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REGULATION OF SYNTHASE PHOSPHATASE AND PHOSPHORYLASE PHOSPHATASE IN RAT LIVER

AGNES W.H. TAN and FRANK Q. NUTTALL

Veterans Administration Hospital, Endocrine-Metabolic Section and the Departments of Biochemistry and Medicine, University of Minnesota, Minneapolis, Minn. 55417 (U.S.A.)

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Summary

Using substrates purified from liver, the apparent K_m values of synthase phosphatase ([UDPglucose—glycogen glucosyltransferase-D]phosphohydrolase, EC 3.1.3.42) and phosphorylase phosphatase (phosphorylase *a* phosphohydrolase, EC 3.1.3.17) were found to be 0.7 and 60 units/ml respectively. The maximal velocity of phosphorylase phosphatase was more than a 100 times that of synthase phosphatase.

In adrenalectomized, fasted animals there was a complete loss of synthase phosphatase but only a slight decrease in phosphorylase phosphatase when activity was measured using endogenous substrates in a concentrated liver extract. When assayed under optimal conditions with purified substrates, both activities were present but had decreased to very low levels. Mixing experiments indicated that synthase D present in the extract of adrenalectomized fasted animals was altered such that it was no longer a substrate for synthase phosphatase from normal rats. Phosphorylase *a* substrate on the other hand was unaltered and readily converted. When glucose was given in vivo, no change in percent of synthase in the I form was seen in adrenalectomized rats but the percent of phosphorylase in the *a* form was reduced. Precipitation of protein from an extract of normal fed rats with ethanol produced a large activation of phosphorylase phosphatase activity with no corresponding increase in synthase phosphatase activity. Despite the low phosphorylase phosphatase present in extracts of adrenalectomized fasted animals, ethanol precipitation increased activity to the same high level as obtained in the normal fed rats.

Synthase phosphatase and phosphorylase phosphatase activities were also decreased in normal fasted, diabetic fed and fasted, and adrenalectomized fed rats. Both enzymes recovered in the same manner temporally after oral glucose administration to adrenalectomized, fasted rats. These results suggest an integrated regulatory mechanism for the two phosphatase.

Introduction

Glycogen synthase (UDP-glucose:glycogen 4-α-glucosyltransferase, EC 2.4.1.11.) and phosphorylase (α-1,4-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1.) exist in liver as active and inactive forms interconvertible by a kinase and a phosphatase. Physiological regulators of glycogen metabolism have frequently been shown to affect both systems simultaneously [1—4]. Glucose stimulates the conversion of synthase D to synthase I and phosphorylase *a* to phosphorylase *b*. The site of control by glucose has been suggested to be at the synthase phosphatase [2—6] and phosphorylase phosphatase [7—9] catalyzed reactions. Several laboratories have reported the isolation of partially purified phosphoprotein phosphatase which had activity on both synthase D and phosphorylase *a* [10—12]. However a partially purified protein which had activity on phosphorylase *a* but not on synthase D has also been reported [13]. At present it is not clear whether synthase phosphatase ([UDPglucose—glycogen glucosyltransferase-D] phosphohydrolase EC 3.1.3.42) and phosphorylase phosphatase (phosphorylase *a* phosphohydrolase, EC 3.1.3.17) are identical or separate enzymes.

Using a concentrated liver extract, Mersmann and Segal [14] reported that the enzyme system which converts glycogen synthase to the active form (synthase I) disappeared from livers of adrenalectomized fasted rats. In alloxan-diabetic fed or fasted rats, a lack of synthase D to I conversion has also been observed [15]. In view of the possibility that the two phosphatase enzymes are identical, it became of interest to us to determine if there were corresponding changes in phosphorylase phosphatase activity under conditions where synthase phosphatase activity has been reported to be low or have completely disappeared.

Since results reported previously were obtained in crude extracts using endogenous substrate, we were particularly interested in distinguishing whether the decrease in synthase phosphatase was the result of a decrease in the phosphatase enzyme or a modification of the synthase D such that it was not suitable as a substrate for the phosphatase. Therefore synthase phosphatase and also phosphorylase phosphatase were determined under more optimal conditions using purified liver substrates. Kinetic constants for both phosphatase reactions were measured and used to compare the results obtained with endogenous and exogenous substrates. Evidence is presented which suggests that synthase phosphatase and phosphorylase phosphatase are regulated similarly.

