

ASSOCIATION OF GLYCOGEN SYNTHASE PHOSPHATASE AND PHOSPHORYLASE PHOSPHATASE ACTIVITIES WITH MEMBRANES OF HEPATIC SMOOTH ENDOPLASMIC RETICULUM

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ABSTRACT

A detailed investigation was conducted to determine the precise subcellular localization of the rate-limiting enzymes of hepatic glycogen metabolism (glycogen synthase and phosphorylase) and their regulatory enzymes (synthase phosphatase and phosphorylase phosphatase). Rat liver was homogenized and fractionated to produce soluble, rough and smooth microsomal fractions. Enzyme assays of the fractions were performed, and the results showed that glycogen synthase and phosphorylase were located in the soluble fraction of the livers. Synthase phosphatase and phosphorylase phosphatase activities were also present in soluble fractions, but were clearly identified in both rough and smooth microsomal fractions. It is suggested that the location of smooth endoplasmic reticulum (SER) within the cytosome forms a microenvironment within hepatocytes that establishes conditions necessary for glycogen synthesis (and degradation). Thus the location of SER in the cell determines regions of the hepatocyte that are rich in glycogen particles. Furthermore, the demonstration of the association of synthase phosphatase and phosphorylase phosphatase with membranes of SER may account for the close morphological association of SER with glycogen particles (i.e., disposition of SER membranes brings the membrane-bound regulatory enzymes in close contact with their substrates).

KEY WORDS smooth endoplasmic reticulum · glycogen synthesis · synthase phosphatase · phosphorylase phosphatase

Early studies on the fine structure of hepatocytes showed a close association of smooth endoplasmic reticulum (SER) and glycogen particles (4, 12, 25). Almost all later investigators have confirmed this close morphological association of SER and glycogen, but the functional implications have been

less clear. Most workers have noted that glucose-6-phosphatase is found in smooth microsomes (9, 19), and the cytochemical localization of the enzyme to rough and smooth endoplasmic reticulum (RER and SER) has been achieved (19, 31). These observations have led to the conclusion that SER is involved in glycogen breakdown and/or glucose release from the cell. However, studies involving animals maintained on a controlled feeding schedule (1, 2) have suggested that SER is associated

with glycogen particles during glycogen deposition. In addition, SER was found closely associated with glycogen particles in hepatocytes of adrenalectomized rats injected with a glucocorticoid (5, 23). Under these conditions, it is clear that the hepatocytes are actively depositing glycogen rather than breaking down the carbohydrate. Thus, some morphological evidence suggests a role for SER in the synthesis of hepatic glycogen (6).

Previous attempts to relate enzymes involved in hepatic glycogen synthesis to the SER have been largely unsuccessful. However, much information has been accumulated on the biochemical mechanisms for hepatic glycogen synthesis. It has been established that the rate-limiting enzymes of glycogen synthesis and degradation are glycogen synthase and glycogen phosphorylase, respectively (14, 27). These enzymes exist in physiologically active and inactive forms with rapid enzymic interconversion between the two forms of each enzyme. The chemical nature of the interconversion reactions of these enzymes involves phosphorylation by specific kinases (16, 17) and dephosphorylation by phosphatases which may be specific or nonspecific (8, 16, 17). The physiologically active form of glycogen synthase is the dephosphorylated or I form, whereas the phosphorylated or D form is inactive under physiological conditions (16–18). Conversely, the physiologically active form of glycogen phosphorylase is phosphophosphorylase (a form) with the dephospho- form of the enzyme (b form) being inactive (16–18).

Luck (21) and several subsequent investigators (15, 18) fractionated liver cells and studied the distribution of glycogen synthase and phosphorylase. These workers concluded that the enzymes are either associated with glycogen particles or found in the soluble component of the cell. No reported evidence exists for the localization of these enzymes in either SER or RER. However, it should be noted that Hizukuri and Larner (15) provided an important early finding when they demonstrated a "converting" factor in a glycogen-free fraction that sedimented after high-speed centrifugation. It was suggested that this fraction was possibly of microsomal origin; however, to our knowledge, no further attempts were made to clarify this point. This fraction catalyzed the conversion of glycogen synthase from inactive to active form in rat liver. Subsequent investigations showed that this enzymatic reaction was a dephosphorylation of glycogen synthase caused by the enzyme synthase phosphatase (29). On the

basis of this information and the morphological evidence cited above, Dallner and Ernster (10) suggested the possibility that synthase phosphatase is associated with membranes of the SER. Cardell (6) also regarded this as a likely possibility.

