Molecular Structure of Glycogens Isolated from the Livers of Foetal Animals

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Although many publications have been concerned with structural determinations of glycogen little attention has been paid to the structure of glycogen obtained from foetal tissues. Bell & Manners (1952) have recorded the β -amylolysis limit of foetal-sheep glycogen. Illingworth, Larner & Cori (1952) determined the average chain length of a sample of this glycogen, supplied by Dr D. J. Bell, and also a sample of foetal guinea-pig-liver glycogen. The other foetal glycogen which has been studied is that from pig's liver (Manners & Archibald, 1957). These authors recorded an end-group assay of this glycogen after oxidation with potassium periodate. Since investigations on foetal glycogen are so few, it was decided to make a study of its structure in samples taken throughout the gestation period. In the present work it was decided to use several methods for splitting liver glycogen in order to study any structural changes which might occur during the course of the gestation period.

The emphasis during this investigation has been on enzymic degradation and the chemical methods have been restricted solely to acid hydrolysis and periodate oxidation. In addition to the use of α -amylase, β -amylase and phosphorylase for enzymic degradation, the preparation and use of amylo-1:6-glucosidase was undertaken. This enzyme, first prepared by Cori & Larner (1951), hydrolyses the α -1:6-linkage in the phosphorylase limit dextrin, producing glucose and a dextrin which is susceptible to further phosphorylase action; the combined action of phosphorylase and amylo-1:6-glucosidase thus results in the complete digestion of glycogen with the formation of glucose 1-phosphate and glucose (Illingworth *et al.* 1952).

MATERIALS AND METHODS

Glycogen. Most of the specimens investigated were extracted from the livers obtained from foetal goats whose foetal age was known (the crown-rump length of each foetus was measured to check the age). The method of isolation, after extraction with alkali and subsequent purification, followed the outline given by Somogyi (1934). The purified glycogen was dried *in vacuo* over phosphorus pentoxide at 100°. Glycogen from other species was prepared in the same way.

α -Amylase. Freeze-dried specimens of human saliva served as the source of this enzyme.

β -Amylase. A commercial specimen of this enzyme was obtained from L. Light and Co. Ltd.

Phosphorylase. Initial preparations of this enzyme followed the method used by Illingworth & Cori (1953); this method was found difficult to reproduce and later preparations were obtained by that of Bot & Zsindely (1956).

Amylo-1:6-glucosidase. This enzyme has yet to be obtained pure. About 80% of the glucosidase present in a crude muscle extract was recovered in the precipitate obtained at 0.41 saturation with ammonium sulphate. It was obtained in a partially purified state from the pooled supernatant fluids after centrifuging the first crystals of phosphorylase (Cori, Illingworth & Keller, 1955). The mother liquor was diluted with 2.7 mM-ethylenediamine-tetra-acetic acid (EDTA), pH 7.2, until the protein concentration was about 1%. Amylase was removed by means of two starch absorptions. Cori *et al.* (1955) reported that repeated starch absorptions decreased the yield of glucosidase but Larner & Schliselfeld (1956) used this stage of purification. Neutral saturated ammonium sulphate solution was then added to 0.3 saturation. The precipitate was dissolved in about half the original volume of a buffer solution containing 2.7 mM-EDTA and 3 mM-cysteine at pH 7.2. The clear solution was dialysed against this buffer at 0° for 1 hr. and then frozen in small portions before storage at -15°. The addition of glutathione (mM) restores the activity lost after repeated freezing and thawing (Larner & Schliselfeld, 1956). The enzyme was assayed by the method of Cori *et al.* (1955), whereby glucosidase is allowed to act on limit dextrin in the presence of an excess of muscle phosphorylase so that it becomes the limiting enzyme in degradation.

Acid hydrolysis. Glycogen solution (20 mg.; 1 ml.) was heated with 5 ml. of 1.5N-sulphuric acid at 100° for 2 hr. Pirt & Whelan (1951) have shown that these are the optimum conditions for minimum destruction of the glucose formed. The method of Nelson (1944) was used to estimate the glucose produced and the degree of hydrolysis was estimated on the weight of glycogen used.

