Structural Changes in Rat Liver Glycogen during Refeeding after Fasting

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Rats starved for 48 hr were refed on a high carbohydrate diet and killed at various intervals (3~24 hr) in order to examine structural changes in liver glycogen. Glycogen was isolated by the HgCl_2 method and thereafter treated with dithizone-chloroform to eliminate HgCl_2 . The extracted glycogen revealed typical α -particles besides β -particles but the KOH-extracted glycogen showed only β -particles. The tissue glycogen level began to increase after refeeding and reached a maximum at 18 hr. However, the iodine adsorption capacity of the HgCl_2 -extracted glycogen (but not the KOH-extracted one) increased by 30% in 6 hr-refed rats compared with control rats fed *ad libitum*, and then decreased to the control level. In the HgCl_2 -extracted samples, changes in glycogen $S_{20,w}$ after refeeding were coincident with the changes in the iodine adsorption capacity and β -amylolysis limit. On the other hand, these structural changes were not observed in the KOH-extracted samples.

These findings supported that the HgCl_2 method is useful for isolating native glycogen and suggested that the liver glycogen structure can be temporarily altered so as to have a high molecular-weight together with high iodine affinity and long outer chains.

Chapman *et al.* observed that glycogen from glycogenesis-activated rat liver could adsorb much more iodine than that in the steady state.¹⁾ Schlamowitz reported that absorption spectra of iodine-glycogen complexes and the number of reducing ends of rat liver glycogen reflected the carbohydrate source fed to the rats.²⁾ Their studies suggested that the distribution of glucose moieties in the glycogen molecule could be changed by the nutritional state. However, these workers examined acid- or alkali-extracted glycogen for structural analyses. Since either acid or alkali results in the degradation of polysaccharide,³⁾ it is essential to obtain glycogen in the native form from tissues for studying the glycogen structure.

Mordoh *et al.* isolated a high molecular-weight glycogen from rat liver by the HgCl_2 method and preliminarily showed that the molecular-weight distribution of the glycogen varied after intubation of a glucose solution to fasting rats.⁴⁾ Bueding and Orrell also reported similar findings in liver fluke, *Fasciola he-*

patica; the high molecular-weight fraction of glycogen disappeared during fasting and reappeared after glucose administration, using cold water instead of KOH for the isolation of glycogen.⁵⁾

However, little attention has been paid to the structure of newly-synthesized glycogen and the mechanisms of its formation. Therefore, in order to investigate in detail the structure of newly-synthesized glycogen *in vivo*, we examined the physical and chemical properties of rat liver glycogen during refeeding after fasting and emphasize the important role of the preparatory procedure for glycogen in this type of study.

MATERIALS AND METHODS

1) *Treatment of rats.* Male Sprague-Dawley strain rats (230~250 g) were used in these experiments. After rats were fasted for 48 hr to deplete their glycogen store, they were refed *ad libitum* on a 25% casein diet from 9:00 hr under a controlled lighting schedule with light from 8:00 to 20:00 hr at 22°C, and then killed at various intervals by

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cervical dislocation. The liver was removed promptly and rinsed in cold saline.

2) Isolation of glycogen.

Method A: Liver (0.5 g) was digested with 2 ml of 30% KOH in a boiling water-bath for 20 min to determine the hepatic glycogen concentration by the method of Hassid and Abraham.⁶⁾

Method B: Liver (5 g) was homogenized with 15 ml of chilled 3% HgCl_2 solution (pH 5.2). Glycogen was isolated from the HgCl_2 soluble fraction by the method of Mordoh *et al.*⁴⁾ Usually only one extraction with HgCl_2 solution was carried out. The recovery of glycogen by the method was about 62% of that by the KOH method, which almost agreed with the previous report.⁴⁾

Method C: The glycogen sample obtained by method B was shaken with 0.01% dithizone-chloroform to remove contaminating HgCl_2 .

