

- Levi-Montalcini, R. & Angeletti, P. U. (1962). *Annu. Rev. Physiol.* **24**, 11.
- Mannik, M. & Kunkel, H. G. (1963). *J. exp. Med.* **117**, 213.
- Markert, C. L. & Hunter, R. L. (1959). *J. Histochem. Cytochem.* **7**, 42.
- Markert, C. L. & Möller, F. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 753.
- Ornstein, L. (1962). *Disc Electrophoresis*. Rochester, N.Y.: Eastman Kodak Co.
- Ouchterlony, O. (1953). *Acta path. microbiol. scand.* **32**, 231.
- Pain, R. H. (1963). *Biochem. J.* **88**, 234.
- Porter, R. R. (1957). In *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Poulik, M. D. & Edelman, G. M. (1961). *Nature, Lond.*, **191**, 1274.
- Putnam, F. W., Migita, S. & Easley, C. W. (1962). In *Protides of the Biological Fluids*, vol. 10, p. 93. Ed. by Peeters, H. Amsterdam: Elsevier Publishing Co.
- Sober, H. A. & Peterson, E. A. (1958). *Fed. Proc.* **17**, 116.
- Stark, G. R., Stein, W. H. & Moore, S. (1960). *J. biol. Chem.* **235**, 3177.

Biochem. J. (1964) **90**, 284

Subcellular Redistribution of Liver α -Glucan Phosphorylase during Alterations in Glycogen Content

By J. R. TATA

National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 21 June 1963)

A large fraction of hepatic glycogen is distributed in the cell as 'particulate' glycogen, which is associated with the smooth endoplasmic-reticulum membranes (Lazarow, 1942; Claude, 1954; Porter & Bruni, 1959; Luck, 1961; Drochmans, 1963). It is also known that glycogen exhibits both intramolecular and intermolecular differences of chemical and metabolic liabilities (Stetten, Katzen & Stetten, 1956; Figueroa & Pfeiffer, 1962; see Stetten & Stetten, 1960). The possibility of localizing particulate and non-particulate glycogen raises the question whether there exists a special relationship between the subcellular distribution of the enzymes concerned with glycogen metabolism and the structural disposition of the polymer. Luck (1961) conclusively demonstrated that UDP-glucose-glycogen glucosyltransferase [uridine diphosphate glucose- α -(1 \rightarrow 4)-glucan α -4-glucosyltransferase, EC 2.4.1.11], a key enzyme in the synthesis of glycogen (see Leloir & Cardini, 1962), was firmly bound to particulate glycogen obtained after a series of cell fractionations. Reliable information, based on similar studies of subcellular distribution, was not available for α -glucan phosphorylase [α -(1 \rightarrow 4)-glucan-orthophosphate glucosyltransferase, EC 2.4.1.1], the major enzyme for the degradation of glycogen (see Stetten & Stetten, 1960; Krebs & Fisher, 1962). Madsen & Cori (1958) had, however, studied the interaction between purified corn glycogen and muscle phosphorylase and described the physico-chemical features of the binding. Some recent studies also suggest that phosphorylase may be associated to some extent with the glycogen-rich liver-microsomal fraction (Sutherland & Wosilait,

1956; Leloir & Goldemberg, 1960; Nigam, 1962; see de Duve, Wattiaux & Baudhuin, 1962). Particulate glycogen was not isolated, nor were enzyme recoveries or the concentration of liver glycogen taken into account in these studies. My interest in systematically defining the relationship between liver phosphorylase and particulate glycogen arose from some observations made on partial glycogen depletion during the early cellular action of thyroid hormones (Tata *et al.* 1963).

The present paper deals with the subcellular localization of rat-liver phosphorylase under different conditions. It is shown that phosphorylase is normally firmly associated with particulate glycogen, but that the enzyme is reversibly redistributed in the soluble fraction if glycogen is depleted by starvation or the administration of puromycin and 3,3',5-tri-iodo-L-thyronine. The binding of phosphorylase to glycogen was also studied in artificial systems with preparations of isolated glycogen of different molecular sizes.

EXPERIMENTAL

Materials. Dog-liver glycogen ($S_{20,w} = 28.0s$), containing less than 0.3% of N, was purchased from Roche Products Ltd., Welwyn, Herts. Rat-liver glycogen of low molecular weight was prepared by the classical KOH extraction followed by precipitation with ethanol (Stetten *et al.* 1956). The dimethyl sulphoxide extraction procedure of Whistler & BeMiller (1962) was adopted for less-degraded or high-molecular-weight preparations of rat-liver glycogen. In such preparations, the main glycogen component had a sedimentation constant, $S_{20,w}$, of 130s, compared with 76s for the alkali-ethanol preparation. Glucose 1-phosphate, glucose 6-phosphate, phosphoenol-

pyruvate and phosphoenolpyruvate kinase were purchased from Boehringer und Soehne, G.m.b.H., Mannheim, Germany. AMP, UDP, UDP-glucose and UTP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. α -Amylase (twice crystallized) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Puromycin was obtained from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y., U.S.A., and dissolved in 0.15M-NaCl. 3,3',5-Tri-iodo-L-thyronine was a gift from Glaxo Laboratories Ltd., Greenford, Middx., and was dissolved in aq. 10% (v/v) propane-1,2-diol at pH 8.2.