Periodate oxidation. These reactions were performed in 5 ml. volumetric flasks in the refrigerator (0-5°). 0.2M-Sodium periodate solution (4 ml.) was added to an aqueous solution of glycogen (1 ml.; 50 mg.). Portions (1 ml.) were taken at suitable time intervals and run into small tubes containing ethylene glycol (0.1 ml.). All apparatus and reactants had before this stage been precooled in the refrigerator. After standing for 1 hr. at room temperature, the formic acid liberated was titrated to a methyl red end-point with 0.01N-barium hydroxide from a carbon dioxide-free microburette fitted with a hypodermic needle. Control

reactions of sodium periodate solution without the glycogen, run parallel to the oxidation, showed no increase in acidity. All glassware used in these experiments was washed in chromic acid and water followed by steaming before drying.

Periodate-oxidized glycogen was also examined by paper chromatography (Partridge, 1949) after acid hydrolysis to detect glucose, which would indicate the presence of anomalous 1:2- or 1:3-linkages in glycogen.

α -Amylolysis. An enzyme digest containing 2 ml. of glycogen solution (40 mg.), 20 ml. of phosphate-citrate buffer (0.16M- Na_2HPO_4 solution adjusted to pH 7.0 with citric acid), 4% sodium chloride solution (1 ml.), salivary amylase solution (1 mg.; 1 ml.) and water (26 ml.) was incubated at 37°. Samples (2 ml.) were withdrawn at suitable intervals and analysed for total reducing sugar.

β -Amylolysis. These digests contained 40 mg. of glycogen solution (2 ml.), 0.2M-acetate buffer at pH 4.6 (5 ml.), 9% sodium propionate solution (1 ml.), water (21 ml.) and β -amylase solution (1 ml.; 1 mg.) and were incubated at 28°. Samples were withdrawn at suitable intervals and the reducing-sugar content of each was determined.

Phosphorolysis. Glycogen solution (40 mg., 2 ml.) was incubated at 35° with 0.4M-phosphate buffer (pH 6.8; 2 ml.), adenylic acid solution (1 ml.; 1 mg.), water (5 ml.) and phosphorylase solution (0.5 ml.; 9500 units/ml.). Samples (2 ml.), withdrawn at suitable intervals, were analysed for glucose 1-phosphate. The inorganic phosphate present in the reaction mixture was precipitated with magnesia mixture (2 ml.). This consisted of 0.5M-magnesium nitrate, 2N-ammonium nitrate and aq. 2N-ammonia solution (Sacks, 1949). The precipitate, which removes the limit dextrins by absorption, was allowed to stand overnight at room temperature before being removed by centrifuging. The glucose 1-phosphate content of the supernatant was determined, after acid hydrolysis in N-sulphuric acid for 5 min. at 100°, as inorganic phosphate by Allen's (1940) method.

End-group determination. The reaction mixture consisted of glycogen solution (1 ml.; 20 mg.), 0.4M-phosphate buffer (prepared from Na_2HPO_4 and KH_2PO_4) at pH 7.0 (5 ml.), 7.5 mM-EDTA at pH 7.0 (1 ml.), 1 mg. of adenylic acid (1 ml.), 9% sodium propionate solution (1 ml.), water (6 ml.), 0.1 ml. of phosphorylase (9500–14 500 units/ml.), and 0.1 ml. of glucosidase (4000 units/ml.). After incubation at 35° for 2 hr., the sum of free glucose and glucose 1-phosphate was determined by the procedure described by Cori & Larnier (1951).

RESULTS

Acid hydrolysis. It was shown by paper chromatography that the sole product of acid hydrolysis of different samples of liver glycogen was glucose. The results of the chemical analyses of these hydrolytic reactions are given in Table 1.

Periodate oxidation. Since no glucose was found in the hydrolysates of solutions of periodate-oxidized glycogens it was concluded that the samples of glycogen investigated contained neither 1:2- nor 1:3-glucosidic linkages. A summary of the average chain lengths deduced from periodate oxidations of the glycogens studied is also given in

Table 1, and a typical example of the course of periodate oxidation of a pooled specimen of foetal-goat-liver glycogen is illustrated in Fig. 1.

