

## Acute Regulation of Hepatic Protein Phosphatases by Glucagon, Insulin, and Glucose\*

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The intravenous administration of glucagon to anesthetized rats resulted within 5 min in a 20% drop in the hepatic phosphorylase phosphatase activity, as measured in a post-mitochondrial supernatant at low dilution, but it did not affect the activity of glycogen-synthase phosphatase. On the other hand, the injection of insulin plus glucose caused increases by about 35% in both phosphatase activities. Upon subcellular fractionation these effects were recovered in the cytosol, but not in the glycogen/microsomal fraction. However, activity changes in the latter fraction were observed after recombination with the liver cytosol from a hormone-treated animal. Preincubation of the liver cytosol with modulator protein (a specific inhibitor of type-1 protein phosphatases) cancelled the activity changes induced by insulin plus glucose. No hormonal effects on hepatic protein phosphatase activities were observed when the fractions were either diluted an additional 10-fold or pretreated with trypsin.

An acute hormonal regulation of protein phosphatases could also be demonstrated in the perfused liver. When added to the perfusion medium, glucose as well as insulin increased the cytosolic protein phosphatase activities by about 25%. Their effect was additive, irrespective of the order of addition. On the other hand, the addition of glucagon and/or vasopressin resulted in a 20% drop in the phosphorylase phosphatase activity. The presence of glucagon did not interfere with the effectiveness of insulin, and vice versa. The changes in the phosphorylase phosphatase activities induced by glucagon, insulin, and glucose represented changes in the  $V_{max}$  only. We propose that the acute control of the hepatic glycogen synthase phosphatase and phosphorylase phosphatase activities is mediated by transferable, cytosolic effector(s).

One of the common criteria in the initial characterization of phosphoserine- and phosphothreonine-protein phosphatases is their sensitivity to inhibition by two heat-stable proteins, inhibitor-1 and modulator protein or inhibitor-2 (1-3). In contrast to the type-2 protein phosphatases, the Mg ATP-dependent or type-1 phosphatases are completely inhibited by these proteins. Thus, the phosphorylase phosphatases

that are associated with the microsomal fraction and with the glycogen particles in the liver have been classified as type-1 (4, 5). The considerable amount of largely latent phosphorylase phosphatase in the nuclei is also inhibitor-sensitive (6, 7). On the other hand, the liver cytosol contains both types of phosphorylase phosphatase (5, 8).

In this laboratory we have studied the characteristics and regulation of the hepatic protein phosphatases that activate glycogen synthase (see Ref. 9 for a recent review). The phosphatase activity toward glycogen synthase is essentially accounted for by type-1 phosphatases in the glycogen fraction and the cytosol, at least when the activity is determined from the rate of activation of purified hepatic synthase *b* (5). In contrast, both type-1 and type-2A (polycation-stimulated) protein phosphatases are able to dephosphorylate muscle glycogen synthase that has been phosphorylated *in vitro* (1, 5). Thus, the former assay provides a method to measure in the liver cytosol specifically the type-1 protein phosphatase activity.

Several studies have indicated that protein phosphatases do not merely serve a "passive" function to reverse the action of protein kinases, but are actually the target of hormonal control. For example, diabetes (as well as starvation of adrenalectomized rats) is associated with a progressive loss of the hepatic glycogen-bound synthase phosphatase activity (9). In addition to this long-term regulation, evidence is accumulating for an acute control of protein phosphatases by hormones. For example, inhibitor-1 is only active when phosphorylated on a threonine residue, and in skeletal muscle this phosphorylation is antagonistically controlled by insulin and adrenaline (10, 11). Since phosphorylase *a* is a potent allosteric inhibitor of the hepatic glycogen-bound synthase phosphatase (9), the latter enzyme is controlled by stimuli that alter the concentration of phosphorylase *a*. Also, the administration of adrenaline causes in skeletal muscle the phosphorylation of the glycogen-bound protein phosphatase, which is thereby translocated to the cytosolic compartment (12).

Shahed *et al.* (13) first reported an acute increase of the phosphorylase phosphatase activity in crude liver extracts after insulin treatment *in vivo*. More recently, Farkas *et al.* (14) showed antagonistic activity changes following the administration of either glucagon or insulin plus glucose. The latter effects appeared unrelated to a change in the activity of a heat-stable protein phosphatase inhibitor. The present work is concerned with the identification of the protein phosphatase(s) that are the target of these acute hormonal effects. We also report data on the underlying mechanism. Part of this work has been published in abstract form (15).