Materials and Methods

Male Holtzman rats of 180—250 gm were used. Bilateral adrenalectomy was performed 5—8 days before the animals were killed. Adrenalectomized animals were maintained with 0.45% NaCl in the drinking water. Animals were made diabetic by intravenous administration of alloxan (40 mg/kg) after a 24 h fast. They were used 3—5 days after treatment. Generally they lost weight and had a blood sugar level greater than 300 mg/100 ml. Insulin was administered either intravenously (2 units/kg) or intraperitoneally (6 units/kg). In experiments

where animals were fasted, the length of fasting was 24 h for normal and diabetic rats and 48 h for adrenalectomized rats.

Preparation of substrates

Liver synthase D was purified from rabbit liver using the method of Lin and Segal [16]. The specific activity obtained was between 5—10 units/mg protein and the enzyme was free of glycogen, phosphorylase *a*, phosphorylase *b*, synthase phosphatase and phosphorylase phosphatase. Liver phosphorylase *a* * was purified from livers of glucagon-treated rats or rabbits using the method of Maddaiah and Madsen [17]. Specific activities of rabbit preparations were generally lower than those of rat and varied between 30—50 units/mg protein. The enzyme was free of glycogen, synthase D, synthase I, phosphorylase phosphatase and synthase phosphatase.

Assay of synthase phosphatase and phosphorylase phosphatase using exogenous substrates

Livers were homogenized 1 : 10 (wt./vol.) in 50 mM glycylglycine, 1% glycogen, 10 mM Na_2SO_4 (pH 7.4) and extracts were obtained by centrifugation at 8000 × *g* for 10 min. In the absence of added glycogen in the extraction buffer, only small amounts of both phosphatase activities could be measured using purified substrates in extracts from normal fasted rats compared to fed rats even though endogenous substrates were converted rapidly in both. Both synthase phosphatase and phosphorylase phosphatase activities increased when the concentration of glycogen in the extraction buffer was increased and the effect was greater in extracts from fasted than from fed animals. Approximately 1% glycogen was optimal; greater than 2% was inhibitory.

Extracts were preincubated at 25° C for 40 min before use. The preincubation served the dual purpose of converting endogenous substrates of phosphatase to products and degrading to low levels metabolites such as ATP and AMP, known modifiers of synthase phosphatase [6,18] and phosphorylase phosphatase [19] respectively. Also, the concentration of phosphorylase *a*, which is inhibitory to synthase phosphatase [20], was decreased to insignificant levels.

Due to the limited supply of substrates, we generally used 1.5 units/ml synthase D in our synthase phosphatase assay. As shown later, this is close to but not at saturating concentration. For the phosphorylase phosphatase assay, a substrate concentration of 20 units/ml, much below the K_m value, was used. Both assays were found to be linear with time and enzyme concentration if less than half of added synthase D and less than one third of added phosphorylase *a* was converted. Because of the limitation in range, a few time points were taken during each assay in order to better approximate the initial rate.

The synthase phosphatase incubation mixture contained: 40 μl of 1 : 10 preincubated liver extract, 10 mM Na_2SO_4, 50 mM glycylglycine (pH 7.4) and 0.15 units of purified synthase D in a final volume of 100 μl. The reaction was started with addition of the extract. At 0, 5, 10 and 15 min of incubation at

* The two forms of phosphorylase in liver are referred to as phosphorylase *a* and phosphorylase *b*, analogous to the muscle and heart enzymes since both of them have measurable activity under proper conditions [22].

25° C, 20 μl was removed and diluted 1 : 6 with 100 mM KF, 10 mM Na_2SO_4, 10 mM EDTA pH 7.0. This diluted sample was assayed for synthase activity at pH 8.8 with and without glucose 6-phosphate using the method of Thomas et al. [21]. The inclusion of Na_2SO_4 in the phosphatase incubation mixture increased the synthase phosphatase activity slightly. The inclusion of Na_2SO_4 in the stopping reagent increased the sensitivity of the assay since sulfate was reported to inhibit synthase D and stimulate synthase I [18]. Because of this, some increase in total synthase activity was observed when a large amount of synthase D was converted to I. In the presence of KF, the total activity remained stable during incubation.

The phosphorylase phosphatase incubation mixture contained: 20 μl of 1 : 10 preincubated liver extract, 50 mM glycylglycine, pH 7.4 and 2 units of purified phosphorylase *a* in a final volume of 100 μl. At 0, 2, 4 and 6 min of incubation at 25° C 20 μl was diluted 1 : 10 with 50 mM 2(*N*-morpholino) ethane sulfonate (MES), 50 mM KF, pH 6.3. This diluted sample was assayed for phosphorylase activity with low and high substrates as described by Tan and Nuttall [22].

