Association of Enzymes with Rat Liver Glycogen Isolated by Rate-zonal Centrifugation*

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SUMMARY

1. Rat liver glycogen was separated into fractions of relatively uniform particle sizes by large volume rate-zonal centrifugation of tissue homogenates with the use of sucrose density gradients.

2. After centrifugation, the distribution curve obtained by plotting the glycogen content of all collected fractions exhibited its maximum a short centrifugal distance from the zone which contained the starting sample. The curve then decreased progressively until an abrupt increase was noted in the zone containing the isopycnically banded membranes.

3. The distribution curves for the α-glucan phosphorylase and of UDP-glucose-glycogen transferase activities were similar to those for glycogen.

4. The phosphorylase-to-glycogen ratios decreased as the molecular weight of the glycogen particles increased, while the opposite was observed with the transferase-to-glycogen ratios.

5. The possible relationship between patterns of preferential binding of enzymes and the molecular weight spectra of glycogen characteristic of their origin are discussed.

Several methods have been used for the isolation of glycogen from mammalian livers. These include extraction with potassium hydroxide (1), trichloroacetic acid (2), phenol (3), hot water (4), and cold water (5, 6). Cold water extraction is a mild procedure which results in the isolation of glycogen particles which are morphologically similar to those seen in liver sections (7, 8), whereas extraction procedures involving the use of KOH and trichloroacetic acid cause considerable degradation of the particles (9). Recently, a rate-zonal centrifugation procedure has been described for separating mildly extracted glycogen into zones of relatively uniform particle size and subsequent collection of these zones as individual fractions (10). Rat liver glycogens separated by rate-zonal centrifugation exhibited a spectrum of particle sizes similar to those observed for the other cold water extraction procedures (6, 9, 10).

Glycogen extracted by mild procedures is associated with both α-glucan phosphorylase (phosphorylase) (11) and UDP-glucose-glycogen transferase (transferase) (12, 13). Former studies were carried out by measuring the enzyme activity and glycogen content of various subcellular fractions obtained by differential centrifugation. Specific associations as related to particle size, however, cannot be established by this method. Rate-zonal centrifugation, on the other hand, provides a rapid method for studies of these specific associations, since the glycogen, which is polydisperse, assumes within the rotor a distribution which is unique among cellular constituents. The B-IV rotor has been extensively used for carrying out rate-zonal centrifugation of large volumes (10). In this study we will discuss the relative distribution of glycogen, α-glucan phosphorylase, and UDP-glucose-glycogen transferase in fractions of rat liver homogenates which were collected after rate-zonal centrifugation.

METHODS

Rat livers were obtained from adult male Sprague-Dawley rats after stunning, decapitation, and exsanguination. Livers were homogenized at a dilution of 1:4, w/v, in a 0.25 M sucrose solution containing 0.06 M NaF, 5 × 10⁻⁴ M MgCl₂, and 4 × 10⁻³ M mercaptoethanol. Homogenization was carried out for 3 to 5 min at 2–4°C in a motor-driven, all-glass homogenizer. Each homogenate was prepared from the livers of two or three animals. The animals used represented the following groups: one group of three young animals weighing 149 to 157 g (Experiment Z-802); two groups of two animals each weighing 260 to 317 g (Experiments Z-803 and Z-805), and one group of animals weighing 268 to 290 g which had received a total dose of 42 mg of cortisone per kg of body weight distributed over the 4 preceding days (Experiment Z-801).

Zonal centrifugation was carried out in the B-IV rotor system, with sucrose as the density gradient material (10). Homogenate (60 ml) was layered directly on 1200 ml of gradient. The

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glucose l-phosphate, to be carried out in the same cuvette. 

enzymatic degradation and the determination of the product, 

matic method (14, 15), which was modified to allow for both the 

zygen concentration between 0.04 to 0.2 mg per ml. 

blue was used as the external indicator) and diluted with water, 

2.5 M phosphoric acid to a pH between 7.0 and 7.5 (bromthymol 

After cooling in an ice bath, the mixture was neutralized with 

o added to incubation mixtures containing no UDP-glucose. 