### EXPERIMENTAL PROCEDURES

**Materials and Buffers**—Glucagon and insulin were obtained from Novo Industri. Modulator protein (inhibitor-2) was prepared according to Yang *et al.* (16). Purified glycogen synthase *b* from dog liver

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was a pool of the forms termed  $b_2$  and  $b_3$  (17). The "standard buffer" used throughout this work contained 0.25 M sucrose, 50 mM imidazole (pH 7.4), 0.5 mM dithiothreitol, 4 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, and 0.5 mM benzamidine.

**Handling of Animals and Livers**—Fed male Wistar rats of about 250 g were anesthetized by an intraperitoneal injection of sodium pentobarbital (15 mg). The abdomen was opened and a thread was loosely placed around one of the two largest liver lobules. The first liver sample was taken from that lobule 30 min after the injection of pentobarbital, and the ligature was closed. Then either glucagon (20  $\mu$ g/kg body weight) or insulin (1 unit/kg) plus glucose (1 g/kg) was injected in the portal vein, and 5 min later the second biopsy was taken from the other major lobe. The liver samples were immediately homogenized in a Potter-Elvehjem tube in 1 volume of buffer and centrifuged at  $10,000 \times g$  for 10 min. The resulting post-mitochondrial supernatant ("extract") was fractionated by centrifugation (35 min at  $220,000 \times g$ ) into a cytosolic fraction and a glycogen/microsomal fraction. The latter pellet was resuspended in standard buffer to the volume of the original extract. Before the assay of protein phosphatases, samples of extract and cytosol (1 ml) were filtered in the cold through a column of Sephadex G-25 ( $5 \times 1.5$  cm), equilibrated in standard buffer. The peak of the colored eluate was pooled, and the dilution caused by the gel filtration (about 1.3-fold) was determined from the protein concentration.

As a control for the treatments *in vivo* phosphorylase *a* and *a + b* were assayed in the liver homogenates. About 15% of the experiments were discontinued because the level of phosphorylase *a* was not clearly increased (after glucagon) or decreased (after insulin plus glucose).

**Liver Perfusions**—Livers were isolated and perfused in a closed circulation system with 100 ml Krebs-Henseleit bicarbonate buffer (pH 7.4), containing washed bovine erythrocytes so as to give a final hematocrit of 0.35. The medium also contained 10 mM glucose (but only 5 mM when the role of glucose itself was under investigation). After a preperfusion of 20 min one of the larger liver lobules was ligated, cut, and immediately homogenized and fractionated as described in the previous section. After the removal of the liver sample the first agent was added to the medium, and 10 min later a second liver sample was ligated and cut. Subsequently the second agent was added, and 10 min later a third liver sample was taken. The agents were added to ensure the following initial concentrations in the perfusate: 50 milliunits/ml for insulin, 0.1  $\mu$ M for glucagon and vasopressin, and 50 mM for glucose.

**Assays**—Phosphorylase phosphatase was assayed with  $^{32}$ P-labeled muscle phosphorylase *a* as substrate (6) in fractions at a final concentration of 30%; *i.e.* 100  $\mu$ l of assay mixture contained the cell fraction (extract, cytosol, or glycogen/microsomes fraction) from 30 mg of liver. The substrate concentration was 1 mg/ml, except in the experiments shown in Table VII. The glycogen-synthase phosphatase activity was determined from the rate of activation of hepatic synthase *b* (17) by liver fractions at a final concentration of 25%. In the latter assay AMP and  $Mg^{2+}$  were included to cancel the inhibition of the glycogen-bound phosphatase by phosphorylase *a*. One unit of protein phosphatase converts 1 unit of substrate into product per minute at 25 °C. Phosphorylase was assayed as described (18). One unit of phosphorylase and of glycogen synthase converts 1  $\mu$ mol of substrate into product per min at 25 °C. Protein was measured according to Bradford (19) with reagent from Bio-Rad Laboratories and bovine serum albumin as standard.

**Computation and Expression of Data**—Results are means  $\pm$  S.E. for the indicated number (*n*) of animals. Except for the data in Table I, the activities measured after treatment are presented as a percentage of the control value (before treatment). Statistical differences were calculated on the absolute values before and after treatment, with the Student's *t* test for paired data.