Subcellular fractionation. Male hooded rats (Mill Hill strain), weighing 140–180 g., were killed by a blow on the head and the livers were homogenized in 0.44M-sucrose containing NaF (0.06M) (1 g. of tissue to 9 ml. of medium) in a Teflon-glass Potter-Elvehjem homogenizer. The presence of NaF in the homogenization medium meant that the active form of phosphorylase (phosphophosphorylase) was studied (see Krebs & Fisher, 1962). The homogenate was fractionated into nuclei, mitochondria, microsomes and cell sap (microsome-free supernatant) by the method of Siekevitz & Watson (1956). Particulate glycogen was separated from the mitochondria-free supernatant or a microsomal suspension by layering it over 2.1M-sucrose and centrifuging at 105000g for 60 min. in the Spinco ultracentrifuge, as described by Luck (1961). Further fractionation was performed by resuspending the glycogen pellet in 1.31M-sucrose and repeating the high-speed centrifuging after layering the suspension over 2.1M-sucrose. Unless otherwise stated, 0.06M-NaF was present in all fractionation media. It was, however, excluded in all preparations used for UDP-glucose-glycogen glucosyltransferase assays. Fractionation procedures were carried out throughout at 0–4°.

Enzyme assays. Liver phosphorylase was assayed by measuring the release of inorganic phosphate from glucose 1-phosphate when the homogenate or subcellular fraction was incubated for 10 min. at 37° in the presence of 0.35% of dog-liver glycogen (Cori & Cori, 1940; Sutherland & Wosilait, 1956). Incubations were performed both in the presence and absence of 1 mM-AMP, and the specific activities were expressed as μ moles of inorganic phosphate released/10 min./mg. of protein. UDP-glucose-glycogen glucosyltransferase was assayed by measuring the UDP

released from UDP-glucose (Leloir & Goldemberg, 1960). Specific activities were expressed as μ moles of UDP released/10 min./mg. of protein.

Binding of phosphorylase to glycogen. The mitochondria-free supernatant or the cell-sap fraction of livers from rats starved for 24 hr. was used as a source of 'soluble' phosphorylase. To 10 ml. of the fraction placed in Spinco ultracentrifuge tubes (rotor no. 40) was added 1.0 ml. of a mixture of sucrose (0.44M) and NaF (0.06M), containing different amounts of rat-liver glycogen. The tubes were allowed to stand at 0–2° for 10 min. before centrifuging at 105000g for 60 min. The phosphorylase activity was then determined as usual in the microsomal or glycogen pellet, the supernatant and a sample of the non-centrifuged mixture, by using an excess of dog-liver glycogen as primer for all samples.

Chemical determinations. Glycogen content was determined by the anthrone method of Seifter, Dayton, Novick & Muntwyler (1950) after the glycogen had been precipitated and washed three or four times with 80% (v/v) methanol. Protein was measured by the biuret method (Gornall, Bardawill & David, 1949) or as described by Lowry, Rosebrough, Farr & Randall (1951). Inorganic phosphate was measured by the method of Fiske & Subbarow (1925), with a Unicam spectrophotometer. UDP was estimated with phosphoenolpyruvate and phosphoenolpyruvate kinase, the pyruvate released being measured colorimetrically with dinitrophenylhydrazine (Leloir & Goldemberg, 1960).

Ultracentrifugal studies. Dr P. Charlwood, National Institute of Medical Research, Mill Hill, kindly performed the ultracentrifugal studies. The sedimentation constants of native glycogen obtained by sucrose-density-gradient centrifuging of different subcellular fractions as well as glycogen extracted by alkali-alcohol and dimethyl sulphoxide procedures were measured.

RESULTS

Subcellular distribution of liver phosphorylase in fed rats. The subcellular distribution of phosphorylase in the nuclear, mitochondrial, microsomal and cell-sap fractions of liver homogenates from fed rats is summarized in Table 1.

Table 1. *Distribution and specific activity of phosphorylase and the amount of glycogen in subcellular fractions of livers of fed rats*

Livers were homogenized in 0.44M-sucrose containing NaF (0.06M) and the homogenates fractionated into the different subcellular constituents by the method of Siekevitz & Watson (1956). Phosphorylase activity, protein and glycogen were measured as described in the text. The values are given as means \pm s.d. of results obtained from six experiments. Only enzyme activities obtained in the presence of AMP are recorded.

Fraction	Distribution (μ moles of phosphate/ 10 min./g. of liver)	Specific activity (μ moles of phosphate/ 10 min./mg. of protein)	Glycogen:protein ratio
Homogenate	196 \pm 13	1.10 \pm 0.06	0.19
Nuclei etc.	16 \pm 6	0.27 \pm 0.05	0.06
Mitochondria	9 \pm 2	0.23 \pm 0.03	0.21
Mitochondria-free supernatant	159 \pm 12	2.04 \pm 0.23	0.32
Microsomes	140 \pm 11	3.67 \pm 0.10	1.17
Supernatant (cell sap)	6 \pm 2	0.08 \pm 0.02	—
Recovery*	91 \pm 6%	—	0.06

* Does not include mitochondria-free supernatant

An average of 90% of the total phosphorylase was recovered after fractionation. Nearly 80% of the recovered enzyme activity was found in the mitochondria-free supernatant and 90% of this could be sedimented with the microsomes. The specific activity of the enzyme in the microsomes was three- to five-fold greater than in the liver homogenate, when compared per mg. of protein. In all other fractions it was four- to ten-fold lower than in the homogenate. The overall pattern of distribution of phosphorylase was similar to that of glycogen in the above major subcellular fractions and the association between these two hepatic constituents is further demonstrated in Table 2.