3) *Determination of sedimentation coefficient of isolated glycogen.* Isolated glycogen was dissolved in 0.1 M potassium phosphate buffer (pH 7.2), and insoluble materials were removed by centrifugation (2000 rpm, 10 min). The resultant supernatant (1% solution) was transferred to single- and wedge-type cells. Centrifugation was carried out at 7000 rpm in an RA60H rotor at 28°C, using a Hitachi-282 analytical ultracentrifuge with a Schlieren optical system.

4) *Sucrose density gradient centrifugation.* A linear gradient of 10~40% sucrose (15 ml) was prepared in a cellulose tube. Glycogen obtained by method C (1% solution, 0.5 ml) was layered over the sucrose solution. The centrifugal force applied was equivalent to 32 min at 15,000 rpm including acceleration and deceleration times calculated as described by Martin and Ames.⁷⁾ After centrifugation, each fraction (1.2 ml) was collected and iodine color intensities at 460 nm of the fractions were measured as described below.

5) *Transmission electron microscopy.* Isolated glycogen was negatively-stained with 4% uranyl acetate (pH 4.1). The specimens were examined with a Hitachi HU-12 microscope at 75 kV.

6) *β -Amylolysis.* Crystalline β -amylase from sweet potato (product of Sigma) was purified by the method of Marshall and Whelan.⁸⁾ Glycogen (1 mg) was dissolved in 0.1 ml of dimethyl sulfoxide (DMSO) at 37°C overnight. Then 0.9 ml of 50 mM acetate buffer (pH 4.8) containing 40 U of β -amylase was added. One unit of the enzyme activity is expressed as the amount which liberates 1 μmol of maltose per min at 37°C. Incubation was carried out for 6 hr at 37°C. Total sugar and reducing power were determined with maltose as a standard.

7) *Determination of average chain lengths ($\overline{CL}$).* A

glycogen sample (2 mg) was incubated with 120 U of *Pseudomonas* isoamylase (product of Hayashibara Biochemical Laboratories, Inc., Okayama, Japan) in 2 ml of 60 mM acetate buffer (pH 3.5) containing 10% DMSO for 24~48 hr at 37°C. One unit of the enzyme activity is expressed as the amount of enzyme causing an increase in $A_{610\text{ nm}}$ of 0.1 in 1 hr as reported previously.⁹⁾ Average chain lengths were calculated from contents of total and reducing sugars in the reaction mixtures with glucose as a standard.

8) *Analytical methods.* Total carbohydrates were determined by the phenol-sulfuric acid method,¹⁰⁾ reducing power was determined by either the method of Somogyi-Nelson^{11,12)} or that of Park-Johnson¹³⁾ modified by Hizukuri *et al.*¹⁴⁾ Protein determination was carried out by the method of Lowry *et al.*¹⁵⁾ with bovine serum albumin as a standard. The iodine-absorption spectra of glycogen were measured by the method of Krisman.¹⁶⁾

RESULTS

1) Changes in hepatic glycogen levels of rats after fasting and refeeding

Though liver glycogen levels of rats decrease to 0.3% of wet tissue weight during 48 hr-fasting, they begin to increase rapidly after refeeding and reach a maximum (14% of wet tissue weight) at 18 hr as shown in Table I. The increases in liver glycogen level were closely correlated with food intake and liver weight till 18 hr after refeeding.

2) Iodine adsorption capacity

The iodine-absorption spectra of glycogen isolated by methods A and C showed an absorption maximum at approximately 460 nm, which was nearly constant among the glycogen samples from fed and refed rats. In the glycogen samples with method C, color intensities at 460 nm per mg glycogen of rats 6~15 hr after refeeding were about 30% higher than those of rats fed *ad libitum* as shown in Table I. However, these increased iodine adsorption capacities of glycogen were not observed when glycogen was isolated with KOH (method A).

3) Morphology of glycogen particles extracted with KOH and HgCl_2

Liver glycogen extracted by a mild pro-

TABLE I. CHANGES IN HEPATIC GLYCOGEN LEVELS AND IODINE ADSORPTION CAPACITIES OF LIVER GLYCOGEN OF RATS AFTER REFEEDING

Rats were fed *ad libitum* or fasted for 48 hr. The fasting rats were fed on a 25% casein diet freely and killed at various times. Liver glycogen was isolated by the KOH and HgCl₂ methods. Contaminating Hg was removed by treatment with dithizone as described in the text. Glycogen contents and absorbance at 460 nm of iodine-glycogen complexes per mg glycogen were determined as described in MATERIALS AND METHODS.