In this paper, we report careful fractionation studies in which highly purified smooth and rough microsomal fractions were prepared and assayed for the rate-limiting enzymes of glycogen metabolism and for the converting enzymes (synthase phosphatase and phosphorylase phosphatase). Our results clearly show significant quantities of both synthase phosphatase and phosphorylase phosphatase in the microsomal fractions of livers from fasted rats. In subsequent and more detailed publications, we will report our findings on the properties of the membrane-bound enzymes and their response to various hormonal and dietary manipulations of the experimental animals.

MATERIALS AND METHODS

Animals

Adult, male Wistar rats (200–250 g) were used in all experiments. Rats were allowed ad lib. access to food and water, but were fasted for 24 h before sacrifice. All rats were maintained on a 12:12 h light-dark cycle.

Electron Microscopy

Animals were decapitated, a portion of the left lateral lobe of each liver was rapidly removed, and the sample was placed in a drop of 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). The tissue was cut into small pieces ~1 mm³ in size and placed in a vial containing the glutaraldehyde fixative. After 2 h of fixation at room temperature, the tissue was rinsed in cacodylate buffer (0.1 M, with 10% sucrose) and postfixed in 1% osmium tetroxide (in 0.1 M phosphate buffer). The tissue was then dehydrated in a graded series of alcohol and embedded in Epon (22). Ultrathin sections were stained with uranyl acetate and lead citrate (26, 32) and examined in a Philips EM-300 electron microscope.

Subcellular Fractionation

Livers from 24-h fasted rats¹ were excised, blotted dry, weighed, and placed in cold 0.25 M sucrose. Homogenization was carried out with a motor-driven Potter-Elvehjem homogenizer in a cold room (0°–4°C). A 20% (wt/vol) homogenate was obtained by making three up-and-down passes of the pestle (900 rpm) in the homogenization vessel. Homogenates were centrifuged twice at 10,000 g for 20 min in a Beckman J-21C (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) preparative centrifuge. The resultant postmitochondrial supernate (PMS) was saved, and the pellet containing nuclei, plasma membrane, mitochondria, and other cellular debris was discarded.

¹ In these initial experiments, fasted animals were used to avoid contamination of the fractions with glycogen particles.

Smooth and rough microsomes were prepared by the Dallner et al. (9, 10) procedure. Briefly, the PMS was made 15 mM Cs⁺ with 1 M stock solution of CsCl. The PMS plus 15 mM Cs⁺ was layered over 15 ml of 1.3 M sucrose plus 15 mM Cs⁺ in an SW27 centrifuge tube (Beckman Instruments). Centrifugation in an L5-50 (Beckman Instruments) ultracentrifuge for 4 h at 105,000 g at 4°C produced a pellet containing rough microsomes beneath the 1.3 M sucrose and a band containing smooth microsomes at the 0.25–1.3 M sucrose interface. The supernate above the band was drawn off (soluble fraction) and saved on ice. The band was drawn off and diluted with distilled water, and the pellet was resuspended in 0.25 M sucrose by gentle homogenization in a glass homogenizer. Both subfractions were centrifuged in a Ti50 rotor at 225,000 g for 1 h. Final pellets were resuspended in 0.25 M sucrose.

Chemical and Enzymatic Determinations

Protein was determined by the method of Lowry et al. (20). Glycogen was determined by extraction of tissue portions in 30% KOH and precipitation of glycogen with 66% ethanol. Glycogen was estimated by hydrolysis via the phenol-sulfuric acid method (11).