Table 2 summarizes the distribution of phosphorylase in the principal fractions obtained by density-gradient centrifugation of particulate glycogen from the mitochondria-free supernatant. After the second sedimentation through 2.1M-sucrose, the phosphorylase:protein ratio was about threefold greater, whereas it was lower in all other submicrosome or cell-sap fractions. About half the enzyme activity was lost after each passage through the high-density sucrose so that the second glycogen pellet contained only 15–20% of the activity of the mitochondria-free supernatant. After correction for this inactivation by 2.1M-sucrose, the relative specific activity of phosphorylase (per mg. of protein) in particulate glycogen would be 25-fold that in the homogenate. The distribution of phosphorylase followed only grossly the pattern of glycogen content in the subfractions as there were substantial differences in the specific

activity/mg. of glycogen from one fraction to another.

Effect of starvation and acute glycogen depletion on the subcellular distribution of phosphorylase. Table 3 shows the distribution and specific activity of phosphorylase in rat-liver nuclei, mitochondria, microsomes and cell sap, 20 hr. after food was withdrawn.

The total phosphorylase activity after overnight starvation was 20% lower than the values in fed control animals. The subcellular distribution of the enzyme was, however, totally different in the two cases. With a total recovery of 85%, only 10–15% of phosphorylase was sedimented with the microsomes but most of the activity was now recovered in the cell sap. The specific activity in the latter fraction was twice that of the whole homogenate whereas it was lower in all the particulate fractions. Subfractionation of the mitochondria-free supernatant with high-density sucrose yielded only very small pellets in the fractions that would correspond to particulate glycogen from fed animals. No phosphorylase activity was detected in such pellets and almost all the enzyme was found in the top 0.44M-sucrose phase.

The glycogen content in our fed animals was 42 ± 5 mg./g. of liver. After overnight starvation it fell to about 10–15% of that of livers from fed animals. The subcellular distribution of this residual glycogen was also different after starvation. Whereas 60–65% of the total glycogen was present in the microsomes, only 5–10% of the total was recovered in this fraction in starved rats.

Table 2. *Specific activity of phosphorylase and the concentration of glycogen in subfractions of mitochondria-free supernatant prepared by serial sucrose-density-gradient centrifugation*

The mitochondria-free supernatant was obtained from livers (fed rats) homogenized in 0.44M-sucrose containing NaF (0.06M). The suspension was layered over 2.1M-sucrose containing NaF (0.06M) and fractionated by the method of Luck (1961). The enzyme activities, glycogen and protein were measured in samples of the top 0.44M-sucrose phase, the 0.44M–2.1M-sucrose interface, the 2.1M-sucrose supernatant and the glycogen-rich pellet. The remainder of the glycogen-rich pellet was suspended in 1.31M-sucrose containing NaF (0.06M) and centrifuged over a layer of 2.1M-sucrose containing NaF (0.06M). The same determinations were made on the top 1.31M-sucrose phase, and on the second glycogen pellet (particulate glycogen) which was suspended in 1.0 ml. of the 2.1M-sucrose layer immediately above it. Values are given as means \pm s.d. of results obtained from four to six experiments. $51 \pm 8\%$ of the phosphorylase in post-mitochondrial supernatant was recovered after the first fractionation and $22 \pm 6\%$ after the refractionation of the first glycogen-rich pellet. These yields should be considered when comparing relative specific activities. Only phosphorylase activities obtained in the presence of AMP are recorded.

Fraction	Specific activity (μ moles of phosphate/ 10 min./mg. of protein)	Glycogen:protein ratio
Mitochondria-free supernatant	196 ± 13	0.32
First centrifuging		
0.44M-Sucrose top phase	14 ± 3	0.06
0.44M–2.1M-Sucrose interface	84 ± 5	0.18
2.1M-Sucrose supernatant	165 ± 10	0.68
Glycogen pellet	346 ± 24	1.91
Second centrifuging		
1.31M-Sucrose top phase	51 ± 5	0.13
Particulate glycogen	273 ± 21	2.42

Unlike preparations from fed animals there was no correlation between the distribution of phosphorylase and glycogen in the different subcellular fractions obtained from starved animals.

The redistribution of phosphorylase from the particulate to the soluble fractions by starvation was rapidly reversed when the animals were re-fed.

Table 4 shows that 18 hr. after food was given, to animals previously starved for 24 hr., the major part of phosphorylase was again recovered in the microsomal fraction. Almost all the activity remained in the cell sap of animals that continued to be starved. There was a gradual decrease in total phosphorylase activity with starvation; re-feeding caused an increase to activities 10–20% above those of the fed control animals.

Hofert, Gorski, Mueller & Boutwell (1962) have shown that administration of puromycin causes a rapid depletion of hepatic glycogen. The subcellular distribution of phosphorylase was therefore studied as a function of time after the administration of the antibiotic to fed rats. Fig. 1 shows that a single injection of puromycin did not affect the total phosphorylase activity of rat liver but markedly altered its distribution between the particulate and the soluble fractions.