Hours after refeeding	Number of rats	Food intake (g)	Liver wet weight (g)	Liver glycogen (g/100 g) ^a	Iodine adsorption			
					HgCl ₂ Method		KOH Method	
					A/mg ^b	(%)	A/mg ^b	(%)
3	3	5.2	8.67	2.48	3.15	104	3.32	117
6	2	9.1	8.34	7.11	3.87	128	3.15	111
9	4	11.5	10.54	8.56	3.67	121	3.12	110
15	2	12.6	12.61	13.28	4.00	132	3.32	117
18	2	12.6	12.55	14.35	3.51	116	3.27	116
24	3	21.6	12.71	10.57	3.54	117	2.91	103
<i>ad libitum</i>	4		13.76	4.65	3.03	100	2.83	100

^a Standard error for each value was less than 13.2% of the mean.

^b Standard error for each value was less than 9.2% of the mean.

FIG. 1. Electron Micrographs of KOH-Extracted (left) and HgCl₂-Extracted (right) Glycogen ($\times 29,000$). Bars indicate 200 nm.

cedure appears as large particles. Acid or alkali has been used to isolate tissue glycogen quantitatively but these solvents decrease the size and molecular weight of glycogen measured by electron microscopy¹⁷⁾ and ultracentrifugation analysis^{3,18)} Therefore we compared the size of glycogen extracted with alkali and HgCl₂ by electron microscopy. As shown in Fig. 1, the KOH-extracted glycogen revealed single spheres of about 35 nm in diam-

eter (β -particles). On the other hand, amorphous and various sizes of particles (60~200 nm in diameter, α -particles) besides β -particles were identified in the HgCl₂-extracted sample and were not affected apparently by the dithizone treatment (data not shown).

4) Sedimentation coefficient of glycogen

To determine the physical properties of gly-

cogen isolated by the present procedures, the sedimentation coefficient of glycogen was determined in Svedberg units. First, for glycogen from the same rat fed *ad libitum*, the effect of the isolation method on $S_{20,w}$ was examined. $S_{20,w}$ of the KOH-extracted sample was calculated to be 113 S (Table II), which agreed with

the value reported previously.¹⁹⁾ The HgCl_2 -extracted one was 198 S and decreased to 156 S on treatment with dithizone. The decrease in $S_{20,w}$ on the treatment may be due to elimination of protein adsorbed on glycogen granules. In fact, the protein concentration in the prepared glycogen fell from an average of 1.23% to 0.31% with the treatment.

Figure 2 shows ultracentrifugal patterns of glycogen extracted by method C. A small and distinct peak (indicated by arrow in Fig. 2), which precedes the main peak, was detected in samples from refed rats but not in those fed *ad libitum*, suggesting that glycogen of the refed rats exhibits heterogeneous and wide molecular-weight distributions.

Figure 3 shows the fractionation of glycogen (method C) by sucrose gradient centrifugation. Heavy fractions (fraction Nos. 6~12) are observed in the samples from 6 hr- and 9 hr-refed rats but have disappeared in the control glycogen. The heavy fraction may correspond to glycogen of higher $S_{20,w}$, seen as the small

TABLE II. SEDIMENTATION COEFFICIENTS OF RAT LIVER GLYCOGEN

Conditions of rats	Method of isolation ^a	$S_{20,w}$	(%)
<i>ad libitum</i>	A	113 ^b	
<i>ad libitum</i>	B	198 ^b	
<i>ad libitum</i>	C	152 ^b	165 (100)
3-hr refed	C	165	176 (108)
6-hr refed	C	218	196 (130)
9-hr refed	C	189	188 (119)

^a A, KOH; B, HgCl_2 ; C, HgCl_2 /dithizone.

^b Glycogen from the same rat liver.

$S_{20,w}$ of glycogen, which corresponded to the main peak shown in Fig. 2, was determined by analytical ultracentrifugation with a Schlieren optical system.