Glycogen synthase phosphatase activity in the various fractions was determined in a two-stage incubation assay. The first incubation involved the conversion of purified glycogen synthase from the D to the I form. This stage was initiated by adding 8 μ l of the subcellular fraction to a mixture of 5 μ l of a solution containing 1.2 U² of muscle glycogen synthase D, 10 mM Tris (pH 7.8), 1 mM EDTA, 0.5 mM EGTA, 0.5 mM phenylmethylsulfonylurea fluoride, 10 mM mercaptoethanol, 5% glycerol, and 8 mg/ml glycogen. To this was added 4 μ l of H₂O or 4 μ l of 42.5 mM MgCl₂. Incubation for varying lengths of time at 30°C was carried out, with the phosphatase reaction stopped by adding 20 μ l of ice-cold 400 mM KF and placing the assay tube on ice. The second stage of the phosphatase assay involved determining the synthase I activity generated in the first stage by the filter paper method of Thomas et al. (30). The synthase phosphatase reaction was linear for all fractions over 20 min. Glycogen synthase D was purified by the method of Takeda et al. (28). Synthase phosphatase is presented as units per gram protein in the subcellular fraction, with 1 U of synthase phosphatase equal to 1 U of synthase D converted to synthase I per minute.

Glycogen phosphorylase phosphatase activity in identical fractions was also determined by a two-stage assay. Enzymatic conversion of phosphorylase a (Sigma Chemical Co., St. Louis, Mo.) to phosphorylase b was determined by incubation for varying lengths of time at 30°C of hepatic subcellular fractions in buffer containing 4 U/ml³ phosphorylase a, 20 mM glycylglycine (pH 7.8), 10 mM MgCl₂, 10 mM caffeine, 0.1% albumin, and 10% glycerol. This reaction was stopped by adding ice-cold KF in MES (pH 6.1) to give final concentrations of 66 mM and 16 mM, respectively, with the tubes subsequently placed on ice. Phosphorylase a activity was then determined on these samples by the filter paper method of Gilboe et al. (13). Phosphorylase phosphatase activity is presented in units per gram protein, with

² Glycogen synthase D is expressed as units (micromoles of glucose incorporated into glycogen from uridine diphosphate glucose per minute per milligram protein in the presence of 10 mM glucose-6-phosphate).

³ Phosphorylase a is also expressed as units (micromoles of glucose incorporated into glycogen from glucose-1-phosphate per minute per milligram protein).

1 U representing 1 U of phosphorylase a converted to phosphorylase b per minute.

Separate portions of each subcellular fraction were also assayed for glycogen synthase activity and phosphorylase activity, as previously described (7, 13, 30).

Significance of differences between means was determined by Student's *t* test. Activities are presented as means \pm standard errors (SEM) of numbers of determinations.

RESULTS

Hepatocytes from 24-h fasted rats contained glycogen particles restricted to distinct regions of the cytosome (Fig. 1). Within the glycogen regions of the cell, numerous tubules and vesicles of SER were closely associated with the particles of glycogen (*inset*, Fig. 1). It is clear from such micrographs that the distribution of SER in the cytosome of hepatocytes is related to the location of glycogen within the cells.

After homogenization and fractionation of rat liver, rough and smooth microsomal subfractions were obtained. Electron microscopy of ultrathin sections of the pellets from these fractions showed that the rough microsomes were isolated predominantly as vesicles containing ribosomes bound to the outer, cytoplasmic surface (Fig. 2), and hence were derived from the RER. The smooth microsomal fraction appeared as smooth-surfaced vesicles, with occasional flattened saccules and sheets of membranes (Fig. 3). We regard this fraction as containing mainly elements of SER; however, contamination by other cellular organelles (Golgi apparatus, plasma membrane, etc.) may represent a minor component (9, 23). Further support for this conclusion was provided by determinations of glucose-6-phosphatase specific activity (data not shown) which showed that the smooth microsomal fraction is rich in this enzyme, and hence contains predominantly membranes of SER. In addition, analysis of RNA content revealed that 95% of the RNA is localized in rough microsomes (data not shown).