## RESULTS

**Characteristics of the Effects *in Vivo***—Fig. 1 illustrates the effect of an intravenous administration of either glucagon or insulin plus glucose on hepatic protein phosphatase activities in subcellular liver fractions. The administration of glucagon resulted after 5 min in a 20% drop of the phosphorylase phosphatase activity in the post-mitochondrial supernatant (Fig. 1, *left*). The glycogen-synthase phosphatase activity was not affected by that treatment. In contrast, the administration of insulin plus glucose caused increases in the activities of

both phosphorylase phosphatase (+30%) and synthase phosphatase (+35%). The protein phosphatase activities were also measured in the cytosolic and the glycogen/microsomal fractions that were obtained by high-speed centrifugation of the liver extracts. Neither treatment did affect the protein phosphatase activities associated with the glycogen/microsomal fraction (Fig. 1, *center*). However, the effects observed in the liver extracts were essentially recovered in the cytosolic compartment (Fig. 1, *right*). The magnitude of the response (expressed as a percentage of the control value) was even more pronounced in the latter fraction. This was especially apparent for the synthase phosphatase activity, which increased by about 50% after injection of insulin plus glucose ( $p = 0.03$  versus the effect in the liver extract;  $n = 7$ ). The glucagon-induced decrease of the phosphorylase phosphatase activity was also more important in the cytosolic fraction than in the extract ( $-28\%$  versus  $-17\%$ ;  $p = 0.004$ ;  $n = 10$ ).

At first glance the data in Fig. 1 might indicate that the acute control of hepatic protein phosphatases by glucagon and by insulin plus glucose is limited to the cytosolic compartment. However, a different picture emerges when the absolute phosphatase activities are considered (Table I). Indeed, only about 7% of the synthase phosphatase activity in a liver extract was cytosolic under the current assay conditions. This means that, if the insulin-induced increase in the liver extract were solely due to a stimulation of a cytosolic phosphatase, the activity of the latter enzyme should have risen from 12 to 67 milliunits/g of liver, rather than to the actual 18 milliunits/g of liver. Furthermore, the magnitude of the increase that was originally measured in the extract was almost completely recovered upon recombination of the cytosol and the glycogen/microsomal fraction (Table I). In this respect it made no difference whether the glycogen/microsomal fraction was derived from a treated or a control rat (not illustrated). The reasoning made for synthase phosphatase applies also to the effects of insulin plus glucose, and of glucagon, on the phosphorylase phosphatase activity, although in this case the cytosolic enzymes contributed more to the overall phosphorylase phosphatase activity in the extract. Another remarkable feature of the results illustrated in Table I is that the sum of the phosphorylase phosphatase activities in the cytosol and the glycogen/microsomal fraction was appreciably larger than the activity in the original extract (and in the recombined fractions). This applied to a lesser extent to the synthase phosphatase activity.

The data presented by Farkas *et al.* (14) and the results illustrated in Fig. 1 and Table I were obtained on liver fractions assayed at unusually high concentrations. We found this to be an essential feature in order to obtain clear hormonal effects. Indeed, when the assays were performed at a 10-fold higher dilution all the effects on protein phosphatase activities became insignificant, in the cytosol (Fig. 2) as well as in liver extracts (not illustrated).

**Types of Protein Phosphatases Involved**—The modulator protein is generally used to differentiate between type-1 and type-2 phosphatases. Yet, Brautigan *et al.* (20) have reported that the catalytic subunit of the type-2A (polycation-stimulated) protein phosphatases was about 80% inhibited by micromolar concentrations of modulator. In contrast, we found that both the catalytic subunit and a type-2A holoenzyme (the polycation-stimulated phosphatase) were virtually unaffected by 8  $\mu$ M homogeneous modulator (5). In the same experiment, the type-1 catalytic subunit was 70% inhibited by 1 nM modulator (5). Thus, the modulator remains an efficient tool to differentiate between type-1 and type-2A protein phosphatases. Table II illustrates results with a con-

FIG. 1. Effects of intravenously injected glucagon (G) or insulin plus glucose (I) on the activities of synthase phosphatase and phosphorylase phosphatase in liver subcellular fractions. The protein phosphatase activities in liver samples obtained 5 min after the injections are expressed as a percentage of the corresponding control activities (before treatment). Asterisks indicate a statistically significant difference from the corresponding control value ( $p < 0.01$  or smaller).