At 3 hr. after the administration of puromycin (the antibiotic was administered just after the peak feeding period of rats), almost all the enzyme activity was present in the soluble fractions. This lasted until about 6 hr., after which a larger fraction was recovered in the particulate fractions until at 18 hr. after the administration of puromycin the pattern had reverted to the normal distribution in the microsomes. The time-course of the reversible redistribution of phosphorylase paralleled the rapid disappearance and reappearance of hepatic glycogen.

An incomplete redistribution of phosphorylase was observed after the injection of small doses (20–50 $\mu\text{g.}/100\text{ g. body wt.}$) of tri-iodothyronine (Fig. 2).

At doses greater than 35 $\mu\text{g.}/100\text{ g.}$, thyroid hormone caused only a 50% drop in hepatic glycogen but, unlike after the administration of puromycin, the low concentration of glycogen was maintained for at least 2 days. Phosphorylase activity was about equally distributed between the particulate and 'soluble' fractions and then gradually increased in the microsomes as glycogen concentrations increased. The administration of tri-iodothyronine did not appreciably affect the total phosphorylase activity.

Table 3. *Distribution and specific activity of phosphorylase and the glycogen:protein ratio in subcellular fractions of livers of rats starved for 18–20 hr.*

Food was withdrawn in the evening, at least 2 hr. before the peak feeding time during which period control animals consumed $10 \pm 3\text{ g.}$ of food/100 g. body wt. Other details were as given in Table 1.

Fraction	Distribution ($\mu\text{moles of phosphate}/$ 10 min./g. of liver)	Specific activity ($\mu\text{moles of phosphate}/$ 10 min./mg. of protein)	Glycogen:protein ratio
Homogenate	168 \pm 7	0.86	0.021
Nuclei etc.	8 \pm 2	0.12	0.031
Mitochondria	5 \pm 1	0.07	0.033
Mitochondria-free supernatant	140 \pm 8	1.58	0.021
Microsomes	20 \pm 3	0.51	0.011
Supernatant (cell sap)	108 \pm 7	1.89	0.039
Recovery*	85 \pm 8%	—	—

* Does not include mitochondria-free supernatant.

Table 4. *Reversibility of phosphorylase redistribution and glycogen concentrations after re-feeding of starved rats*

Rats weighed 160–170 g. at the start of the experiment. The values are given as means \pm S.D. of results from four experiments, there being three rats in each group in each experiment. Phosphorylase and glycogen determinations and subcellular fractionation were as given in Table 1 and the text. Animals of the last group in this Table were killed 24 hr. after being re-fed.

Nutritional status	Wt. of liver (g.)	Phosphorylase per liver (m-moles of phosphate/ 10 min.)	Enzyme (%)		Total glycogen (mg./g. of liver)	Glycogen in microsomes (% of total)
			In microsomes	In supernatant		
Fed throughout	7.5 \pm 0.4	1.48 \pm 0.11	79	6	44.2	64
Starved for 20 hr.	6.1 \pm 0.4	1.02 \pm 0.10	8	78	4.6	9
Starved for 48 hr.	5.4 \pm 0.3	0.80 \pm 0.15	2	81	1.8	5
Starved for 20 hr., then re-fed	10.1 \pm 0.7	1.72 \pm 0.16	84	4	68.8	49

Interaction between 'soluble' phosphorylase and microsomal and isolated glycogen. The above experiments suggested indirectly that phosphorylase was

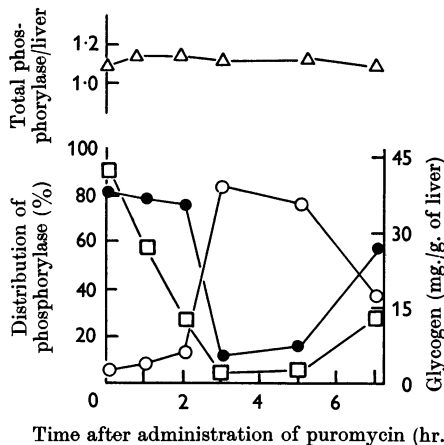


Fig. 1. Reversible redistribution of hepatic phosphorylase in microsomes and supernatant, and total glycogen content of the liver, after a single injection of 20 mg. of puromycin to groups of four rats of 140-160 g. body wt. The antibiotic was injected soon after the peak feeding period, and the fractions of total phosphorylase in the homogenate recovered in microsomes and supernatant were compared. About 85-90% of the enzyme was recovered after fractionation. The separate curve at the top representing total phosphorylase per liver is expressed as m-moles of inorganic phosphate released/10 min. The values at zero time were from determinations made on uninjected controls. ●, Phosphorylase recovered in microsomes; ○, phosphorylase recovered in supernatant; □, glycogen; △, total phosphorylase per liver.