We measured the activity of glycogen synthase in four subcellular fractions: PMS, soluble, rough microsomes, and smooth microsomes. The enzyme was found in both PMS and soluble fractions, whereas no enzyme activity beyond trace amounts was detected in microsomal subfractions (Fig. 4). A similar distribution of glycogen phosphorylase was observed (Fig. 5). Thus, specific activity of these rate-limiting enzymes was detected in PMS and soluble fractions of the livers, but not in significant quantities in the rough and smooth microsomal fractions.

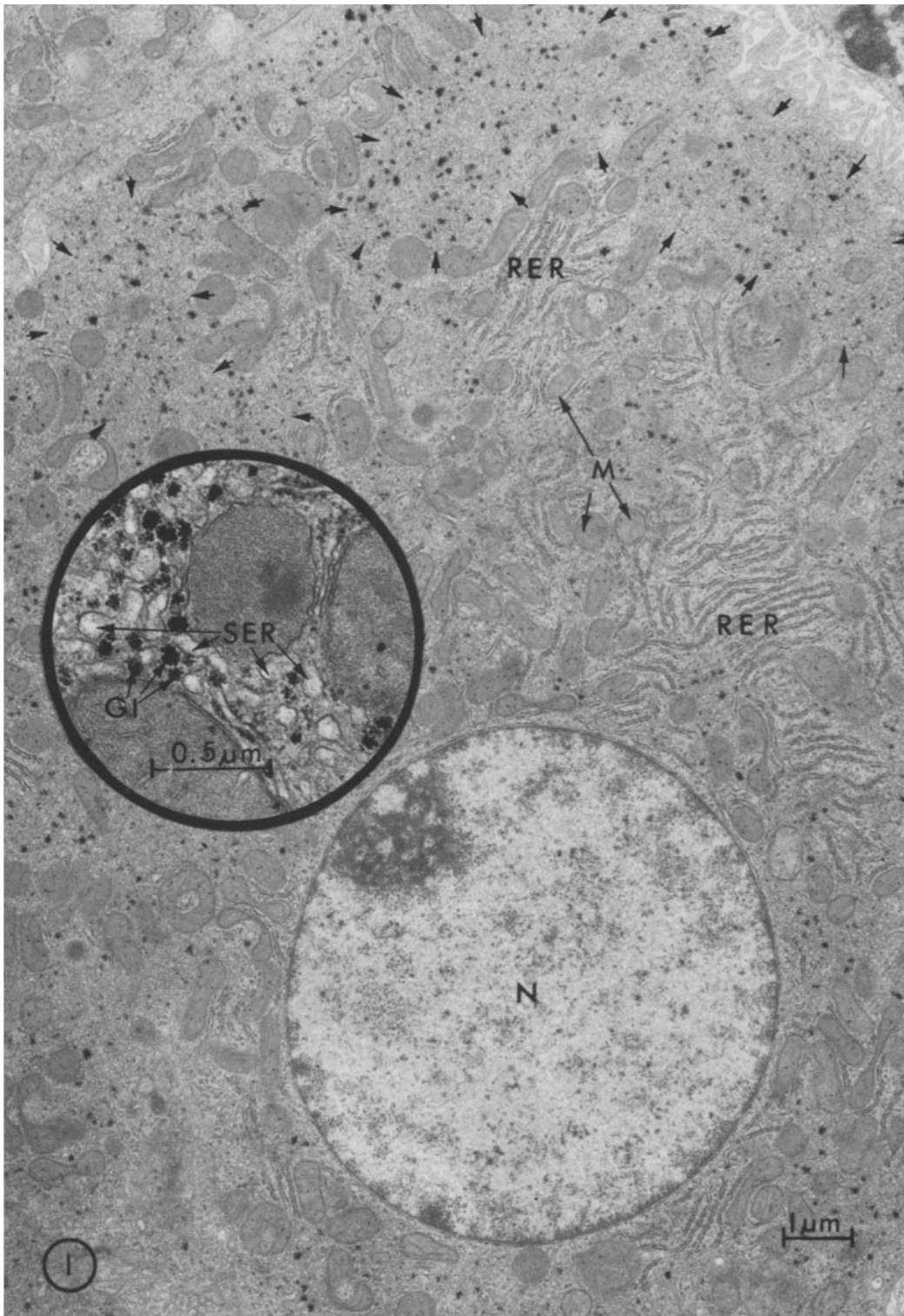


FIGURE 1 Low magnification ($\times 10,000$) electron micrograph of centrilobular hepatocyte from overnight-fasted normal rat. Note glycogen area (arrows) containing numerous tubules and vesicles of SER and glycogen particles. Numerous areas of cytoplasm rich in RER are obvious. Mitochondria (*M*) and nucleus (*N*) are also identified. A higher magnification ($\times 36,000$) of a glycogen region from a centrilobular hepatocyte is displayed in the *inset*. Note close proximity of glycogen (*GI*) to SER.

