissues were not affected) and the results (Table 3) show that the accumulated glycogen had, in general, a normal-type structure, although the β-amylolysis limits were slightly lower than for most other glycogens. The glycogenosis is not, therefore, of Type I, II, III, or IV, and since the tissues had considerable phosphorylase activity, Type VIb can also be eliminated. This case would appear to be the rare Type V disease, only one other case being on record.[3]

The micro-methods have also been used to confirm the results of analyses obtained by conventional methods. In 1937, Haworth, Hirst, and Isherwood [15] reported that, according to methylation analysis, a sample of rabbit-liver glycogen (British Drug Houses, Ltd.) had $\overline{CL}$ 18, and therefore differed significantly from another sample (hereafter referred to as sample X) examined in 1932 by Haworth and Percival [16] and found to have $\overline{CL}$ 12. The latter value has since been confirmed by periodate oxidation.[9] We have now confirmed the previous conclusion that the commercial sample had a lower degree of branching than sample X since by α-amylolysis the $\overline{CL}$ is 15. We have also noted that the B.D.H. sample has a greater β-amylolysis limit (55%) than that of sample X (49%) and most other glycogens. The glycogen value (0·95) and iodine-staining properties ($\lambda_{max.}$ 460 mμ; *E* 0·42) of the commercial sample fall within the normal range of values.

Floridean starches from *Dilsea edulis* have also been analysed by α-amylolysis. Previous $\overline{CL}$ determinations by periodate oxidation (Part IV [17]) on three different samples gave values in the range 9—13 glucose residues, and Peat and his co-workers [18] found a $\overline{CL}$ of 15 for another sample by the same method. Greenwood and Thomson [19] recently reported $\overline{CL}$ 18·6 for a sample of Floridean starch, and suggested that comparison with our earlier results was not possible owing to the presence of protein impurity in these samples, and consequent interference with periodate oxidation. The apparent protein contents of our samples were 5·7, 2·1, and 3·3%, respectively, whereas Anderson and Greenwood [20] have shown that, with oat starch, up to 3% of protein does not produce any error during periodate oxidation, although discrepancies arise when the protein content exceeds 23%. Sample II has therefore been re-examined; on α-amylolysis, the P_M value was 78, equivalent to $\overline{CL}$ 13 (compare $\overline{CL}$ 12 by periodate oxidation). An additional sample (V) of Floridean starch has been analysed; the P_M value was 79, again corresponding to $\overline{CL}$ 13. We conclude, therefore, that the $\overline{CL}$'s of individual Floridean starch samples may vary in the range 10—18 glucose residues, and include samples with $\overline{CL}$ values similar to those of many animal glycogens. In agreement with this conclusion, Meeuse and his

[13] Mommaerts, Illingworth, Pearson, Guillory, and Seraydarian, *Proc. Nat. Acad. Sci.*, 1959, **45**, 791.
[14] Schmid, Robbins, and Traut, *Proc. Nat. Acad. Sci.*, 1959, **45**, 1236.
[15] Haworth, Hirst, and Isherwood, *J.*, 1937, 577.
[16] Haworth and Percival, *J.*, 1932, 2277.
[17] Fleming, Hirst, and Manners, *J.*, 1956, 2831.
[18] Peat, Turvey, and Evans, *J.*, 1959, 3341.
[19] Greenwood and Thomson, *J.*, 1961, 1534.
[20] Anderson and Greenwood, *J. Sci. Food Agric.*, 1955, **6**, 587.

co-workers [21] have isolated Floridean starch from other species of red algæ, and by periodate oxidation obtained $\overline{CL}$ values of 12, 14, and 10 for three samples. Since Floridean starch functions as a reserve carbohydrate, some variation in $\overline{CL}$ is not unexpected as this property will vary with the metabolic state of the algæ. Although the degree of branching in Floridean starches and glycogens may be similar, it must be noted that the polysaccharides differ in other respects, *e.g.*, viscosity, iodine-staining,[11] reaction with concanavalin-A,[1] and susceptibility to potato phosphorylase [22] and R-enzyme.[18]