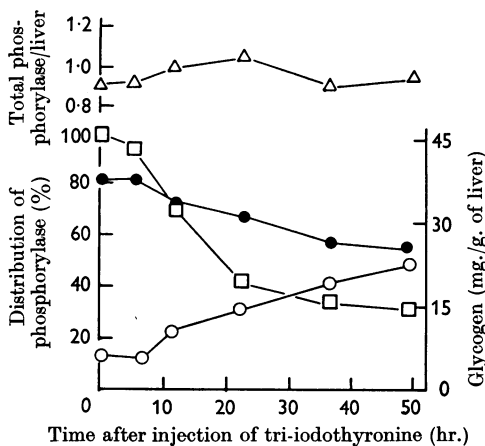


Fig. 2. Effect, as a function of time, of a single injection of 52 μ g. of 3,3',5-tri-iodo-L-thyronine on the distribution of phosphorylase between microsomes and supernatant, and hepatic glycogen. All other details and symbols are as in Fig. 1, except that male Wistar albino rats were used instead of hooded rats.

bound to particulate glycogen. When liver microsomes or particulate glycogen subfractions from fed rats were added to 'soluble' phosphorylase present in the cell sap of starved rats, all the enzyme activity could be sedimented with the microsomes on ultracentrifuging. It was not known whether phosphorylase in the cell sap was really 'free' or bound to some other constituent(s). However, the relationship between the amount of microsomes or particulate glycogen added and the fraction of total enzyme activity that could be sedimented indicated a phenomenon of reversible binding (Fig. 3).

It also suggested that the particulate glycogen or the binding component(s) of microsomes from fed rats was about one-third saturated with respect to phosphorylase.

Further evidence was obtained when chemically isolated rat-liver glycogen was added to 'soluble' phosphorylase present in the mitochondria-free supernatant of animals starved for 24 hr. Different results were obtained according to the preparation of glycogen used. With high-molecular-weight

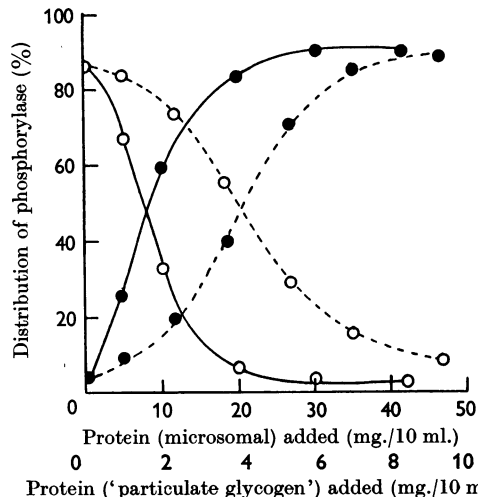


Fig. 3. Binding of 'soluble' liver phosphorylase in the cell sap from starved rats to microsomes or particulate glycogen from fed rats. Microsomes or particulate glycogen (second glycogen pellet after sucrose-density-gradient centrifuging), obtained from the livers of fed rats, were added in different amounts to 10 ml. of microsome-free supernatant (equivalent to 1.5 g. of liver) from rats starved for 22 hr. After 10-15 min. the suspension was centrifuged at 105000g for 60 min. and the phosphorylase activities in the sedimented particles and supernatant were measured. Values in this Figure represent the sum of phosphorylase originally in the microsome-free supernatant of the starved rats and that present in the added microsomes or particulate glycogen of fed animals. ●, Phosphorylase in the sediment after the addition of microsomes (solid line) or particulate glycogen (broken line); ○, phosphorylase left in the supernatant after the addition of microsomes (solid line) or particulate glycogen (broken line).

Table 5. *Sedimentation constants of the major components of glycogen extracted chemically or obtained by sucrose-density-gradient centrifuging of subcellular fractions*

The numbers in parentheses refer to the percentages of total glycogen in the samples estimated to represent the sedimentation-constant value. Other details are given in the text.

Nutritional status	Glycogen preparation	$S_{20,w}$ of the major components* (s)
Fed	Alkali-ethanol	76.3
	Dimethyl sulphoxide	127.6, 130.3
	Dimethyl sulphoxide, followed by sucrose-density-gradient centrifuging	
	(a) Particulate glycogen	129.4 (64)
	(b) In 2.1M-sucrose supernatant	83.4 (11); 115.4 (25)
Starved	'Native', sucrose-density-gradient centrifuging	
	Particulate glycogen	75.0, 131.0
Starved, then re-fed	Mitochondria-free supernatant	~60.0
	'Native', sucrose-density-gradient centrifuging	
	(a) Particulate glycogen	194.0 (51)
	(b) In 2.1M-sucrose supernatant	64.6 (15); 88.6 (23)

* All preparations were polydisperse and values for only the major components are given.

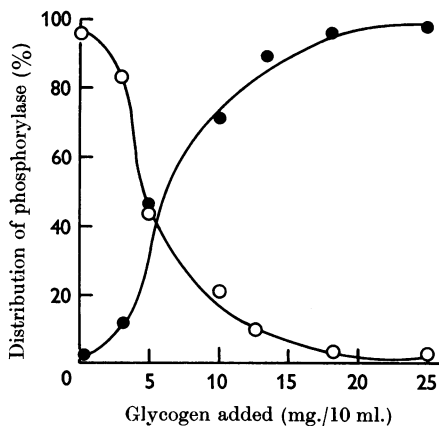


Fig. 4. Sedimentation of phosphorylase with the microsomal pellet after the addition of high-molecular-weight glycogen to mitochondria-free supernatant from livers of rats starved for 20 hr. High-molecular-weight glycogen was extracted from livers of fed rats by dimethyl sulphoxide and the major component (at least 50%) was 128s. The glycogen content of the mitochondria-free supernatant of starved rats was 1.8 mg./10 ml. At least 80% of the added high-molecular-weight glycogen was recovered in the microsomes after centrifuging. ●, Percentage of phosphorylase of the mitochondria-free supernatant sedimenting with microsomes; ○, percentage of phosphorylase left in the supernatant.

glycogen ($S_{20,w}$ of main component = 130s) obtained by extraction of rat liver with dimethyl sulphoxide, half the 'soluble' phosphorylase sedimented with the pellet when 4 mg. of glycogen was added/10 ml. of the mitochondria-free supernatant (Fig. 4).