α -Amylolysis. The course of α -amylolysis was expressed as percentage conversion into maltose. The limit of such degradation is shown in Table 1.

β -Amylolysis. The β -amylolysis limits of the different glycogens examined are recorded in Table 1 as percentage conversions into maltose. The average chain lengths of all these glycogens have been determined by means of periodate oxidation (see Table 1), and so the exterior chain length for all the specimens can be calculated.

It has been shown by Peat, Whelan & Thomas (1952) that the side chains in an amylopectin β -limit dextrin contain two or three glucose residues. Summer & French (1956), after studying the action of β -amylase on model substrates, concluded that the exterior chain 'stubs' in a glycogen or amylopectin β -limit dextrin could never be shorter than 2.5 glucose units.

The exterior chain length of the glycogen specimens quoted in Table 1 have therefore been calculated on the assumption that the β -amylase action on glycogen ceased at the second or third residue from the outermost branch points. The exterior chain length by the definition of Manners (1955) is equal to the number of glucose residues removed by β -amylase plus 2.5. The interior chain length is then obtained by subtracting the exterior chain length from one less than the average number of glucose units as given in Table 1.

Phosphorolysis. Limit dextrins have been prepared from different glycogen specimens by the action of crystalline rabbit-muscle phosphorylase. The extent of phosphorolysis is recorded as the percentage conversion of glycogen into glucose 1-phosphate in Table 1.

The results obtained for phosphorolysis of foetal-goat-liver glycogen suggest a trend towards increasing degradation with increasing foetal age. This is illustrated in Fig. 2, in which age is plotted against percentage phosphorolysis.

End-group determination. The results obtained by the combined action of crystalline muscle phosphorylase and amylo-1:6-glucosidase are shown in Table 1.

Cori & Larnier (1951) postulate that the ratio of free glucose to total glucose (i.e. free glucose plus phosphorylated glucose) in a digest of a polysaccharide with phosphorylase plus glucosidase should be a measure of the degree of branching and, conversely, of the average number of glucose units per branch. This has been used to calculate the average number of glucose units per branch.

An ion-exchange resin (De-Acidite FF) in the chloride form was used to separate an enzymic digest of liver glycogen after treatment with muscle

Table 1. *Structural analysis of glycogens*

Sources of glycogen*	Conversion into glucose by acid (%)	[α] _D in water	Action of phosphorylase + amylo-1:6-glucosidase			Action of β -amylase			Conversion by phosphorylase (% as glucose 1-phosphate)	Conversion by α -amylase (% as maltose)
			Average chain length (periodate assay)	Glucose (%)	Free + phosphorylated glucose (%)	Average chain length	Conversion into maltose (%)	Exterior chain length	Interior chain length	
Male rabbit	95.8	202°	12.6	7.0	96.4	13.8	49.7	8.9	3.4	75.0
Female goat	98.6	200	10.8	9.1	94.0	10.3	49.7	7.8	2.3	79.3
Pregnant goat	97.5	—	12.4	7.9	95.6	12.1	53.3	9.10	1.2	75.8
Pregnant rabbit (11 days)	103.5	199	11.7	8.1	97.5	12.0	52.7	8.9	2.3	80.3
Pregnant guinea pig	—	193	—	7.9	91.4	11.5	43.2	7.8	3	—
Pregnant rabbit (25 days)	—	—	12.3	—	—	—	—	—	—	—
Foetal cat	99.0	200	13.1	7.9	94.5	12.0	46.4	8.9	3.4	80.5
Pregnant monkey	104.0	—	12.0	—	—	—	42.4	7.8	3.4	—
F.G. (pool)	95.5	198	12.3	8.8	99.0	11.3	52.3	9.10	2.3	80.7
F.G. (146)	97.5	200	12.8	8.8	96.0	11.0	52.3	8.9	2.3	80.5
F.G. (139)	94.5	202	12.6	—	—	—	51.7	9	3	79.0
F.G. (135)	94.8	—	15.7	8.4	98.3	11.7	44.0	9.10	5.6	—
F.G. (134)	95.5	—	15.3	6.8	95.3	14.0	53.3	11.12	2.3	82.5
F.G. (128)	102.0	196	11.5	7.8	94.4	12.4	50.6	8.9	2.3	80.4
F.G. (127)	—	—	12.6	6.9	98.7	14.2	49.7	8.9	3.4	77.4
F.G. (120)	101.3	—	14.2	—	—	—	48.8	9.10	3.4	83.5
F.G. (119)	—	—	12.8	7.8	98.1	12.6	—	—	—	—
F.G. (116)	94.5	203	15.3	6.8	98.9	14.6	52.7	10.11	3.4	75.3
F.G. (115)	96.3	—	12.3	7.0	96.6	13.8	—	—	—	78.8
F.G. (111)	—	—	13.8	8.1	98.5	12.2	—	—	—	—
F.G. (110)	98.5	198	14.6	6.9	93.3	13.5	51.4	10	4	81.0
F.G. (106)	—	197	12.4	8.2	98.7	12.1	51.6	8.9	2.3	80.3
F.G. (103)	—	—	12.6	—	—	—	40.0	7.8	4.5	—
F.G. (100+)	—	—	12.4	8.0	93.6	11.7	52.0	8.9	2.3	81.7
F.G. (95)	99.2	200	12.1	8.8	97.0	11.1	43.3	7.8	3.4	—