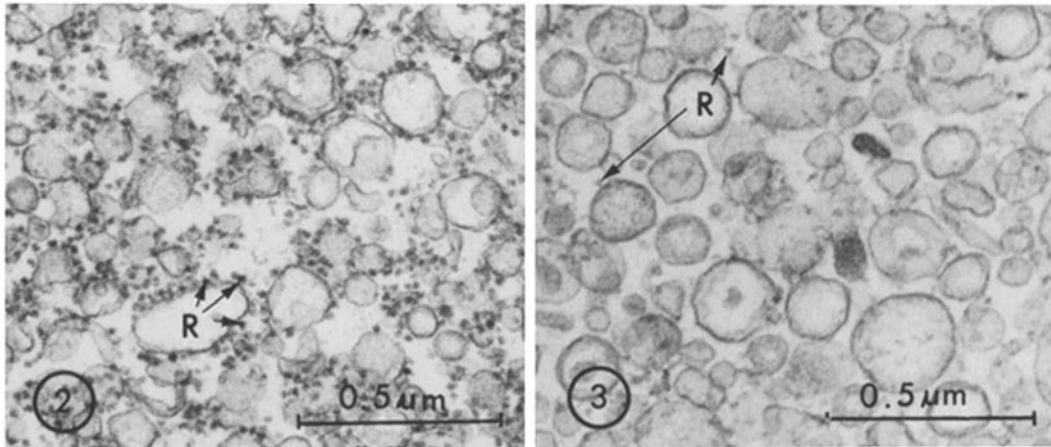


FIGURE 2 Electron micrograph of a section through the rough microsomal pellet showing ribosomes (*R*) on the outer (cytoplasmic) surface of vesicles. $\times 54,500$.

FIGURE 3 Electron micrograph of section through the smooth microsomal pellet. Only occasional vesicles have ribosomes (*R*) attached to membranes. $\times 54,500$.

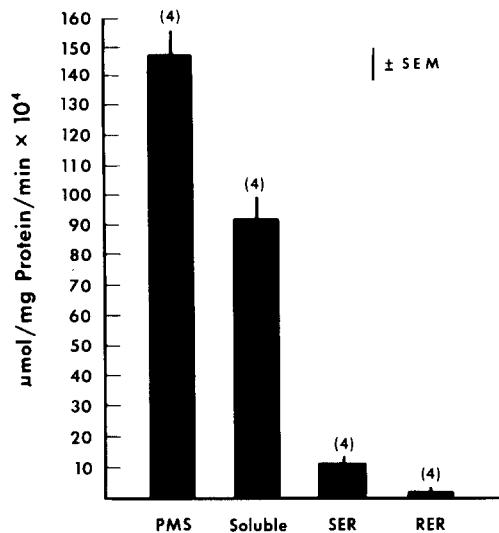


FIGURE 4 Glycogen synthase activity in hepatic subcellular fractions from overnight-fasted normal rats. Enzyme-specific activity is presented as micromoles product formed per milligram protein per minute. Each point represents the mean of four determinations \pm SEM. See text for details of assay procedure. *PMS*, postmitochondrial supernate; *Soluble*, postmicrosomal supernate; *SER*, smooth microsomes; *RER*, rough microsomes.