FIG. 2. Sedimentation Patterns of Liver Glycogen (extracted by method C) of Rats Fed *ad libitum* (I), 6-hr Refed (II), 9-hr Refed (III), and 3-hr Refed (IV).

Photographs were taken at 6 min intervals.

FIG. 3. Fractionation of Glycogen by Sucrose Density Gradient Centrifugation.

Glycogen (0.5mg, by method C) was fractionated on a 10~40% linear sucrose gradient and absorbance at 460 nm of iodine-glycogen complexes of each fraction was measured as described in the text. ($\blacktriangle$), rats fed *ad libitum*; ($\circ$), 6-hr refed; ($\square$), 9-hr refed.

peak in Fig. 2.

5) β -Amylolysis

Next we determined β -amylolysis limits of glycogen which showed different physical characteristics as observed above. As shown in Fig. 4, β -amylolysis limits (%) of the KOH-extracted glycogen from both fed and refed rats are similar at about 40% as reported previously.²⁰⁾ On the other hand, the HgCl_2 -extracted one showed lower β -amylolysis limits than those of the KOH-extracted samples but were dependent on the duration of refeeding. When these samples were treated with hot alkali (100°C, 10 min), the β -amylolysis limit increased to return to that seen in the original alkali-extracted samples (see Table III and Fig. 4).

6) Average chain lengths ($\overline{CL}$) of debranched glycogen

To examine whether the glycogen character-

FIG. 4. β -Amylolysis Limit (%) of Liver Glycogen from Rats after Refeeding and from Control (C) Rats Fed *ad libitum*.

Glycogen was isolated by the KOH method (A) and by the HgCl_2 method/dithizone treatment (B). Symbols and bars are average $\pm$ SE of 3~4 rats.

TABLE III. β -AMYLOLYSIS LIMITS (%) OF THE HgCl_2 -EXTRACTED GLYCOGEN WITH AND WITHOUT ALKALI TREATMENT

	Non-treatment	Alkali-treatment ^a
<i>ad libitum</i>	33	38
	33	37
9-hr refed	36	45
	37	45

^a 1 M KOH, 100°C for 10 min.

istics (higher in $S_{20,w}$, iodine-adsorption capacity, and β -amylolysis limit compared with those of the control) are related with the distribution of α -1,4 chains of glycogen, glycogen obtained by method C was debranched by isoamylase. As shown in Fig. 5, $\overline{CL}$ of debranched glycogen from 9~15 hr refed rats were about 13, longer by 1.5 glucose units than that of the control. Similar results were obtained with samples extracted by method B and by the TCA-method.* The products after debranching by isoamylase were sepa-

* We previously obtained similar results with the periodate oxidation method for the determination of $\overline{CL}$. (H. Fuwa, unpublished data.)

FIG. 5. Average Chain Lengths ($\overline{CL}$) of Liver Glycogen from Refed and Control (C) Rats Fed *ad libitum*.

Glycogen samples (method C) were debranched by isoamylase to determine $\overline{CL}$ as described in the text. Symbols and bars are average $\pm$ SE of 3~4 rats.

rated by Sephadex G-75 column chromatography, giving one peak (data not shown) as reported before.²¹⁾

DISCUSSION

Glycogen is generally assumed to be synthesized by the cooperative action of glycogen synthase (EC 2.4.1.11) and branching enzyme (EC 2.4.1.18), and is catabolized mainly by phosphorylase (EC 2.4.1.1), debranching enzymes (EC 3.2.1.33 and EC 2.4.1.25), and lysosomal α -glucosidase (EC 3.2.1.3). The glycogen structure is considered to be dependent essentially on glycogen synthesizing enzymes. For example, the structure of glycogen from tissues of patients with several distinct glycogen storage diseases has been extensively studied.^{22,23)} Among them glycogen from a patient with Type IV glycogenosis, in which branching enzyme was deficient, had an abnormal structure like amylopectin.^{24,25)} In other types, the structure of glycogen was almost normal.²²⁾

