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THE MOLECULAR STRUCTURES OF SOME NEWLY FORMED RAT-LIVER GLYCOGENS

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ABSTRACT

Glycogen has been isolated from the livers of rats which had been fasted and then intubated with D-glucose. The structure of the glycogen, as determined by iodine staining and enzymic methods, was shown to be very similar to that from control animals. There were slight differences in the iodine-staining properties, but not as marked as that previously reported in the literature.

INTRODUCTION

In vivo glycogenic procedures, such as administration of D-glucose to starved animals, have been used to study the control of glycogen synthesis. In an earlier report[1], differences in the iodine-adsorption characteristics between the rapidly formed liver-glycogen and "normal" liver-glycogens were observed. It was suggested that these differences were due to structural differences between the two types of glycogen. In order to investigate the possibility further, normal and rapidly formed glycogens were isolated and their structures compared by both iodine-staining and enzymic-degradation methods. The results of this study are now presented.

MATERIALS AND METHODS

Streptozotocin was obtained from I.C.N. Pharmaceuticals, Surrey. Protamine zinc insulin (40 units/ml) and regular insulin (40 units/ml) were obtained from Burroughs Wellcome & Co., London. Sweet-potato beta-amylase (Type 1B) was obtained from the Sigma Chemical Co. Salivary alpha-amylase was isolated from human saliva by the acetone fractionation procedure of Bernfeld[3]. Isoamylase was partially purified from a *Cytophaga* culture filtrate (Glaxo Research Ltd. Batch No. 1E1A/R69F) by chromatography on DEAE cellulose using 0.01M Tris-HCl buffer at pH 8.0. Controlled-pore glass granules (CPG-10, 1250 Å) were obtained from BDH Chemicals Ltd.

Experimental procedures with animals. — A high carbohydrate diet containing 58% of D-glucose, 22% of casein, 11% of cellulose, 6% of Hawk–Oser salt mixture[3],

2% of liver extract and vitamin mixture[4], and 1% of calcium stearate was prepared and compressed to form 1-g pellets.

Albino rats aged between two and three months were obtained from the Centre for Laboratory Animals, Edinburgh, and maintained on an adequate stock-diet. Control animals were maintained on the stock diet until sacrifice. Intubated animals were starved for 18–24 h and then intubated with 60% (w/v) aqueous D-glucose (5 ml) by means of a stomach tube under ether anaesthesia. They were then maintained on the high carbohydrate diet until sacrifice.

Diabetic animals were given a single, intraperitoneal injection of streptozotocin [55 mg/kg body weight in 0.01M sodium citrate (pH 4.5)] one week before the experiment. During this period, they showed increased urine excretion, glucose in the urine, and high blood-sugar levels. They were maintained on the stock diet until 3 days before the experiment and thereafter on the high carbohydrate diet. Insulin-treated, diabetic animals received an intraperitoneal injection of protamine zinc insulin (40 units/kg body weight) 24 h before sacrifice, and the same dose of regular insulin exactly 2 h before sacrifice. Throughout the experimental period, they were maintained on the high carbohydrate diet.

Removal of the liver and extraction of glycogen. — Immediately after sacrifice, the liver was removed and rapidly frozen in liquid nitrogen. Pieces of frozen liver were weighed and then digested in 40% (w/v) aqueous potassium hydroxide (5 ml) on a boiling water-bath. After 40 min, the digest was cooled and two volumes of ethanol were added. The glycogen was allowed to precipitate overnight at 4°.

Estimation of glycogen. — After centrifugation, the precipitated glycogen was redissolved in a known volume of water, and aliquots were taken for the determination of carbohydrate by the phenol–sulphuric acid method[5].

Purification of glycogen. — The glycogen was purified by five reprecipitations with ethanol, washed twice with boiling ethanol, and finally dried with ether or acetone.

Iodine-staining procedure. — The iodine-staining procedure used was that described by Archibald et al.[6]. The spectrum of a solution containing 0.01% of glycogen, 0.02% of iodine, and 0.2% of potassium iodide in half-saturated ammonium sulphate was recorded against an iodine–iodide control using a Unicam SP800 spectrophotometer. From the spectrum, the wavelength of maximum absorption (λ_{max}) and the absorption at that wavelength corrected for the exact carbohydrate content (E_{max}) were determined. Under these conditions, the average chain-length of the glycogen (c.l.) is given[6] by the relationship:

$$\text{c.l.} = 16 + 0.114(\lambda_{max} - 500).$$

alpha-Amylolysis procedure. — Digests containing glycogen (0.5 mg), salivary amylase (2 units/ml), and 0.05% sodium chloride in 4 ml of 0.01M sodium citrate–phosphate buffer (pH 7.0) were incubated at 37° for 18–24 h under an atmosphere of toluene. After incubation, the production of reducing sugars was determined as maltose by the Nelson–Somogyi method[7], and the total carbohydrate was determined