Glucose was determined by the method of Nelson [23] and protein by a modified Lowry procedure [24]. Bovine serum albumin was used as standard.

One unit of synthase or phosphorylase activity is defined as 1 μmol of UDP-glucose or glucose 1-phosphate respectively incorporated into glycogen per min at 30° C. One unit of phosphatase activity is defined as 1 unit of substrate converted per min at 25° C under the conditions described.

Porcine insulin and glucagon hydrochloride were obtained from Eli Lilly Company. Alloxan monohydrate, and other biochemicals of the highest grades were purchased from Sigma and Calbiochem Companies.

Results

Synthase phosphatase and phosphorylase phosphatase activities in liver extracts using endogenous substrates

Endogenous synthase D in liver extracts from normal fed rats was completely converted on incubation to synthase I (Fig. 1A). In normal fasted rats and in adrenalectomized fed rats, the synthase D to synthase I converting activity was similar. However in extracts from adrenalectomized, 48 h-fasted rats no conversion was observed (Fig. 1B), confirming the results of Mersmann and Segal [14]. Phosphorylase *a* decreased rapidly upon incubation of extracts prepared from livers of normal fed rats (Fig. 1A). This phosphorylase phosphatase activity was reduced in livers from adrenalectomized fasted rats but did not disappear completely as did the synthase phosphatase activity. Total phosphorylase was lower in fasted rats, either normal or adrenalectomized, as previously reported [22].

The conversion of synthase D to synthase I was also studied in livers of diabetic rats. The extent of synthase D to synthase I conversion varied with the nutritional state of the animals. In extracts prepared from diabetic fed animals, some activation of synthase was present, but the rate was low and the reaction stopped before 50% of the synthase D was utilized. Endogenous synthase D from diabetic rats fasted 16 h, on the other hand, was rapidly and completely

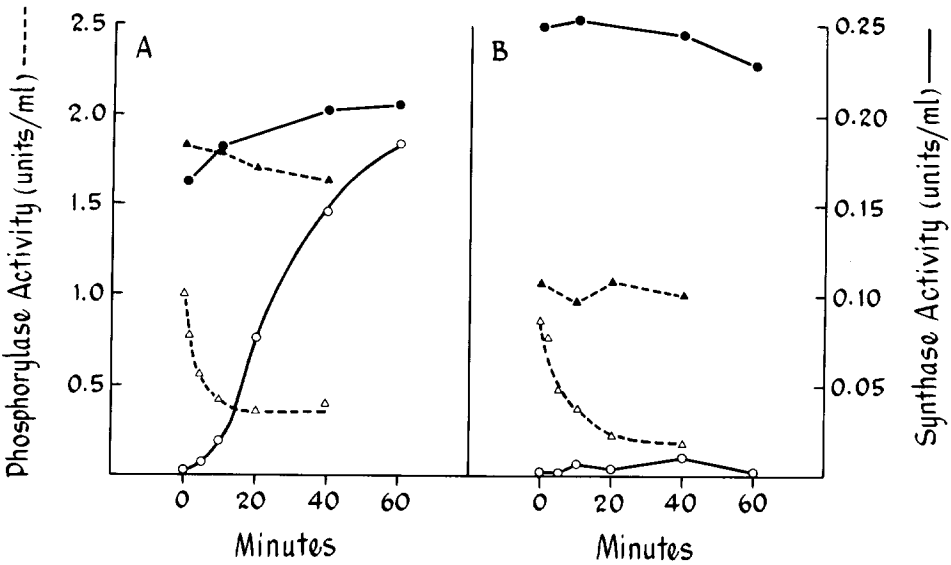

Fig. 1. Synthase phosphatase and phosphorylase phosphatase activities on endogenous substrates in liver extracts of normal fed (A) and adrenalectomized rats fasted 48 h (B). Tissue preparation and incubation conditions were modified from those of Hers et al. [2]. Liver was homogenized 1 : 3 in 50 mM glycylglycine pH 7.2 and centrifuged at 8000 × *g* for 10 min. The extract was passed through a Sephadex G—25 column (50 ml bed volume) and eluted with 50 mM glycylglycine pH 7.2. The column-treated extract (now 1 : 6) was incubated at 25°C. The extract was kept cold and the phosphatase assays were started about 30 min after killing the rats. At different time points, aliquots were diluted with 100 mM KF, 10 mM EDTA and assayed for synthase and phosphorylase activities. Symbols: synthase I activity ○; total synthase activity, ●; phosphorylase *a* activity, △; total phosphorylase activity, ▲.

converted in this system. In general, phosphorylase phosphatase activity measured simultaneously was slightly lower in diabetic fed compared to normal fed controls. Usually the phosphorylase phosphatase was so active that much of the substrate was converted during preparation of the tissue for assay. With this low level of substrate the rate was highly variable and greatly dependent on the amount of phosphorylase *a* present as can be seen from later kinetic studies.