No activity was retained in the supernatant after centrifuging when amounts exceeding 10 mg.

of glycogen/10 ml. were added. The addition of high-molecular-weight glycogen did not alter the total phosphorylase activity.

With low-molecular-weight glycogen ($S_{20,w}$ of main component = 28s) obtained from dog liver by extraction with alkali and ethanol, no more than 50% of the total phosphorylase activity was carried into the pellet even at concentrations exceeding 20 mg./10 ml. However, the addition of this preparation of low-molecular-weight glycogen to cell sap led to recoveries of enzyme activity in excess of the phosphorylase initially present. Thus, in terms of absolute number of units of phosphorylase, more enzyme activity was sedimented with the pellet with increasing amounts of glycogen added. The cause of this increase in total phosphorylase activity with degraded glycogen preparations is not known.

Molecular size of glycogen and distribution of phosphorylase. Glycogen is present in tissue in a whole spectrum of molecular sizes (see Stetten & Stetten, 1960; Bueding, 1962). The difference observed in the binding of phosphorylase according to the degree of degradation of glycogen prompted the measurement of the sedimentation constant of native glycogen (obtained by sucrose-density-gradient centrifuging) present in the different subcellular fractions of livers of fed and starved rats. Determinations were made both on preparations obtained by chemical extractions as well as on the major components of native glycogen present in the centrifugal subfractions of livers from fed, starved and re-fed animals.

Table 5 summarizes some of the values on the same samples of chemically prepared or 'native' glycogen used in the experiments described above (Table 2 and Fig. 4). The alkali-ethanol extraction procedure gives a more degraded product than the glycogen extracted with dimethyl sulphoxide. In

fact the sedimentation constant of the latter product was very close to the value of 130s for particulate glycogen obtained by differential centrifuging of mitochondria-free supernatant from livers of fed rats. After 24 hr. of starvation the size of residual glycogen present in the mitochondria-free supernatant was smaller, the major component having a sedimentation constant of about 60s. The re-feeding of starved rats resulted after 24 hr. in an increase in the average size of particulate glycogen ($S_{20,w} = 194.0s$). From the subcellular distribution of phosphorylase and the binding studies *in vitro*, it was apparent that glycogen of higher molecular weight bound phosphorylase more firmly than did molecules of smaller size.

Glycogen of higher molecular weight was also metabolically the more labile as seen by the relatively more rapid disappearance and appearance of microsomal or particulate glycogen after starvation and re-feeding (see Table 4). Glycogen is present in tissues in a variety of molecular sizes, and the sedimentation-constant values given above refer only to the major component discernible during ultracentrifuging.

DISCUSSION

Almost all phosphorylase activity in the livers of fed animals was recovered in microsomes when the tissue was homogenized and fractionated in 0.44M-sucrose containing sodium fluoride (0.06M). Although there was appreciable loss of activity on further fractionation through high-density sucrose, the major part of the enzyme was recovered in particulate glycogen. The distribution of phosphorylase in other subcellular fractions paralleled that of glycogen. Although similar fractionation studies based on total recoveries were not reported by other workers, our results are compatible with the association between phosphorylase and glycogen suggested in previous studies (Sutherland & Wosilait, 1956; Leloir & Goldemberg, 1960; Nigam, 1962; see de Duve *et al.* 1962). Nigam (1962) also observed the highest specific activity of phosphorylase/mg. of protein to be in glycogen-rich particles (but separated by centrifuging the mitochondria-free supernatant at only 25000g for 10 min.); however, he ruled out any relationship between the enzyme and glycogen.

When much of the glycogen was depleted by starvation, the major part of the phosphorylase was recovered in the microsome-free supernatant. This redistribution of enzyme from the particulate to the soluble fractions was almost complete after overnight starvation (16–20 hr. after withdrawal of food) and the pattern was maintained as long as food was withheld. The rats were usually starved overnight, which is the period of maximal food

intake. There was a return to normal pattern on re-feeding (Table 4). Table 4 also shows that microsomal glycogen was depleted more rapidly than non-microsomal glycogen. The microsomal phosphorylase:cell-sap phosphorylase specific activity ratio dropped from 45.5 in fed rats to 0.26 in starved rats, which represents a 175-fold change. The reversible redistribution depended only on the time taken to lower the glycogen concentration and the extent of lowering. Thus the administration of puromycin, which very rapidly but transiently depletes the liver of almost all its glycogen, causes reversible redistribution of the enzyme (see Fig. 1). Hofert *et al.* (1962), who were the first to describe the glycogenolytic activity of puromycin, had demonstrated that glycogen depletion was unrelated to the well-known inhibition of protein synthesis by this antibiotic. If glycogen loss occurred more gradually or incompletely, as after the administration of tri-iodothyronine (Tata *et al.* 1963), phosphorylase was recovered in both the microsomal (or particulate-glycogen) and cell-sap fractions, and this pattern was maintained for 2 days after a single injection of the hormone (Fig. 2). This partial glycogenolytic effect of tri-iodothyronine is not believed to be directly related to the mechanism of hormonal action.