* F.G. indicates that the source of glycogen was the liver of a foetal goat of the estimated age (in days) shown in parentheses. All results are calculated in terms of weight of glycogen.

phosphorylase and glucosidase. The free glucose passed through the resin whereas the phosphorylated sugar was retained by it. A molar solution of sodium chloride was used to elute the glucose 1-phosphate. The results obtained by this procedure were in agreement with those given by the method of Cori & Larnar (1951).

Specific rotations. These were measured in a semi-microtube of length 0.5 dm. with aqueous 1% (w/v) solutions of glycogen, and are shown in Table 1.

DISCUSSION

The experimental results recorded above show no marked departure from the results obtained in other structural investigations of liver glycogen. With the exception of phosphorolysis, there is no obvious variation in the behaviour of the glycogen

with respect to degradation as the foetal age of the goat increases. With phosphorolysis, however, there are indications of increased susceptibility to degradation after 130 days (Fig. 2). This increased susceptibility to phosphorolysis disappears in the mature goat (Table 1). It is interesting to speculate whether the susceptibility to phosphorolysis decreases quickly after parturition.

At this stage attention may be drawn to the work of Illingworth *et al.* (1952), who found that the degree of phosphorolysis decreased progressively in glycogens isolated from the livers of embryonic, new-born and young adult guinea pigs. Unfortunately only a single specimen of embryonic liver glycogen was quoted with no indication of the approximate foetal age. These authors reported differences in structure for embryonic, new-born and young adult guinea pigs with phosphorylase degradations of 42.7, 40.8 and 39.9% respectively. The present work suggests that these figures are well within the range of normal variation for a particular age grouping of the same species.

The same authors also quote figures for the enzymically determined end group. This is measured by the free glucose formed after glycogen has been subjected to the combined actions of crystalline muscle phosphorylase and amylo-1:6-glucosidase. The figures given are 7.7, 6.9 and 6.5% of end groups in foetal, new-born and young adult liver glycogen of guinea pigs. With the same method of analysis it was found during the present investigation that the end-group content of foetal-goat-liver glycogen varies from 8.8 to 6.8% in an irregular manner within the range of foetal ages 95–146 days (full term, approx. 150 days) (Table 1). In the light of these results the conclusions drawn by Illingworth *et al.* (1952) may need modification.

A third discrepancy exists between the present work and that of Illingworth *et al.* (1952). They have shown that the values for end groups obtained by the enzymic method of phosphorylase plus glucosidase degradation showed about 20% constant deviation from the values obtained by periodate oxidation. They found that the enzymic values were the higher in all cases, but the present study has shown that the enzymic values, with few exceptions, are lower than the values derived from periodate oxidation. The arithmetic mean of the average chain lengths obtained by enzymic studies is 12.4, whereas that obtained from the mean of the periodate values is 12.9. It is suggested that amylo-1:6-glucosidase can now be prepared in a much purer state than was possible for the earlier preparations of this enzyme by Illingworth *et al.* (1952). Traces of amylases in their preparations would appreciably affect their end-group determinations and this is probably the major cause of the discrepancies.