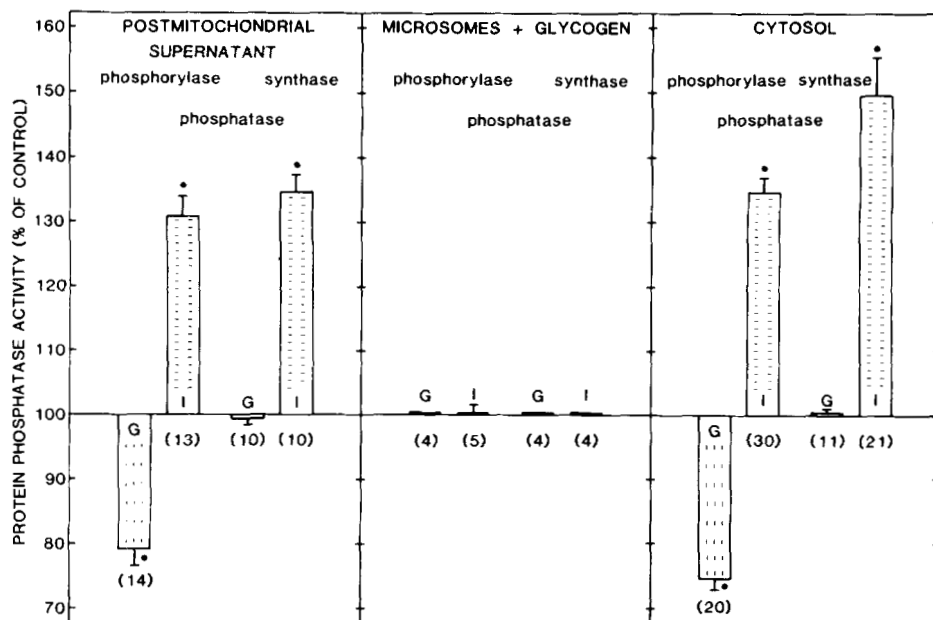


TABLE I  
Effect of glucagon and insulin plus glucose on protein phosphatase activities in isolated and recombined subcellular liver fractions

Enzyme activities were determined in the indicated cell fractions before (0 min) and 5 min after intravenous treatment. The values in parentheses are the mean differences caused by the treatment.

Treatment (n)	Cell fraction	Synthase phosphatase		Phosphorylase phosphatase	
		0 min	5 min	0 min	5 min
<i>milliunits/g liver</i>					
Insulin plus glucose (5)	Extract	169 ± 44	224 ± 57 <sup>a</sup>	6.24 ± 0.44	7.76 ± 0.59 <sup>a</sup>
	Cytosol	12 ± 3	18 ± 4 <sup>a</sup>	1.55 ± 0.23	2.15 ± 0.30 <sup>a</sup>
	Glycogen/microsomes	183 ± 73	183 ± 73 <sup>b</sup>	7.70 ± 0.53	7.70 ± 0.51 <sup>b</sup>
	Glycogen/microsomes plus cytosol	154 ± 53	205 ± 63 <sup>a</sup>	5.41 ± 0.35	6.86 ± 0.46 <sup>a</sup>
<i>units/g liver</i>					
Glucagon (4)	Extract	—	—	6.71 ± 0.54	5.42 ± 0.33 <sup>a</sup>
	Cytosol	—	—	2.28 ± 0.33	1.61 ± 0.34 <sup>a</sup>
	Glycogen/microsomes	—	—	5.85 ± 0.51	5.92 ± 0.43 <sup>b</sup>
	Glycogen/microsomes plus cytosol	—	—	6.11 ± 0.45	4.57 ± 0.29 <sup>a</sup>

<sup>a</sup> Significantly different from the corresponding control value ( $p = 0.03$  or smaller).

<sup>b</sup> Not significantly different from the corresponding control value ( $p = 0.5$  or greater).

centration of modulator that depressed the cytosolic synthase phosphatase activity to one-third. This concentration of modulator lowered the synthase phosphatase activity to the same final value when a treatment with insulin plus glucose had first increased the activity by 56%. The same was true for the phosphorylase phosphatase activity, although the adopted concentration of modulator protein decreased the latter activity by only 50%. The different sensitivity of the cytosolic synthase phosphatase and phosphorylase phosphatase activities to inhibition by modulator is in agreement with previous observations that the synthase phosphatase activity in this fraction stems entirely from a type-1 phosphatase, whereas both type-1 and type-2A phosphatases contribute to the cytosolic phosphorylase phosphatase activity. The effect of modulator on the control of the phosphorylase phosphatase activity by glucagon is more difficult to interpret. Indeed, the modulator decreased the latter activity by about 40%, both

before and after administration of glucagon. Results similar to those illustrated in Table II for cytosolic fractions were also obtained with liver extracts (not illustrated).