In all the redistribution studies as a function of the glycogen content, the total amount of phosphorylase in the liver generally did not alter appreciably. After 24 hr. of starvation there was a 15–20% decrease in total phosphorylase which increased by about the same amount over the normal values 24 hr. after starved rats were re-fed. These values are similar to those obtained by Niemeyer, González & Rozzi (1961) for comparable periods of time. The omission of AMP from the incubation medium resulted in a decrease of 10–20% of the activity, a value similar to that observed by Sutherland & Wosilait (1956). However, phosphorylase when present in the cell sap of starved animals exhibited a higher response to AMP than that bound to the particulate fractions, thus indirectly indicating that glycogen might preferentially bind the active form or stabilize it.

The exact nature of forces involved in the interaction between hepatic glycogen and phosphorylase is not known. Phosphorylase present in the cell sap is assumed to be free or, if bound, the affinity of binding of cell-sap constituents is extremely low compared with the binding to microsomal or particulate glycogen. It would be reasonable to conclude that the binding reported above is a reversible phenomenon of simple equilibrium between free (or 'soluble') and bound ('particulate') enzyme, with steric factors probably playing an important role. Madsen & Cori (1958), who used purified corn glycogen and muscle phos-

Table 6. *Reversible redistribution between microsomal and supernatant fractions of phosphorylase and UDP-glucose-glycogen glucosyltransferase during alterations in the glycogen content by starvation and re-feeding*

Phosphorylase was assayed in subcellular fractions as described in Table 1. UDP-glucose-glycogen-glucosyltransferase activity was measured as described by Leloir & Goldemberg (1960) in subcellular fractions from samples of liver homogenized in the absence of NaF.

Nutritional status	Glycogen (mg./g. of liver)	Phosphorylase* distribution (%)		UDP-glucose-glycogen- glucosyltransferase* distribution (%)	
		Microsomes	Supernatant	Microsomes	Supernatant
Fed	38.5	77	6	61	3
Starved for 20 hr.	2.8	7	73	4	78
Starved for 24 hr., then re-fed for 20 hr.	54.6	83	5	72	5

* The variation in enzyme activity/g. of liver in the different groups of animals was within $\pm 6\%$ for UDP-glucose-glycogen glucosyltransferase whereas the fluctuations for phosphorylase were higher (see Tables 1-4).

phorylase, calculated that 1 molecule of glycogen could bind a maximum of 22-33 molecules of phosphorylase. Though such calculations were not possible in our saturation studies, the overall capacity of hepatic glycogen under normal conditions is about three times the phosphorylase present in the tissue. The ultracentrifugal results suggest a relationship between binding affinity and the size of the glycogen molecule. Glycogen is present in tissues in a whole range of molecular weights and metabolic lability.

Digestion of particulate glycogen for 1 hr. with α -amylase as described by Luck (1961) resulted in the release of 30-50% of the phosphorylase activity originally associated with the glycogen pellet. Attempts to dissociate the enzyme from glycogen with detergents (sodium deoxycholate, sodium dodecyl sulphate) were not successful because of extensive inactivation of phosphorylase. For example, treatment of particulate glycogen with 0.4% sodium deoxycholate for 10 min. at 0-2° resulted in the loss of 55-90% of enzyme activity after ultracentrifuging.

There are marked similarities between the localization of UDP-glucose-glycogen glucosyltransferase in particulate glycogen described by Luck (1961) and that of phosphorylase observed in the present work. When redistribution studies were performed on the two enzymes determined in portions of the same liver samples, both enzymes simultaneously reversibly passed from the particulate to the soluble fractions during alterations in glycogen content (see Table 6).

In some experiments, the localization of 'inactivating enzyme' (phosphorylase phosphatase) was also followed by omitting sodium fluoride from the high-density sucrose used for the isolation of particulate glycogen. On incubation, much of the tissue's 'inactivating-enzyme' activity could also be recovered with glycogen and phosphorylase.

Among the wider implications of the present studies are the following two considerations: (1) The two most important enzymes in the synthesis and breakdown of glycogen are both bound to the polymer itself. One could speculate that this relationship constitutes a part of an intracellular glycogen-regulatory mechanism if the following assumptions were valid: that the binding affinity increases with the size of the glycogen molecule and that it is the active phosphorylase that is preferentially bound to particulate glycogen. (2) The binding of UDP-glucose-glycogen glucosyltransferase and phosphorylase may be of some consequence in the study of glycogen metabolism during development. It is now known that glycogen can be detected in developing tissues at the same time as, or soon after, UDP-glucose-glycogen glucosyltransferase makes its first appearance but that phosphorylase can only be detected after some time has elapsed (Grillo & Ozone, 1962). This raises two equally plausible suggestions as to the initiation of phosphorylase activity: (a) first, that glycogen, synthesized by UDP-glucose-glycogen glucosyltransferase, induces the synthesis of phosphorylase according to the now classical ideas of substrate induction by interaction with genetically controlled repressors (see Knox, 1961). Niemeyer, Pérez, Garces & Vergara (1962) have, in fact, suggested that the increase in phosphorylase activity after prolonged starvation may result from such a substrate-induced enzyme synthesis; (b) secondly, that phosphorylase is already formed when glycogen first makes its appearance but that it is preponderantly in the 'soluble' inactive form (dephosphophosphorylase). The preferential binding of the small amount of active phosphorylase to particulate glycogen could then push the equilibrium in favour of an accumulation of the active form (phosphophosphorylase) so that the presence of the enzyme could then be easily detected.