Fig. 1. Effect of varying the length of time of periodate oxidation on the calculated chain length of foetal-goat-liver glycogen. A pooled specimen of foetal-goat-liver glycogen was oxidized for different times and the degree of oxidation estimated by titration of the liberated formic acid with 0.01 N-Ba(OH)₂.

Fig. 2. Variation of the limit of phosphorolysis of foetal-goat-liver glycogen with foetal age. The degree of phosphorolysis is recorded as glucose 1-phosphate expressed as a percentage of that theoretically available. Phosphorolysis was carried out at 35° with 0.4M-phosphate buffer (pH 6.8) for up to 4 hr.

Furthermore, Illingworth *et al.* (1952) point out that the glycogen content of such organs as liver and muscle is subject to a rapid turnover and that the composition of a particular glycogen sample depends on the previous history of the animal from which the tissue is taken. Pioneer experiments which resulted in this suggestion have been performed by Bell (1936), who determined end-group values by the methylation method of glycogen formed after ingestion of galactose by starved rabbits. Average chain lengths of 18 were found after galactose feeding. Later Bacon, Baldwin & Bell (1944) showed by the same technique that glycogen with average chain lengths of 12 was formed after the feeding of both glucose and fructose and after the injection of fructose. Feeding with sucrose was found to result in the formation of glycogen having a unit chain of 18 glucose radicals.

Schlamowitz (1951) has repeated Bell's (1936) work and attempted to find out whether the type and level of carbohydrate administered reflects itself in the production of glycogens with different branching characteristics. Glucose, galactose and fructose were administered to starved rabbits by stomach tube and by continuous intravenous infusion. Analyses for end groups by periodate oxidation on the purified liver glycogen from these animals and from normal controls showed that the values obtained after infusion of carbohydrate were greater than those obtained after administration by stomach tube. These, in turn, had end-group values greater than those for the glycogen from animals fed on rabbit pellets or sucrose or carrots. Schlamowitz considers end-group values of 19 as 'normal' whereas Bell and other workers consistently report values of 12. It is clear that the previous history of the animal studied and its feeding habits before experimentation must be taken into account.

Knowledge of the effect of anaesthetics on glycogen structure is meagre. During the present investigation many of the foetal goats and pregnant animals from which the liver glycogen was obtained were under Nembutal anaesthesia for as long as 7 hr. in some cases, but this time was not constant. There is no evidence to show whether the anaesthetic has any effect on the configuration of glycogen.

End-group assays of glycogen by periodate oxidation have been subjected to a great deal of criticism and discussion. The most recent publication on this subject is by Manners & Archibald (1957), who oxidized 15 samples of glycogen by potassium metaperiodate and also studied the method used by Potter & Hassid (1948). The latter oxidized their specimens, dissolved in sodium chloride solution, by sodium metaperiodate for 25 hr. at 2°. Manners &

Archibald found that under these conditions oxidation is incomplete. Schlamowitz (1951) produced experimental evidence that oxidation under these conditions was complete. However, the average chain lengths of glycogen calculated from his results are higher than any recorded by other workers on similar specimens and suggest incomplete oxidation.

Periodate oxidations carried out during the course of this work used the conditions of Poglase, Smith & Tyler (1952). These workers used an aqueous solution and not a 1.5 % sodium chloride solution of glycogen for the sodium metaperiodate oxidation at 2°. Our experimental evidence indicates that under the conditions described, the oxidation by sodium metaperiodate reached completion. Furthermore, 24 specimens of glycogen were assayed after oxidation under these conditions and, of these, 18 had average chain lengths within the range 10–14. These results agree with those of Abdel-Akher & Smith (1951), who for another 37 individual glycogens found average chain lengths of 10–14, and with those of Manners & Archibald (1957), who assayed 30 specimens of glycogen and found that the average chain lengths of 23 of these were within this range. Thus there is evidence that, under the present conditions, oxidation is complete after 72 hr. The present work is, however, in agreement with Manners & Archibald that different samples of glycogen are oxidized, under identical conditions, at slightly differing rates. The present experiments suggest that the recommendation of Manners & Archibald to continue periodate oxidation for 7–10 days is perhaps over-generous for many glycogen samples.