P-mixture was established by the quantitative recovery of UDP 

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In order to obtain quantitative recoveries, glycogen concentra- 

cations and UDP-glucose-glycogen transferase activities were de- 

termined as soon as each sample had been collected. On the 

other hand, glycogen phosphorylase activities, which were found 

n samples was heated for 10 to 15 min in a boiling water bath with 

12 ml of 5 N KOH in a graduated tube (covered with a marble). 

After cooling in an ice bath, the mixture was neutralized with 

5 M phosphoric acid to a pH between 7.0 and 7.5 (bromthymol 

blue was used as the external indicator) and diluted with water, 

to yield a glycogen concentration between 0.04 to 0.2 mg per ml. 

Glycogen was determined by a previously described microenzy- 

matic method (14, 15), which was modified to allow for both the 

catalytic degradation and the determination of the product, 

glucose 1-phosphate, to be carried out in the same cuvette. To 

0.05 ml of the sample, containing 0.002 to 0.01 mg of glycogen, 

were added 0.1 ml of a degradation mixture containing 60 μM 
potassium phosphate buffer (pH 7.0), 0.45 μM AMP, 1.05 μM 
EDTA, 0.05% bovine serum albumin, 6 units of eight-times 
recrystallized phosphorylase b (16), and 4 units of amylo-1,6- 
glucosidase (14). After incubation for 1 hour at room tempera- 
ture, glucose 1-phosphate was determined as described previously 
(14). Analysis of preformed glucose 6-phosphate or glucose 
1-phosphate was carried out in the same manner, except that 
phosphorylase b and amylo-1,6-glucosidase were omitted. 

Active glycogen phosphorylase activity of each appropriately 
diluted fraction was assayed at room temperature by measuring the rate of formation of glucose 1-phosphate in the presence of 
glycogen and inorganic phosphate. The reaction mixture con- 
tained, in 0.8 ml, 1.6 mg of glycogen (extracted with KOH), 60 
μM potassium phosphate buffer (pH 7.2), 2 μM AMP, 40 μM imid- 
zazole buffer (pH 7.2), 5 μM MgCl₂, 48 μM NaF, 0.05 μM glucose 
1,6-diphosphate, 0.2 unit of glucose 6-phosphate dehydrogenase 
0.2 units of phosphoglucomutase, and 0.3 μM NADP. Reduc- 
tion of NADP was determined spectrophotometrically at a wave 
length of 340 μν. When necessary, appropriate turbidity con- 
trols, without added enzymes and phosphate, were run simulta- 
genously. Phosphorylase activities were expressed in micro- 
moles of glucose 1-phosphate produced per min. 

Total UDP-glucose-glycogen transferase activity was deter- 
mained by measuring the production of UDP in the presence of 
UDP-glucose and glycogen (12). Of each collected sample 0.05 
ml (or a dilution thereof) was incubated for 30 min at 30°C, in a 
total volume of 0.2 ml containing 1.4 μM UDP-glucose, 0.4 mg 
of glycogen (KOH-extracted), 1 μM glucose-6-P, 10 μM potassium 
acylglycine buffer (pH 7.8), 1.6 μM mercaptoethanol, and 0.4 
μM EDTA. After incubation, the mixture was heated in a boil- 
ing water bath for 1 min. Controls were similarly treated but 
without incubation. The stability of UDP in the reaction 
mixture was established by the quantitative recovery of UDP 
added to incubation mixtures containing no UDP-glucose. 
After centrifugation at 20,000 X g for 15 min, UDP was deter- 
mained in an aliquot of the supernatant by coupling the dephos- 
phorylation of P-enolpyruvate, catalyzed by pyruvic kinase, 
with the oxidation of NADH, and catalyzed by lactic dehydro- 
genase. The reaction mixture, in a total volume of 0.78 ml,
TABLE I
Glycogen concentrations and phosphorylase and UDP-glucose-glycogen transferase activities of rat liver homogenates

All measurements are per g wet weight of liver.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glycogen</th>
<th>Phosphorylase</th>
<th>Transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount</td>
<td>Recovery a</td>
<td>Activity</td>
</tr>
<tr>
<td></td>
<td>µg/g</td>
<td>%</td>
<td>µM/min</td>
</tr>
<tr>
<td>Z 802</td>
<td>55.7</td>
<td>94</td>
<td>3.71</td>
</tr>
<tr>
<td>Z 803</td>
<td>56.4</td>
<td>91</td>
<td>7.98</td>
</tr>
<tr>
<td>Z 805</td>
<td>38.8</td>
<td>90</td>
<td>5.54</td>
</tr>
<tr>
<td>Z 801b</td>
<td>75.8</td>
<td>88</td>
<td>4.82</td>
</tr>
</tbody>
</table>

a The total collected from the gradient times 100, divided by the total present in the homogenate.  
b Livers from cortisone-treated rats.