In contrast, phosphorylase phosphatase activity was measured in eight subfractions, and was clearly present in hepatic microsomal membrane fractions obtained from the livers of fasted rats (Table I). The enzyme was detected with highest

specific activity in both rough (42.4 ± 4.5 U/g) and smooth (31.1 ± 6.2 U/g) microsomes. It is noteworthy that a significant amount of enzyme was demonstrated in the soluble fraction (9.2 ± 2.2 U/g) as well.

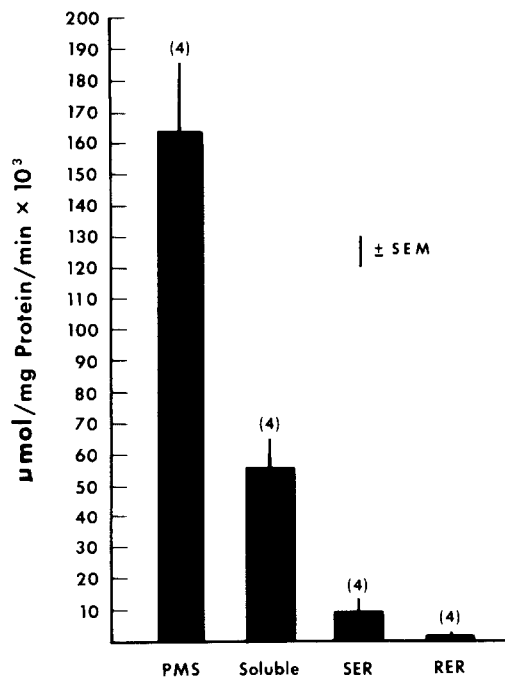


FIGURE 5 Glycogen phosphorylase activity in hepatic subcellular fractions from overnight-fasted normal rats. See Fig. 4 and text for details.

The protein concentrations, total protein, phosphorylase phosphatase specific activity, total phosphorylase phosphatase activity, and enzyme recovery in each fraction of a representative experiment are presented in Table I. As can be seen, a significant amount of both total protein and phosphorylase phosphatase activity were lost in the first two 10,000 g centrifugations (65 and 48%, respectively). However, and more importantly, the majority of the phosphorylase phosphatase activity recovered (114% of activity applied) from the discontinuous sucrose gradient centrifugation step was associated with the SER and RER fractions. Thus, although it is clear that the very mild ho-

mogenization procedure employed in these experiments to minimize contamination of ER membrane fractions with other cellular membranes results in an appreciable loss of phosphorylase phosphatase activity in the initial centrifugation steps, it is also clear that the majority of this activity sediments in nearly equal amounts in the SER and RER fractions.

Synthase phosphatase was also localized in the hepatic microsomal fractions where 1.29 ± 0.11 U/g of enzyme activity was observed in rough microsomes and 5.09 ± 0.45 U/g in smooth microsomes (Table II). The soluble fraction displayed 2.70 ± 0.22 U/g enzyme specific activity.

TABLE I
Subcellular Distribution of Phosphorylase Phosphatase Activity in Liver

	Protein concentration	Total protein	Phosphorylase phosphatase activity	Phosphorylase phosphatase activity	Enzyme recovery	Supernate II activity
	mg/ml	g	U/g*	U	%	%
20% homogenate (10,000 g for 20 min)						
Supernate	17.0	0.493	13.0	6.41	49	
Pellet	35.5	0.553	12.0	6.63	51	
Supernate I (10,000 g for 20 min)						
Supernate II	15.3	0.367	18.6	6.83	52	
Pellet	12.6	0.126	15.2	1.92	15	
Supernate II (105,000 g for 2 h on sucrose gradient)*						
Supernate (soluble)	5.2	0.186	9.2	1.71	13	22
SER	3.9	0.070	31.1	2.17	16	28
RER	3.5	0.063	42.4	2.66	20	34
1.3 M sucrose	3.0	0.053	23.2	1.23	9	16

* See text for details.