by the phenol–sulphuric acid method[5]. The apparent percentage conversion into maltose (alpha-amylolysis limit) is related to the average chain-length under these conditions[8] by the expression:

$$100/\text{c.l.} = 23.3 - 0.21 \text{ (alpha-amylolysis limit)}.$$

beta-Amylolysis procedure. — Digests containing glycogen (1 mg) and beta-amylase (50 units) in 0.8 ml of 0.02M sodium acetate buffer (pH 4.8) were incubated at 37° for 18–24 h under an atmosphere of toluene. After incubation, the percentage conversion into maltose (beta-amylolysis limit) was determined in a similar manner to the determinations of alpha-amylolysis limit. The average, exterior chain-length (e.c.l.) was calculated from the average chain-length determined by alpha-amylolysis, and the beta-amylolysis limit, by the expression:

$$\text{e.c.l.} = \text{beta-amylolysis limit} \times \text{c.l.} + 2.0.$$

The average, interior chain-length (i.c.l.) was calculated from the expression:

$$\text{i.c.l.} = \text{c.l.} - (\text{e.c.l.} + 1).$$

Chain-length determination by isoamylase digestion. — The isoamylase method for the direct determination of average chain-length was used[9]. Digests containing glycogen (2.5 mg) and *Cytophaga* isoamylase (1.6 units/ml) in 0.5 ml of 0.05M sodium acetate buffer (pH 5.5) were incubated at 37° for 18–24 h. After incubation, the reducing sugars were determined as glucose by the Nelson–Somogyi method[7], and the total polysaccharide content by the phenol–sulphuric method[5]. Since isoamylase only cleaves the (1 → 6)-α-D-glucosidic inter-chain linkages, the ratio of total glucose content to the free reducing-groups gives a direct measurement of the average chain-length.

Molecular-weight studies by controlled-pore glass chromatography. — Aqueous glycogen (0.5 ml, 4 mg/ml) was applied to a column (93 × 1 cm) of glass granules (pore size, 1250 Å) equilibrated in 0.05M Tris-HCl (pH 7.2). The column was eluted with the same buffer at a flow rate of 0.3 ml/min, and 1-ml fractions were collected. The carbohydrate content of the fractions was determined by the phenol–sulphuric acid method[5]. The exclusion volume of the column and the total inclusion volume were determined by using a glycogen of high molecular weight (extracted with cold mercuric chloride from rat liver[10]) and D-glucose, respectively. The polydispersity of the glycogen results in a wide peak which can be described in terms of its elution volume for the maximum point, and its breadth at half the maximum value. The elution volume at the maximum value was expressed as the K_{av} value where:

$$K_{av} = (V_e - V_t)/(V_0 - V_t),$$

and V_e = elution volume; V_0 = exclusion volume; and V_t = inclusion volume.

RESULTS AND DISCUSSION

Rats were starved for 18–24 h, intubated with D-glucose, and then fed the high carbohydrate diet. At various intervals after intubation, the animals were sacrificed and the glycogen content of the liver was determined. Fig. 1 shows the variation in liver-glycogen content with post-intubation time. It can be seen that the starvation period depleted the liver glycogen and that there was a rapid resynthesis of glycogen in the first twelve hours after intubation. Thereafter, the glycogen level remained approximately constant, but considerably elevated above the control values.

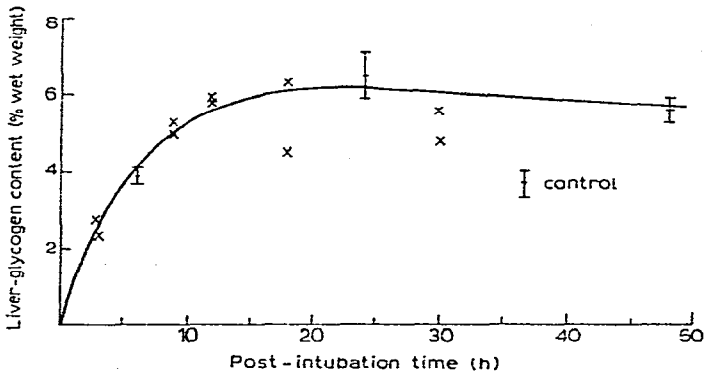

Fig. 1. Variation in liver-glycogen content with post-intubation time; where more than two experiments were performed, values are shown as a mean value ±standard error.

Glycogens obtained 6, 24, and 48 h after intubation were purified; iodine-staining, and alpha- and beta-amylolysis procedures were then carried out. The results (Table I) showed that there was no significant difference between the alpha- and beta-amylolysis limits of the intubated glycogens and control glycogens, but that there appeared to be a small increase in the λ_{max}. However, there was little variation in the E_{max} values, whereas the results of Chapman, Felts, and Chaikoff[1] had shown up to a twofold variation in the iodine adsorption.