Results

Rate-zonal centrifugation of rat liver homogenates in sucrose density gradients sedimented glycogen, phosphorylase activity, and transferase activity throughout the gradient (Fig. 1). The curves representing the distributions of all three were similar but not identical. The maxima of all three curves were in the zone between Fractions 8 and 13, and all three exhibited a progressive decrease from Fractions 14 through 31. An abrupt increase was noted in all three curves in the zone between Fractions 32 and 37. This increase resulted from a centrifugal artifact rather than the presence of a large amount of specific glycogen of high molecular weight. (The artifact is due to the large amounts of material present in the original homogenate which sediment rapidly from the starting zone to their isopycnic zone within the gradient (17).)

Glycogen concentrations and phosphorylase and transferase activities of the livers used for zonal centrifugation are summarized in Table I. The recoveries, as measured by comparing the total amounts collected after zonal centrifugation with those present in the starting homogenate, were satisfactory. Excessive losses of transferase activity occurred unless the samples were analyzed immediately after their collection from the centrifuge. The liver glycogen content of the animals used varied from 38.8 mg per g of liver in the young animals (Z-805) to 75.8 mg per g in the cortisone-treated animals. However, the distribution of glycogen within the gradient after centrifugation was similar in all experiments. Also, in spite of the variations in total phosphorylase and transferase activities in different livers, the distributions of each were similar. There was no correlation between the glycogen content of livers and the total activities of either phosphorylase or transferase, nor was there any between the total activities of the two enzymes.

The ratio of phosphorylase activity to glycogen concentration was highest in the low-numbered fractions; whereas the ratio of transferase activity to glycogen concentration was lowest in these fractions (Fig. 2). These fractions contain the smaller glycogen particles (10). The phosphorylase-to-glycogen ratio progressively decreased with increasing fraction number; whereas the opposite occurred with the transferase-to-glycogen ratios. These differences in the distributions of phosphorylase and transferase are better shown in the curves obtained by plotting phosphorylase to transferase ratios against fraction number (Fig. 3). The curve of phosphorylase to transferase ratios in experiments using liver homogenates from the cortisone-treated animals falls within the shaded area of Fig. 3, which indicates no change in...
Enzyme-to-glycogen ratios of three arbitrary subdivisions of fractions collected following zonal centrifugation of rat liver homogenates

Values were calculated from the sums of all the measurements made in the fractions indicated.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fractions combined</th>
<th>Ratio of phosphorylase to glycogen</th>
<th>Ratio of transferase to glycogen</th>
<th>Ratio of phosphorylase to transferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-802</td>
<td>8-13</td>
<td>0.087</td>
<td>0.016</td>
<td>5.32</td>
</tr>
<tr>
<td></td>
<td>14-23</td>
<td>0.066</td>
<td>0.024</td>
<td>2.71</td>
</tr>
<tr>
<td></td>
<td>24-33</td>
<td>0.024</td>
<td>0.035</td>
<td>0.65</td>
</tr>
<tr>
<td>Z-803</td>
<td>8-13</td>
<td>0.200</td>
<td>0.036</td>
<td>4.82</td>
</tr>
<tr>
<td></td>
<td>14-23</td>
<td>0.146</td>
<td>0.043</td>
<td>3.28</td>
</tr>
<tr>
<td></td>
<td>24-33</td>
<td>0.008</td>
<td>0.052</td>
<td>1.50</td>
</tr>
<tr>
<td>Z-805</td>
<td>8-13</td>
<td>0.190</td>
<td>0.051</td>
<td>3.70</td>
</tr>
<tr>
<td></td>
<td>14-23</td>
<td>0.119</td>
<td>0.077</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>24-33</td>
<td>0.059</td>
<td>0.083</td>
<td>0.01</td>
</tr>
<tr>
<td>Z-801b</td>
<td>8-13</td>
<td>0.103</td>
<td>0.032</td>
<td>3.29</td>
</tr>
<tr>
<td></td>
<td>14-23</td>
<td>0.092</td>
<td>0.047</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>24-33</td>
<td>0.020</td>
<td>0.062</td>
<td>0.57</td>
</tr>
</tbody>
</table>

a Micromoles per min per milligram.
b Livers from cortisone-treated rats.