Incubation of type-1 phosphorylase phosphatases with trypsin results in the destruction of the inhibitory regulatory subunit(s) and yields a fully active free catalytic subunit (5). Table III shows that an incubation of the liver cytosol with trypsin cancelled the response of the phosphorylase phosphatase activity to glucagon as well as to insulin plus glucose.

*Studies on Isolated Perfused Livers*—The *in vivo* experiments did not allow us to delineate the relative importance of glucose and of insulin, nor did they exclude indirect effects through the release of extrahepatic mediators. Therefore, we turned to the isolated perfused liver. Table IV illustrates that the addition of insulin to the perfusion medium resulted in an increase of the synthase phosphatase and phosphorylase phosphatase activities by about 30%. However, subsequent

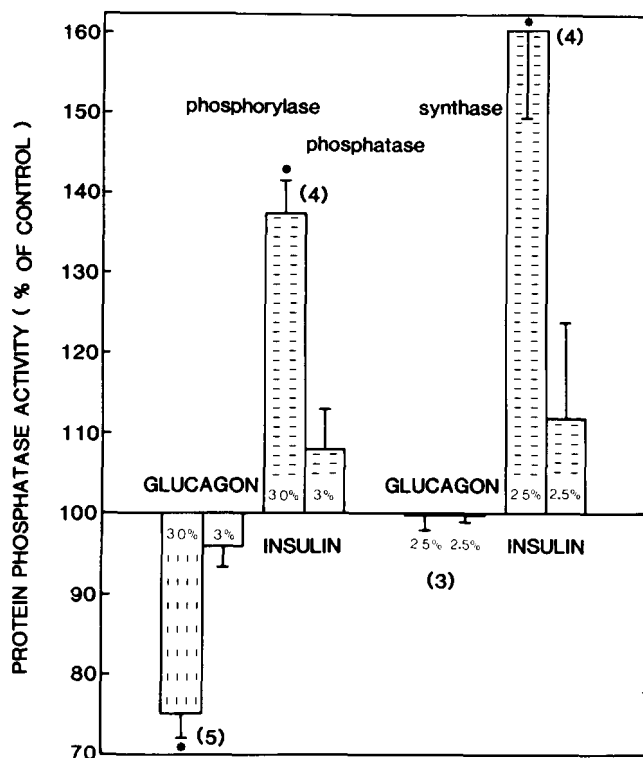


FIG. 2. Effect of dilution of the liver cytosol on the measured response of protein phosphatase activities to intravenous glucagon or insulin plus glucose. Conditions and symbols are as in the right panel of Fig. 1. The final concentration of the cytosol is indicated in each condition. Asterisks indicate a statistically significant difference from the corresponding control value ( $p < 0.01$  or smaller).

TABLE II

Effect of modulator on the response of cytosolic protein phosphatase activities to insulin plus glucose or glucagon in vivo

Before the assay of protein phosphatase activities the cytosolic fractions were incubated for 15 min at 25 °C, either as such or in the presence of 5  $\mu$ M modulator. The activities are expressed as a percentage of the activity measured at 0 min in the absence of modulator.

Time after treatment min	Modulator	Insulin plus glucose		Glucagon, phosphorylase phosphatase
		Synthase phosphatase	Phosphorylase phosphatase	
0	-	100	100	100
0	+	34 $\pm$ 5 (8)	49 $\pm$ 4 (11)	57 $\pm$ 4 (6)
5	-	156 $\pm$ 9 (8) <sup>a</sup>	135 $\pm$ 3 (11) <sup>a</sup>	73 $\pm$ 4 (6) <sup>a</sup>
5	+	36 $\pm$ 2 (8)	53 $\pm$ 2 (11) <sup>a</sup>	43 $\pm$ 2 (6) <sup>a</sup>

<sup>a</sup> Statistically significant difference from the corresponding value before treatment ( $p < 0.01$  or smaller).

addition of 50 mM glucose further increased these activities by 20–30%. Conversely, the presence of 50 mM glucose alone increased the phosphatase activities by about 30% and a supplementary increase of 20% was observed after addition of insulin.