Table II shows the values of average chain-length derived from the results in Table I, together with the values obtained from the isoamylase method. It can be seen that there is good agreement between the three procedures. The values of average interior and exterior chain-lengths are also shown.

Although there was little structural difference between the intubated glycogens and control glycogens, there was considerable overall variation in the glycogens with average chain-lengths carrying between 10–15 D-glucose residues. This variation was greater than that expected from experimental error. Fig 2. shows the variation in average, interior chain-length and average, exterior chain-length with varying chain-length. This result demonstrates that most of the variation in the glycogen chain-

TABLE I

STRUCTURAL ANALYSIS OF GLYCOGENS FROM CONTROL AND STARVED–INTUBATED ANIMALS[a]

Treatment	*Number of animals*	*Liver-glycogen content* (% *wet weight*)	*Iodine-staining properties*		*beta-Amylolysis limit* (%)	*alpha-Amylolysis limit* (%)
			λ_{max} (*nm*)	E_{max}		
Control	8	3.7 ±0.3	464 ±1	1.10 ±0.03	45.1 ±1.9	75.3 ±1.5
6 h Post intubation	6	3.9 ±0.2	480 ±5**	1.10 ±0.06	46.8 ±1.2	75.4 ±2.1
24 h Post intubation	7	6.5 ±0.6**	477 ±3**	1.20 ±0.02**	46.3 ±1.0	78.6 ±0.6
48 h Post intubation	4	5.6 ±0.3	459 ±5	1.07 ±0.04	42.1 ±1.9	77.5 ±1.1

[a]The results are expressed as a mean ±standard error of the mean. The significance of the difference between control and experimental means was assessed by using a t test. The probability of a null hypothesis is shown by asterisks ($p>0.05$, no asterisk; $p<0.05$, one asterisk; $p<0.01$, two asterisks).

TABLE II

CHAIN-LENGTH PARAMETERS OF GLYCOGENS FROM CONTROL AND INTUBATED ANIMALS

Treatment	*Number of glycogens*[a]	*Average chain-length*[b] (*A*)	(*B*)	(*C*)	*External chain-length*	*Internal chain-length*
Control	8	11.9 ±0.1	12.0 ±0.4	12.8 ±0.5(6)	7.5 ±0.5	3.6 ±0.1
6 h Post intubation	6	13.7 ±0.6	12.0 ±0.7	12.8 ±0.4(3)	7.7 ±0.4	3.5 ±0.2
24 h Post intubation	7	13.4 ±0.3	13.0 ±0.2	13.6 ±0.2(6)	8.0 ±0.2	4.0 ±0.1
48 h Post intubation	4	11.3 ±0.6	12.7 ±0.3(3)	12.5 ±0.5(2)	7.2 ±0.1(3)	4.5 ±0.5(3)

[a]Number of glycogens analysed as stated or given in brackets. [b](*A*), determined from iodine-staining data; (B), determined from alpha-amylolysis data; (C), determined using isoamylase.

length was due to variations in the exterior chain-length. This probably reflects the fact that exterior chain-length depends upon the relative activities of three enzymes, namely, glycogen synthetase, phosphorylase, and branching enzyme, whereas the interior chain-length is dependent primarily on the specificity of the branching enzyme. Liver branching-enzyme has been shown to act on glycogen having an average, exterior chain-length of 11–21 D-glucose residues[11], and to transfer a maltosaccharide containing six or more D-glucose residues[12]. However, no detailed studies have been made on the position of transfer. The corresponding muscle-enzyme has received more study, and has been shown to preferentially transfer chain segments of seven