In higher plants [especially in maize mutants having a starch modifying gene, *amylose-extender*, *dull*, and *sugary-1*^{26~28)}], the exis-

tence of isozymes of branching enzyme has been demonstrated which affect the fine structure of amylopectin.^{29~31)} Though branching enzymes in animals and plants are considered to have an essential role as to structural characteristics of glycogen and starch, the mechanism of regulation of the enzyme in animal tissue is still unknown even though two forms (free and RNA-bound) of rat liver branching enzyme have been recently identified.³²⁾

In the present study, we characterized the glycogen structure of rat liver which was changed transiently by a nutritional effect. First, with the HgCl_2 -method for isolation of glycogen, we confirmed that the newly-synthesized glycogen during refeeding after fasting could adsorb much more iodine as observed by Chapman *et al.*¹⁾ However, glycogen with the KOH method failed to show the phenomenon, which differed from the results of Chapman *et al.*¹⁾ This discrepancy may be due to the differences in experimental conditions used including the assay for iodine absorption spectra. Second, glycogen having a high iodine adsorption capacity showed a relatively higher $S_{20,w}$ and β -amylolysis limit. In general, $S_{20,w}$ determined by us was rather smaller than that reported previously.⁴⁾ Mordoh *et al.* did not examine the effect of dithizone treatment, and failed to observe the distinct correlation between $S_{20,w}$ and liver glycogen content.⁴⁾ Our results clearly show that $S_{20,w}$ is correlated with the duration of refeeding (see Table II). Elimination of HgCl_2 by dithizone treatment may result in minimization of deviation of an individual value compared with that of non-treated samples.

β -Amylolysis limits of the HgCl_2 -extracted glycogen were estimated to be characteristic of the structure of glycogen obtained at different stages of refeeding. Increased β -amylolysis limits of glycogen from 6~9-hr refed rats suggested the possibilities that these glycogens have longer outer chains and/or a higher degree of branching in their exterior chains. But the latter possibility is not acceptable because the iodine color intensity per unit weight of

glycogen varied inversely to the degree of branching.²⁾ We rather think that the outer chain lengths of these glycogens are relatively longer and thus capable of helix formation, resulting in the observed high iodine adsorption capacity. As described before, when the HgCl_2 -extracted glycogen was treated with hot alkali, the β -amylolysis limit of the sample increased (Table III). The rate of the increase was higher in 9-hr refed rats than in the control ones. This suggests that the outer chains of glycogen from the refed rats were much more folded in the glycogen molecules since these glycogens are of high molecular-weight and are composed of α -particles as shown by electron microscopy.

Drochmans observed that filament-like glucoside linkage chains sprouted from β -particles when β -particles were incubated with phosphorylase and G-1-P.¹⁹⁾ It is therefore now assumed that the glycogen α -particle is an associate of some β -particles, linked in series by an α -1,4 linear chain which is 20~40 glucose residues, as calculated by electron microscopy.^{33,34)}

We tried to separate the 20~40 glucose unit chain by Sephadex G-75 column chromatography from the products after debranching of glycogen (60 mg), but could not detect peaks corresponding to the chains on measuring $\overline{DP}$ and λ_{max} of iodine absorption spectra. The reason for this is that the chains have less glucose residues compared with β -particle glycogen (55,000 glucose residues).^{4,35)} Therefore, the small difference in $\overline{CL}$ of debranched glycogen from refed and control rats (Fig. 5) may be reflected in $\overline{CL}$ of β -particle glycogen themselves.

It has been shown that animal tissue glycogen is metabolically inhomogeneous with respect to molecular weight.^{36,37)} Namely, high molecular-weight glycogen in the liver may be preferentially attacked by glycogen catabolizing enzymes,³⁸⁾ and furthermore labeled glucose could be less incorporated into high molecular-weight glycogen than into a low molecular-weight one.³⁹⁾ Thus it is likely that the glycogen level in tissues is controlled by

increased susceptibility of the newly-synthesized glycogen, which is of high molecular-weight, to catabolizing enzymes and, as a result, transient changes in the glycogen structure take place. However, further work is needed on the details of the phenomenon.

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