Experimental

Analytical Methods.—Glucose (1—9 μg.) and maltose (2—14 μg.) were determined by the method of Park and Johnson.[8] The reagents required are potassium ferricyanide solution (0·5 g./l.), carbonate–cyanide solution (sodium carbonate 5·3 and potassium cyanide 0·65 g./l.) and ferric ion solution [ferric ammonium sulphate 1·5 g. and Duponol 1 g. (B.D.H. reagent, purified by Soxhlet extraction with ethanol and recrystallized three times from ethanol) in 1 l. of 0·05N-sulphuric acid]. Sugar solution (2 ml.), ferricyanide solution (1 ml.), and carbonate–cyanide solution (1 ml.) were mixed and heated in a boiling-water bath for 18 min., then cooled, and ferric ion solution (5 ml.) was added. After 20 min., the optical densities were measured on a Unicam S.P. 600 spectrophotometer at 700 mμ against a water–reagent control solution. When samples from enzyme digests were being examined, known amounts of maltose were also estimated to confirm the calibration graph.

In certain experiments, reducing sugars were determined by the Somogyi method.[7]

The concentration of glycogen was determined by acid hydrolysis (2N-sulphuric acid at 98° for 2·25 hr.) of a sample (*ca.* 0·5—1·0 mg.) and measurement of the liberated glucose.

Glycogen values were determined as described previously,[1] the results being standardized against a sample of rabbit-liver glycogen (G.V. 1·00) kindly provided by Professor F. Smith.

The absorption spectra of the glycogen–iodine complexes (0·01% of polysaccharide and 0·02% of iodine in 0·2% aqueous potassium iodide) were measured on a Unicam S.P. 600 spectrophotometer, as described previously.[11]

Micromethod for α-Amylolysis.—α-Amylase was prepared from human saliva as described previously.[6] The enzyme preparation was freeze-dried in 0·1M-phosphate–citrate buffer pH 7·0 and had an activity [6] of 16 units/mg., and was free from maltase. The apparent percentage conversion into maltose (P_M) produced by the incubation of the α-amylase with a series of starch-type polysaccharides of known degree of branching ($P_{1,6}$) was measured [6] and the following relationship obtained: $P_{1,6} = 23{\cdot}4 - 0{\cdot}20P_M$. This equation differs only slightly from that obtained previously [6] with a different preparation of α-amylase.

In the previous experiments, enzyme digests contained 4—5 units of α-amylase per mg. of glycogen, this enzyme concentration being sufficient to bring about the second stage of α-amylolysis.[23] It has now been found that variation of the enzyme concentration within the range 4—8 units per mg. of glycogen has no significant effect on the P_M value. Dilution of the enzyme and substrate caused no significant change in P_M value, as shown by the following experiment. Glycogen (*Ascaris lumbricoides* II; 14·2 mg.), 0·5% sodium chloride solution (2·5 ml.), 0·1M-phosphate–citrate buffer of pH 7·0 (2·5 ml.), α-amylase (6·0 mg.; 96 units), and water to a final volume of 25 ml. were mixed to give solution A and incubated at 36°. Samples (2 ml.) were removed at intervals and diluted to 250 ml. before analysis in duplicate. Other samples were removed as follows: 5 ml. diluted to 20 ml. with water (solution B); 5 ml. diluted to 100 ml. (solution C); 2 ml. diluted to 250 ml. (solution D). These solutions were also incubated at 36° and analysed at intervals, after appropriate dilution.

	P_M value		
Time of incubation (hr.)	4	7	24
Solution A	76	80	79
A (Somogyi method)	77	78	78
B	75	80	80
C	75	78	78
D	77	78	78

[21] Meeuse, Andries, and Wood, *J. Exp. Bot.*, 1960, **11**, 129.
[22] Liddle, Manners, and Wright, *Biochem. J.*, 1961, **80**, 304.
[23] Walker and Whelan, *Biochem. J.*, 1960, **76**, 257.