The presence of glucagon in the perfusion medium led to a 20% drop in the phosphorylase phosphatase activity, but, again, it did not alter the synthase phosphatase activity (Table V). Subsequent addition of vasopressin did not further affect either activity. However, when vasopressin was added in the first place it did decrease the phosphorylase phosphatase activity by some 30%, and then glucagon did not induce further activity changes.

Table VI illustrates that the presence of an excess of

TABLE III

Effect of trypsin on the response of the cytosolic phosphorylase phosphatase activity to glucagon and insulin in vivo

Before the assay of the protein phosphatase activities the cytosolic fractions ( $n = 9$ ) were incubated for 20 min at 25 °C, either as such or in the presence of trypsin (1.5 mg/ml). In the latter condition the proteolysis was stopped by addition of soybean trypsin inhibitor (8 mg/ml). The subsequent phosphorylase phosphatase assays were performed at 5 °C instead of the usual 25 °C.

Treatment	Phosphorylase phosphatase activity	
	Spontaneous	After trypsin
	% $\pm$ S.E.	
Control	100	100
Glucagon	69 $\pm$ 3 <sup>a</sup>	98 $\pm$ 5
Insulin plus glucose	141 $\pm$ 8 <sup>a</sup>	110 $\pm$ 5

<sup>a</sup> Statistically significant difference from the corresponding control value ( $p < 0.001$  or smaller).

TABLE IV

Effects of sequential additions of insulin and glucose on the protein phosphatase activities in the perfused liver

See "Experimental Procedures" for details. Protein phosphatase activities were measured in the liver cytosol. The results are means  $\pm$  S.E. of six experiments. They are expressed as a percentage of the activity measured at 0 min.

First addition (at 0 min)	Second addition (at 10 min)	Phosphorylase phosphatase	Synthase phosphatase
		% of control	
Insulin		126 $\pm$ 7 <sup>a</sup>	134 $\pm$ 4 <sup>a</sup>
	Glucose	144 $\pm$ 8 <sup>a,b</sup>	164 $\pm$ 7 <sup>a,b</sup>
Glucose		130 $\pm$ 5 <sup>a</sup>	135 $\pm$ 5 <sup>a</sup>
	Insulin	151 $\pm$ 6 <sup>a,b</sup>	155 $\pm$ 9 <sup>a,b</sup>

<sup>a</sup> Significantly different from the corresponding value at 0 min ( $p = 0.01$  or smaller).

<sup>b</sup> Significantly different from the corresponding value at 10 min ( $p = 0.02$  or smaller).

TABLE V

Effects of sequential additions of glucagon and vasopressin on the protein phosphatase activities in the perfused liver

See "Experimental Procedures" for details. Protein phosphatase activities were measured in the liver cytosol. The results are means  $\pm$  S.E. of six experiments. They are expressed as a percentage of the activity measured at 0 min.

First addition (at 0 min)	Second addition (at 10 min)	Phosphorylase phosphatase	Synthase phosphatase
		% of control	
Glucagon		80 $\pm$ 3 <sup>a</sup>	99 $\pm$ 3
	Vasopressin	74 $\pm$ 4 <sup>a,b</sup>	102 $\pm$ 6 <sup>b</sup>
Vasopressin		69 $\pm$ 4 <sup>a</sup>	103 $\pm$ 3
	Glucagon	74 $\pm$ 2 <sup>a,b</sup>	111 $\pm$ 6 <sup>b</sup>

<sup>a</sup> Significantly different from the corresponding value at 0 min ( $p = 0.004$  or smaller).

<sup>b</sup> Not significantly different from the corresponding value at 10 min ( $p = 0.14$  or greater).

glucagon did not interfere at all with the effectiveness of subsequently added insulin, and vice versa. Indeed, insulin was still able to increase both phosphatase activities by about 35% after a preperfusion with glucagon. Conversely, when insulin was first allowed to increase the phosphatase activities, glucagon was still able to decrease the phosphorylase phosphatase activity by 25% without affecting the synthase phosphatase activity.

TABLE VI

Effects of sequential additions of glucagon and insulin on the protein phosphatase activities in the perfused liver

See "Experimental Procedures" for details. Protein phosphatase activities were measured in the liver cytosol. The results are means  $\pm$  S.E. of six or seven experiments. They are expressed as a percentage of the activity measured at 0 min.