It is interesting to compare our results after β -amylolysis of 21 samples of glycogen with the results obtained after similar degradation by Liddle & Manners (1957*a*). These workers degraded 25 samples of glycogen, with average chain lengths varying from 8 to 18 glucose residues, with barley β -amylase. They found that the percentage conversion into maltose was 46 ± 7 in all but two instances. In a later paper Liddle & Manners (1957*b*) report average chain lengths of 7–15 in 15 samples of glycogen. β -Amylolysis during the present work has covered 21 specimens, all of which showed a percentage conversion into maltose within the range 47 ± 7 . Earlier work by Bell & Manners (1952) showed that 11 glycogens (chain length about 12) on β -amylolysis with crystalline sweet-potato enzyme lost about 45 % of the molecule solely as maltose. These authors also tabulate the results of β -amylolysis on 16 samples of glycogen by other workers. In this case the range of percentage conversion into maltose was 20–53 with enzyme from either wheat or barley. The β -amylase used during the present investigation was

an amorphous commercial sample, (L. Light & Co. Ltd.). The results illustrate the variation in molecular structure that exists in glycogen and this is in agreement with the work of Bell & Manners (1952) and also of Liddle & Manners (1957*a, b*). Afanas'eva & Stepanenko (1956) compared glycogens from rabbit and frog livers. Although the β -amylolysis limit and the point of branching were shown to be the same in both species, the frog-liver glycogen showed greater compactness in that the outer branches were shorter. In the present work this shortening of the outer chains has been observed in liver glycogens from pregnant rabbit, female goat and foetal goat.

Manners (e.g. Manners, 1954) consistently examines α -amylase digests of different glycogens (rabbit liver, brewer's yeast and human muscle from a case of von Gierke's disease) by paper chromatography with the orcinol-spray reagent (Bevenue & Williams, 1951) or the acid resorcinol reagent (Bell, 1955). In all cases he has failed to show the presence of any ketose. The α -amylase digests of liver glycogen from pregnant doe, pregnant goat and foetal goat at varying stages of gestation have been repeatedly examined by paper chromatography in this laboratory. Neither maltulose nor ketose-containing oligosaccharides could be detected. We therefore conclude, like Manners, that fructose is not a constituent of the above glycogens.

Stetten & Stetten (1954) have shown by injecting radioactive glucose intraperitoneally into rats that liver glycogen, although yielding only glucose upon hydrolysis, is metabolically inhomogeneous. They showed that the peripheral tier, determined by β -amylolysis, from liver glycogen initially accounted for the bulk of the ^{14}C . With the passage of time, however, an increasing fraction of the labelled glucose was found in the β -amylase limit dextrin until, by the twelfth hour, this fraction exceeded that in the periphery. Glycogen turnover *in vivo* is therefore not merely a replacement of pre-existing molecules by newly formed glycogen molecules. This is compatible with the inhomogeneous structures found by us and other workers.

SUMMARY

1. Glycogen was isolated from livers of goat foetuses over a wide range of foetal ages and its structure investigated chemically and enzymically.

2. Average chain lengths were calculated from the results of periodate oxidation.

3. α -Amylase, β -amylase, phosphorylase and amylo-1:6-glucosidase were employed in the enzymic degradations and average chain lengths calculated from the results of the combined actions of phosphorylase and amylo-1:6-glucosidase.

4. The average chain lengths obtained by the chemical and enzymic methods were compared.

5. The interior and exterior chain lengths were calculated from the results of β -amylolysis and periodate oxidation.

6. The 25 specimens of glycogen investigated resembled the majority of mammalian glycogens in their degree of branching.

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