the ratios after cortisone administration. The differences between the distributions of phosphorylase and transferase are immediately evident when calculations of the enzyme-to-glycogen ratios are made on the following combined samples: 8 through 13; 14 through 23; and 24 through 33 (Table II). These combinations were selected because they represent clearly identifiable regions of the distribution curves (see Fig. 1) and avoid the confusing influence of fractions containing primarily either soluble materials (Fractions 4 to 7) or isopycnically banded membranes (Fractions 34 to 37). The ratios of phosphorylase activity to glycogen content decrease markedly as the molecular size of glycogen increases (increase in fraction number). On the other hand, the ratios of transferase activity to glycogen content increase as the molecular size of glycogen increases.

**DISCUSSION**

Rat liver glycogen, isolated by mild extraction procedures, is polydisperse and exists as a continuous spectrum of particle sizes up to 150 to 160 nm, with the largest particles having molecular weights of many hundreds of millions. Polydisperse glycogen has been separated into zones of relatively uniform particle size with the B-IV rotor system (10). The distribution of rat liver glycogen within the B-IV rotor after zonal centrifugation is unique and is different from the distributions of other major subcellular fractions such as nuclei, mitochondria, and microsomes (10, 17).

Two enzymes studied in this report do not themselves sediment appreciably under the centrifugal conditions used. Therefore, their presence in samples collected from zones located centrifugally to the starting zone is due to their associating with particles that sediment away from the starting zone. The curve representing the concentration of glycogen throughout the gradient decreases gradually with increase in particle size, but increases abruptly in the zone containing the membranes and cell fragments which band isopycnically. This second peak of glycogen concentration has been shown to be a sedimentation artifact introduced by the presence of large amounts of rapidly sedimenting material and must be considered in zonal centrifuge studies using large amounts of tissue homogenate.

Madsen and Cori (18) measured the binding of purified 200 S corn glycogen with phosphorylase. Furthermore, the binding of both phosphorylase and transferase to rat and mouse liver glycogen has been demonstrated (11–13, 19, 20). The zonal centrifuge techniques used in the present study indicate a preferential binding of these enzymes as a function of the molecular weight of glycogen.

The curve of phosphorylase-to-glycogen ratios (Fig. 2) suggests that phosphorylase has a higher affinity for low molecular weight glycogen. This conclusion is based on the assumption that no relocation occurred during the centrifugation. Since the possible occurrence of this phenomenon cannot be excluded, the concentration of phosphorylase in any fraction obtained from rate-zonal centrifugation could be different from the amount of phosphorylase originally (i.e. in the homogenate) bound to the glycogen present in that fraction. However, the positive slope of the curve of transferase-to-glycogen ratios indicates a preferential binding of the transferase to glycogen of higher molecular weight. The possibility of redistribution of transferase does not obviate this conclusion. On the contrary, if it did occur, it would indicate that the dependence of transferase binding on the molecular weight of glycogen is even greater than was observed.

In addition, a specific relationship between the size of the glycogen molecule and the enzymes associated with its metabolism is indicated by the differential incorporation of uniformly labeled 14C-labeled glucose into the high and low molecular weight fractions of glycogen in the vestode *Hymenolepis diminuta* (21). Furthermore, when UDP-glucose-14C is incubated with polydisperse glycogen obtained from this tapeworm and with a preparation of transferase from the same organism, the specific radioactivity of the high molecular weight fraction is greater than that of the lower molecular weight fraction.2 This again suggests a higher affinity of the transferase for glycogen of the larger molecular size.

Cold water-extracted glycogens exhibit molecular weight distributions highly characteristic of a given species, tissue, and physiopathological state (9, 22–25). These distinctive differences in molecular weight spectra have not been explained. If patterns of enzyme binding as a function of the molecular weight of glycogen are found also to be characteristic of the tissue of origin, an attempt should be made to correlate such differences with the distinctive characteristics in the molecular weight spectra of glycogens.

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