The effect of the sodium chloride concentration was also examined. Glycogen (*Ascaris lumbricoides* II; 13·9 mg.), 0·5% sodium chloride solution (2·5 ml.), buffer of pH 7·0 (2·5 ml.), α-amylase (5·2 mg.; 83 units), and water to 25 ml. were mixed (solution E) and incubated at 36°. Samples were removed as follows: 5 ml. diluted to 20 ml. (solution F); 5 ml. and 2 ml. of sodium chloride solution diluted to 20 ml. (solution G); 5 ml. and 10 ml. of sodium chloride solution diluted to 20 ml. (solution H); 2 ml. and 10 ml. of sodium chloride solution diluted to 250 ml. (solution I). The following P_M values were obtained after suitable dilution of aliquot portions:

Solution	E	F	G	H	I
After 6 hr. incubation	78	77	77	45	56
After 24 hr. incubation	80	79	79	68	73

Since higher concentrations of sodium chloride were inhibitory, a concentration of 0·005% was used in the later analyses.

The gastrocnemius muscle glycogens were analysed by incubating a sample (2·5—3·0 mg., concentration determined by acid hydrolysis), 0·5% sodium chloride solution (0·25 ml.), α-amylase (1·6 mg.; 25 units), and water to 25 ml. at 36°. After 15 and 24 hr., samples (3 ml.) were removed and diluted to 50 ml., and the P_M values were determined. With the soleus and pectoralis major samples, 1·5 and 0·75 mg., respectively, were incubated with 0·9 and 0·5 mg. of α-amylase in a final volume of 20 ml., and samples (3 or 4 ml.) were diluted to 25 ml. for analysis.

A sample of *Ascaris lumbricoides* II glycogen of P_M value 78 was analyzed with each muscle glycogen; all the *Ascaris* results fell within the range P_M 77—78.

Micromethod for β-Amylolysis.—A digest was prepared containing *Ascaris* glycogen (16·0 mg.), 0·2M-acetate buffer of pH 4·6 (4 ml.), β-amylase (825 units), and water to 20 ml., and incubated at 36°. A sample (5 ml.) was removed and diluted to 25 ml. The percentage production of maltose after 24 hr. was 49·5 (Park and Johnson), 49·2 (Somogyi) in the original digest, and 51·0 (Park and Johnson) in the diluted digest. After 42 hr., the results were 49·5, 49·5, and 50·4, respectively.

The muscle glycogens (2·5—3·0 mg.), 0·2M-acetate buffer of pH 4·6 (1 ml.), and β-amylase (160 units) were incubated at 36° in a total volume of 25 ml. Samples (4 ml.) were removed after 20 and 38 hr. and diluted to 50 ml. and the maltose contents were measured. *Ascaris* glycogen was similarly analysed, the results being 48—49% (compare the previous value [10] of 49%).

Isolation of Glycogens.—All biopsy samples were stored at −20° before extraction.

(*a*) *Patient* G. R. The sample (0·95 g.) was digested with 40% potassium hydroxide (5 ml.) at 98° until the tissue had dissolved. After cooling, water (3 ml.) was added and the glycogen precipitated by the addition of ethanol (10 ml.). It was purified by four further precipitations with ethanol, giving 39 mg. (by acid hydrolysis of a portion, equiv. to a glycogen content of 4·1%).

(*b*) *Patient* J. W. The muscle samples, together with some blood, were extracted with 40% potassium hydroxide solution (3—5 ml.) and purified as above. The yield of gastrocnemius glycogen (48 mg. from 530 mg. sample) was 9·1%; of soleus glycogen (5·2 mg. from 170 mg.) was 2·4%; and of pectorialis major glycogen (1·2 mg. from 200 mg.) was 0·6%. The difference between the glycogen contents of the tissues and the above yields was due to the presence of some blood plasma in the frozen samples.

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