SUMMARY

1. About 70–80 % of α -glucan phosphorylase was recovered in the microsomal fraction of livers from fed rats. After subfractionation of microsomes by sucrose-density-gradient centrifuging, the majority of the recoverable enzyme activity was associated with the 'particulate' glycogen fraction.

2. Starvation for 18–20 hr. (overnight) led to a drop of 15–20 % in total phosphorylase activity per liver and 80 % of the enzyme was now present in the 105 000g supernatant. Then, 24 hr. after the re-feeding of starved rats the enzyme reappeared in the particulate fraction, with a 15–20 % increase in total phosphorylase. The reversible redistribution of phosphorylase between the particulate and 'soluble' fractions paralleled the depletion and reappearance of glycogen.

3. The administration of puromycin to fed rats caused a rapid and reversible subcellular redistribution of the enzyme; this followed the course of depletion and reappearance of glycogen in the liver. With 3,3',5-tri-iodo-L-thyronine, the redistribution was incomplete but its effect persisted for a longer time than after puromycin.

4. The association between particulate glycogen and phosphorylase appears to be a phenomenon of reversible binding. It was studied by measuring, in artificial systems, the distribution of phosphorylase present in the 105 000g supernatant from starved rats after the addition of liver microsomes and particulate glycogen from fed rats or chemically isolated glycogen preparations. Particulate glycogen in livers from fed rats was about one-third saturated with respect to phosphorylase.

5. The binding of phosphorylase to glycogen exhibited a specificity with regard to the size of the glycogen molecule. Some physiological implications of this association have been discussed.

REFERENCES

- Bueding, E. (1962). *Fed. Proc.* **21**, 1939.
 Claude, A. J. (1954). *Proc. Roy. Soc. B*, **142**, 177.
 Cori, G. T. & Cori, C. F. (1940). *J. biol. Chem.* **135**, 733.

- de Duve, C., Wattiaux, R. & Baudhuin, P. (1962). *Advanc. Enzymol.* **24**, 291.
 Drochmans, P. (1963). *Symp. biochem. Soc.* **23**, 127.
 Figueroa, E. & Pfeiffer, A. (1962). *Arch. Biochem. Biophys.* **99**, 357.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **277**, 751.
 Grillo, T. A. I. & Ozone, K. (1962). *Nature, Lond.*, **195**, 902.
 Hofert, J., Gorski, J., Mueller, G. C. & Boutwell, R. K. (1962). *Arch. Biochem. Biophys.* **97**, 134.
 Knox, W. E. (1961). In *Synthesis of Molecular and Cellular Structure*, p. 13. Ed. by Rudnick, D. Chicago: The Ronald Press Co.
 Krebs, E. G. & Fisher, E. H. (1962). *Advanc. Enzymol.* **24**, 263.
 Lazarow, A. (1942). *Anat. Rec.* **84**, 31.
 Leloir, L. F. & Cardini, C. E. (1962). In *The Enzymes*, vol. 6, p. 317. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
 Leloir, L. F. & Goldemberg, S. H. (1960). *J. biol. Chem.* **235**, 919.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Luck, D. J. L. (1961). *J. biophys. biochem. Cytol.* **10**, 195.
 Madsen, N. B. & Cori, C. F. (1958). *J. biol. Chem.* **233**, 1251.
 Niemeyer, H., González, C. & Rozzi, R. (1961). *J. biol. Chem.* **236**, 610.
 Niemeyer, H., Pérez, N., Garces, E. & Vergara, F. E. (1962). *Biochim. biophys. Acta*, **62**, 411.
 Nigam, V. N. (1962). *Nature, Lond.*, **196**, 478.
 Porter, K. R. & Bruni, C. (1959). *Cancer Res.* **19**, 997.
 Seifter, S., Dayton, S., Novick, B. & Muntwyler, E. (1950). *Arch. Biochem. Biophys.* **25**, 191.
 Sjökevitz, P. & Watson, M. L. (1956). *J. biophys. biochem. Cytol.* **2**, 653.
 Stetten, DeW., jun. & Stetten, M. R. (1960). *Physiol. Rev.* **40**, 505.
 Stetten, M. R., Katzen, M. M. & Stetten, DeW., jun. (1956). *J. biol. Chem.* **222**, 587.
 Sutherland, E. W. & Wosilait, W. D. (1956). *J. biol. Chem.* **218**, 459.
 Tata, J. R., Ernster, L., Lindberg, O., Arrhenius, E., Pedersen, S. & Hedman, R. (1963). *Biochem. J.* **86**, 408.
 Whistler, R. L. & BeMiller, J. N. (1962). *Arch. Biochem. Biophys.* **98**, 120.