TABLE II
Subcellular Distribution of Glycogen Synthase Phosphatase Activity in Liver

	Synthase phosphatase activity				Enzyme recovery		Supernate II activity	
	U/g	U/g	U	U	%		%	
20% homogenate (10,000 g for 20 min)	+*	-	+	-	+	-	+	-
Supernate I	4.37	2.62	2.15	1.29	65	69		
Pellet	1.36	1.02	0.75	0.56	35	31		
Supernate I (10,000 g for 20 min)								
Supernate II	3.79	1.67	1.39	0.61	48	33		
Pellet	4.20	3.45	0.51	0.43	18	15		
Supernate II (105,000 g for 2 h on sucrose gradient)								
Supernate (soluble)	4.42	2.70	0.82	0.50	28	27	66	42
SER	3.07	5.09	0.21	0.36	7	19	17	31
RER	0.77	1.29	0.05	0.08	2	4	4	7
1.3 M sucrose	3.13	4.38	0.16	0.23	6	12	13	20

See text for further details.

* Activities were determined in the presence (+) or the absence (-) of 10 mM Mg⁺².

Several reports exist (3, 14, 17) of effects of divalent cations on phosphoprotein phosphatase activities in crude extracts made from muscle and liver. Consequently, we investigated the effects of divalent cations on our hepatic subcellular fractions and found that magnesium had dramatic and opposite effects on enzyme-specific activity, depending on whether the enzyme was soluble or membrane associated. Thus, when Mg^{+2} was present, enzyme activity was decreased to 0.77 ± 0.22 U/g in rough and 3.07 ± 0.56 U/g in smooth microsomal fractions. In marked contrast, in the presence of Mg^{+2} , enzyme activity increased in the soluble (4.42 ± 0.50 U/g) fractions. Moreover, when synthase phosphatase was solubilized by high salt treatment, the solubilized enzyme retained its sensitivity to Mg^{+2} and thus could still be distinguished from the soluble form of the enzyme. In preliminary studies, we have found that the K_a of soluble enzyme for Mg^{+2} is 2 mM, whereas the K_i of the SER enzyme is 1.5 mM.⁴

The quantitative distribution of glycogen synthase phosphatase activity measured in the presence and absence of 10 mM Mg^{+2} in the same liver fractions shown for phosphorylase phosphatase activity in Table I is shown in Table II. Similar to phosphorylase phosphatase activity, ~50% of the synthase activity measured in the presence of magnesium was lost during the initial low-speed centrifugation steps. In contrast, only one-third of the activity measured in the absence of magnesium was recovered in the PMS. The recovery of synthase phosphatase activity applied to the discontinuous sucrose gradient was 89% measured in the presence of added 10 mM magnesium and 192% measured in the absence of the cation. The distribution of synthase phosphatase activity among the various fractions differed markedly from that of phosphorylase phosphatase in that nearly all of the former activity was associated with the SER, whereas the latter was distributed equally between SER and RER. The data also demonstrate that >50% of the synthase phosphatase activity in the PMS is associated with ER membranes.

DISCUSSION

The most significant observations reported in this communication are that significant amounts of synthase phosphatase and phosphorylase phosphatase activities are present in ER membranes.

⁴ Curnow, R. T., and R. N. Margolis. Manuscript in preparation.

These findings assume particular importance because these enzymes serve as potential regulators of hepatic glycogen synthesis and breakdown. Their localization in ER membranes, particularly the SER, may partially explain the close morphological association between glycogen particles and SER membranes during glycogenesis and glycogenolysis in liver. This is especially true for synthase phosphatase activity which is present almost exclusively in these membranes.

Previous investigations into the subcellular localization of these enzymes (15, 29) indicated that a small amount of phosphoprotein phosphatase (i.e., "converting") activity resided in a fraction that sedimented after high-speed centrifugation. Hizukuri and Larner (15) suggested that this fraction was of microsomal origin, but no steps were taken to substantiate this initial finding. Subsequent reports have dealt mainly with characterization and purification of soluble or glycogen-associated phosphoprotein phosphatases from crude homogenates of either skeletal muscle or livers (7, 13, 16, 17) from rats and other laboratory animals.