First addition (at 0 min)	Second addition (at 10 min)	Phosphorylase phosphatase	Synthase phosphatase
		% of control	
Glucagon		82 $\pm$ 2 <sup>a</sup>	100 $\pm$ 1
	Insulin	118 $\pm$ 6 <sup>b</sup>	138 $\pm$ 5 <sup>b</sup>
Insulin		134 $\pm$ 2 <sup>a</sup>	129 $\pm$ 5 <sup>a</sup>
	Glucagon	104 $\pm$ 5 <sup>b</sup>	125 $\pm$ 3

<sup>a</sup> Significantly different from the corresponding value at 0 min ( $p = 0.002$  or smaller).

<sup>b</sup> Significantly different from the corresponding value at 10 min ( $p = 0.003$  or smaller).

TABLE VII

Effects of glucagon, insulin, and glucose on the activity of phosphorylase phosphatase assayed at different substrate concentrations

Liver samples were taken before and 10 min after the addition of glucagon, insulin, or glucose to the perfusate. The activity of phosphorylase phosphatase was measured in the cytosolic fraction at the indicated substrate concentrations. The results are expressed as a percentage  $\pm$  S.E. of the activities at 0 min, from which they are statistically significantly different ( $p = 0.04$  or smaller). The absolute activities at 0 min ( $n = 14$ ) were 106  $\pm$  6 and 4.1  $\pm$  0.3 units/g of protein at the high and low substrate concentration, respectively.

Phosphorylase <i>a</i>  mg/ml	Phosphorylase phosphatase activity		
	Glucagon (4)	Insulin (4)	Glucose (6)
	% of control		
3.0	86 $\pm$ 3	138 $\pm$ 8	120 $\pm$ 3
0.125	84 $\pm$ 3	138 $\pm$ 3	118 $\pm$ 6

The small magnitude of the hormonal effects precluded an exact kinetic quantification of the observed changes in terms of  $K_m$  and  $V_{max}$ . However, in a series of perfusion experiments, we have measured the activity of phosphorylase phosphatase at two widely different and non-saturating substrate concentrations (Table VII). At either substrate concentration identical activity changes were recorded, whether induced by glucagon, insulin, or glucose. This clearly shows that these agents affect specifically the  $V_{max}$ .

#### DISCUSSION

**Significance of the Acute Control of Protein Phosphatases**—We can discard the possibility that the reported effects on protein phosphatase activities are an artifact, due to a changing contribution of endogenous substrate (phosphorylase *a*, synthase *b*) to the phosphatase assays. Indeed, the effects were generally more important in the cytosolic fraction, where the interference of endogenous substrates is minimal because of their strong binding to the glycogen particles (21). For this reason the effects on the perfused liver were routinely assessed from the changes in cytosolic phosphatase activities.

The possible physiological implications of the effects that we describe are obvious. Glycogenolytic hormones could activate phosphorylase not only by increasing the activity of phosphorylase kinase, but also by decreasing phosphorylase phosphatase activity. Insulin is known to antagonize the hepatic effects of physiological glucagon concentrations (22, 23) and of  $\alpha$ -adrenergic agents (24–26). However, the hormone

as such can also activate glycogen synthase (26) and inactivate phosphorylase (27) in isolated liver cells. The latter effects could be adequately explained by the insulin-induced increase in the activities of synthase phosphatase and phosphorylase phosphatase (Table IV).

An unexpected finding in our studies on the isolated perfused liver was that a high glucose concentration did also enhance both protein phosphatase activities, independently of insulin (Table IV). Glucose can directly promote the inactivation of phosphorylase (9) and possibly also the activation of glycogen synthase (28). However, the present observations are not due to a direct effect of glucose or its metabolites, since all our results were obtained with preparations that had been filtered through Sephadex G-25 before the assay of protein phosphatase activities.

The overall magnitude of the effects on protein phosphatase activities ranged from 20 to 50% in tissue preparations at a concentration of 25–30%. However, since the effects were lost upon dilution (Fig. 2), their magnitude could be larger in the intact liver.