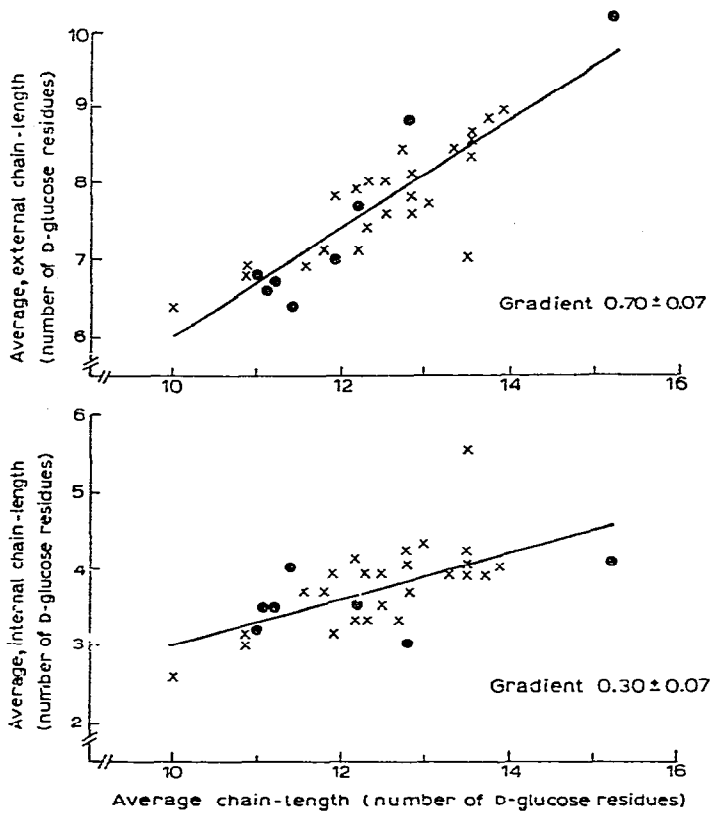

Fig. 2. Variation in external and internal chain-length with the overall, average chain-length of rat-liver glycogens: glycogens from intubated rats (×) and control rats (●).

TABLE III

STRUCTURAL ANALYSIS OF GLYCOGENS FROM CONTROL AND TREATED ANIMALS[a]

Treatment	*Number of animals*	*Liver-glycogen content (% wet weight)*	*Iodine-staining properties*		*beta-Amylolysis limit (%)*	*alpha-Amylolysis limit (%)*
			λ_{max} *(nm)*	E_{max}		
Control	8	3.7 ±0.3	464 ±1	1.10 ±0.03	45.1 ±1.9	75.3 ±1.5
A[b]	2	12.3 ±1.7**	477 ±2**	1.12 ±0.02	46.0 ±0.6	81.5 ±2.5
B[c]	2	5.8 ±0.7	447 ±3*	1.07 ±0.07	45.8 ±0.2	72.5 ±1.5
Diabetic	2	3.4 ±0.3	458 ±2	1.10 ±0.02	41.5 ±0.5	76.0 ±4.0
Insulin-treated diabetic	4	6.5 ±1.5	461 ±4	1.00 ±0.09	44.7 ±3.1	74.0 ±1.0

[a]See Table I for details. [b]A, animals starved for 36 h, then intubated, and re-fed on high carbohydrate diet; the animals were then sacrificed after 24 h and the glycogen was extracted (see text for details). [c]B, Animals starved for 18 h, then intubated, and re-fed on high carbohydrate diet for 6 h. This procedure was repeated, and then the animals were sacrificed and the glycogen was extracted.

D-glucose residues, and to place the segment in such a way as to maintain the average, interior chain-length at about three D-glucose residues[13].

Other starvation–refeeding procedures and the insulin treatment of diabetic animals were tried (Table III). Although the liver-glycogen content was high, there was no significant variation in the alpha- and beta-amylolysis limits of the isolated glycogens. However, there was some variation in the λ_{max} values of their iodine complexes. The mechanism of iodine binding by glycogen is not clearly understood, although the adsorption to linear oligosaccharides has recently been clarified[14] and may involve a series of maltosaccharide helices, each of which may contain six D-glucose residues. Although a similar mechanism may exist for the exterior chains of glycogen, the nature of the weaker adsorption to the interior, branched regions is uncertain.

Chapman et al.[1] suggested that their abnormal iodine-adsorption results might have resulted, in part, from glycogens having a molecular weight higher than normal. To test whether the small iodine-adsorption variations observed here could be due to molecular-weight differences, three intubated glycogens that showed the greatest variation in λ_{max} were fractionated on a glass-granule column together with two control glycogens. The results (Table IV) showed that there was little difference in both the average molecular-weight and the polydispersity of the glycogens. It is unlikely, therefore, that the differences in λ_{max} result from molecular-size differences. The cause of the variation in iodine-staining power remains unknown; it may be due to other structural variations in the glycogen molecule or to the presence of other associated molecules.

TABLE IV

CONTROLLED-PORE GLASS CHROMATOGRAPHY OF GLYCOGENS

Glycogen	*Iodine-staining properties*		K_{av}[a]	V_{50}[b] *(ml)*
	λ_{max} *(nm)*	E_{max}		
Control	466	1.14	0.60	15.7
Control	463	1.16	0.67	15.8
Intubated	480	1.23	0.60	16.1
Intubated	489	1.20	0.67	17.0
Intubated	492	1.30	0.63	16.2

[a] $K_{av} = (V_e - V_t)/(V_o - V_t)$, where V_e = elution volume; V_o = exclusion volume; and V_t = inclusion volume (see text for further details). [b] V_{50} = Breadth of peak at half the maximum height.

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