The methods of subcellular fractionation and enzyme assay reported in this paper have permitted a clear determination of the precise localization of synthase phosphatase and phosphorylase phosphatase in the hepatocyte. Accordingly, the presence of synthase phosphatase and phosphorylase phosphatase in the soluble fraction obtained from the hepatocyte confirms previously cited reports (7, 13, 16, 17); however, the localization of both enzymes in the microsomal fraction and particularly the smooth microsomal fraction is a new and significant finding. Furthermore, our observation of a stimulation by Mg^{+2} of the soluble form of synthase phosphatase, in accordance with reports cited in the literature (17), is countered by the observed inhibition of membrane-bound synthase phosphatase by Mg^{+2} . Indeed, the membrane-bound enzyme exhibited higher specific activity in the absence of Mg^{+2} . Magnesium thus provides us a probe for delineating between soluble and membrane-associated forms of synthase phosphatase. Indeed, situations in which significant amounts of enzyme are activated by Mg^{+2} may be indicative of the presence of large amounts of soluble enzyme as opposed to membrane-associated enzyme, as is the case in the PMS fraction. Because ~32% of the intracellular Mg^{+2} in liver is associated with ER membranes (24), it is possible to speculate that Mg^{+2} could have an *in vivo* role in the regulation

of this enzyme, perhaps with respect to binding of the enzyme and/or controlling the activity once it is bound to the membrane.

The localization of both synthase phosphatase and phosphorylase phosphatase to rough and smooth microsomal fractions may provide the link between the morphological observations of increased SER during glycogen synthesis (2, 5), close association of SER membranes and glycogen particles (2, 4, 5, 23), and the well-established biochemical pathways of hepatic glycogen synthesis (18, 27). In addition, preliminary investigations in our laboratories suggest that both membrane-bound forms of synthase phosphatase and phosphorylase phosphatase are increased in amount and/or activity in fed rats. Therefore, it is conceivable that during periods of glycogen deposition both synthase phosphatase and phosphorylase phosphatase are activated, with the balance of enzymatic interconversion of glycogen synthase and glycogen phosphorylase subsequently shifted toward glycogen synthesis. Conversely, during periods of glycogen depletion, both synthase phosphatase and phosphorylase phosphatase are inactivated, with the balance then shifted toward glycogen breakdown.

In summary, we envision a situation in which SER forms a localized microenvironment within certain areas of the hepatocyte and in which the regulatory enzymes synthase phosphatase and phosphorylase phosphatase are bound to the cytoplasmic surface of the SER. It is difficult at this time to reconcile the presence of phosphorylase phosphatase activities and, to a much lesser extent, of synthase phosphatase activities, in RER, as well, with the clear-cut finding of glycogen deposits which are exclusively opposite elements of the SER. However, we feel that it is both the presence of these enzymes on SER and the location of the SER in the cytoplasm that are crucial factors. In either eventuality, it is the position of these enzymes on SER membranes and the location of the membranes in the cytoplasm that are the crucial factors. The location of the SER places the enzymes in close proximity to their substrates (glycogen synthase and glycogen phosphorylase) and also allows for hormonal and nutritional factors, as well as divalent cations, to exert regulatory influences on synthase phosphatase and phosphorylase phosphatase activities within the localized microenvironments. The overall result is the establishment of certain specified areas within the cell that provide optimal conditions for glycogen syn-

thesis. As glycogen synthesis progresses, accumulations of glycogen appear in the hepatocyte and are identified in the electron microscope as "glycogen-rich" areas of the cytosome.

The evidence presented in this communication suggests that these glycogen regions are rich both in converting enzymes (synthase phosphatase and phosphorylase phosphatase) and in the rate-limiting enzymes of glycogen synthesis and depletion (glycogen synthase and glycogen phosphorylase). Obviously, much more work is required to fully characterize the hormonal, nutritional, and metabolic factors that regulate synthase phosphatase and phosphorylase phosphatase activation, synthesis, and association with SER. However, we feel that the results presented in this paper clearly demonstrate a functional connection between SER and glycogen synthesis in the rat hepatocyte.

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