**Protein Phosphatases Involved in the Hormonal Effects**—In the current assay conditions the synthase phosphatase activity stems exclusively from type-1 phosphatases. This, together with our observation that the effects of insulin plus glucose are cancelled by the modulator protein (Table II) proves unambiguously that insulin and glucose activate type-1 phosphatases, apparently by increasing their  $V_{max}$  (Table VII). The effects of modulator on the phosphorylase phosphatase activity allow us to conclude furthermore that insulin and glucose did not affect the activity of type-2A protein phosphatases. Insulin was recently reported to increase the activity of pyruvate-kinase phosphatase, which has been classified as an  $Mg^{2+}$ -dependent (type-2C) protein phosphatase (29). Our observations do not detract from the latter work, since the protein phosphatases 2C and 2B (calcineurin) do not contribute to our assays (5).

A number of observations indicate that glucagon does not simply reverse the effects of insulin (and glucose). The modulator decreased, but did not abolish the effect of glucagon (Table II). Furthermore, glucagon did not have any effect on the synthase phosphatase activity. Quite strikingly, the effects of glucagon and insulin on the cytosolic phosphatases were not mutually exclusive (Table VI). Altogether, these data indicate that glucagon affects also type-2A phosphorylase phosphatases, and that there are little if any common elements in the action mechanism of insulin and glucagon. It seems likely that glucagon and vasopressin affect the same protein phosphatases, since a preperfusion with either hormone rendered the second hormone completely inefficient (Table V).

**Possible Mechanisms for the Changes in Protein Phosphatase Activities**—Although the available data do not allow us to propose a detailed mechanism for the acute regulation of protein phosphatases, we can at least exclude some mechanisms that could account for the observed changes in  $V_{max}$ . All the active type-1 protein phosphatases can be converted *in vitro* into an inactive Mg ATP-dependent form, which is a 1:1 complex of catalytic subunit and modulator protein (3, 30). Trypsin treatment of the active or activated forms in the absence of  $Mn^{2+}$  yields a stable, fully active catalytic subunit, whereas the activity of the Mg ATP-dependent enzyme is not recovered. Since in our experiments the effect of either *in vivo* treatment was cancelled by incubation with trypsin (Table III), we can exclude a conversion of the active subunit into an inactive Mg ATP-dependent form or vice versa. The same conclusion was reached in a study on the acute resto-

ration by insulin of protein phosphatase-1 activity in the diabetic liver (31).

Redistribution of type-1 phosphatases between different subcellular compartments has been proposed as a distinct mechanism for control of these enzymes in skeletal muscle (12). However, since the total catalytic activity of the cytosolic type-1 phosphatase(s)—as measured after incubation of the cytosol with trypsin—was not affected by either treatment *in vivo*, translocation of type-1 phosphatases from or to the soluble fraction does not appear to play a role. Neither were the phosphatase activities of the isolated glycogen/microsomal fraction affected (Fig. 1). Finally, we have also checked that neither treatment changed the phosphorylase phosphatase activity in the washed 10,000 × *g* pellet, which consists mainly of nuclei and mitochondria (not illustrated).

**Evidence for Transferable Cytosolic Mediator(s)**—A key observation was that the effects of treatment *in vivo* were not expressed in the isolated glycogen/microsomal fraction, and that the effects could be transferred to that fraction by simple addition of cytosol from a treated animal (Table I). Taken together with the need for high tissue concentrations (Fig. 2), these results suggest the existence of one or more hormone-controlled cytosolic effectors of protein phosphatases.

When considering the possible nature of putative mediators, we should bear in mind that all the effects were observed in gel-filtered preparations. Unless it binds very tightly to its target enzyme, an effector of at least several kilodaltons should therefore be expected. Glucagon, vasopressin, glucose, and insulin can all modify the hepatic concentration of phosphorylase *α*, which inhibits glycogen-synthase phosphatase (9, 32–34). However, this mechanism cannot account for the present effects since the assays of synthase phosphatase were performed under conditions (addition of AMP) in which the inhibition by phosphorylase *α* is cancelled (33).

The present knowledge argues against the heat-stable inhibitor-1 as a mediator. Farkas *et al.* (14) did not find any evidence for a hormone-induced change in the amount of heat-stable inhibitory activity in liver extracts. Also, boiling of the cytosolic fraction cancelled its ability to induce activity changes in a glycogen/microsomal fraction (not illustrated). Finally, a changed phosphorylation state of inhibitor-1 cannot account for the effects of both glucagon and insulin, since they were not mutually exclusive (Table VI). The aim of further work will be to identify the putative soluble effectors of protein phosphatases.

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