Kinetic studies of synthase phosphatase and phosphorylase phosphatase using purified substrates

The affinity of synthase phosphatase and phosphorylase phosphatase for their respective liver substrates was determined. The apparent K_m of synthase phosphatase for purified liver synthase D was found to be 0.7 units/ml. This is significantly lower than the 3—6 units/ml reported for the dog liver phosphatase when muscle synthase D substrate was used [25]. In the present study the maximal velocity of the enzyme was about 1.5 units/gm, wet weight. Phosphorylase phosphatase had a much higher K_m for liver phosphorylase, approximately 60 units/ml and the V was also much higher, 225 units/gm, wet weight. This explains the difficulty in estimating phosphorylase phosphatase activity in a concentrated tissue extract when the endogenous substrate concentration was between 2—4 units/ml.

Changes in synthase phosphatase and phosphorylase phosphatase activities using exogenous substrates in liver of rats under different physiological conditions

Results of determinations using the assay conditions described are shown in Table I. Synthase phosphatase activity was significantly higher in normal fed than in fasted rats. Some synthase phosphatase was present in adrenalectomized rats fasted for 48 h, but the activity was greatly reduced. In adrenalectomized fed rats the activity was similar to that of the normal fasted rat. Synthase phosphatase activity was low in diabetic rats. However, in diabetic fed rats the activity was less than in diabetic rats fasted overnight. Insulin given for 2 h before killing brought about a partial recovery of activity whereas insulin given for 15 min was ineffective. Phosphorylase phosphatase activity in livers of these same rats changed in a manner similar to the synthase phosphatase, but the differences frequently were not proportional.

The time course of recovery of synthase phosphatase activity in adrenalectomized fasted rats gavaged with glucose was followed and compared with that of phosphorylase phosphatase activity (Fig. 2). Synthase phosphatase activity was not clearly increased until after 2 h. Results from another experiment indicated that in 4 h the activity reached the level of the fed rats. It is striking that the recovery of the phosphorylase phosphatase followed closely that of synthase phosphatase, suggesting that both enzymes were under the same set of physiological controls.

Nature of synthase D in livers of adrenalectomized fasted and diabetic fed rats

The low synthase phosphatase activity in livers from adrenalectomized fasted and diabetic fed rats could account for the lack of and decreased activation respectively of endogenous synthase noted in crude extracts (Fig. 1) but it does not rule out the possibility that the substrate synthase D also was modified. To investigate this possibility mixing experiments were done. When an extract from a normal fed rat was mixed with an extract from an adrenalectomized

TABLE I

SYNTHASE PHOSPHATASE AND PHOSPHORYLASE PHOSPHATASE ACTIVITIES IN LIVERS OF RATS UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS

Values represent mean ± S.E.

Rats (number in parentheses)	Blood glucose (mg %)	Phosphorylase phosphatase (units/g)	Synthase phosphatase (units/g)
Normal fed (19)	101 ± 7	59 ± 3	0.89 ± 0.04
Normal fasted 24 h (13)	77 ± 6 [a]	43 ± 4 [a]	0.47 ± 0.04 [a]
Adrenalectomized fed (3)	98 ± 5	35 ± 2 [a,b]	0.54 ± 0.08 [a,b]
Adrenalectomized fasted 48 h (11)	64 ± 4 [a]	14 ± 1 [a]	0.07 ± 0.01 [a]
Diabetic fed (10)	482 ± 30	30 ± 4 [a,b]	0.17 ± 0.03 [a,b]
Diabetic fasted 24 h (3)	307 ± 39 [c]	42 ± 6 [a]	0.33 ± 0.10 [a]
Diabetic fed + insulin 2 h (5)	162 ± 48 [c]	47 ± 2 [c]	0.38 ∓ 0.03 [c]

[a] $P < 0.05$ compared to normal fed.
[b] $P < 0.01$ compared to adrenalectomized fasted.
[c] $P < 0.05$ compared to diabetic fed.

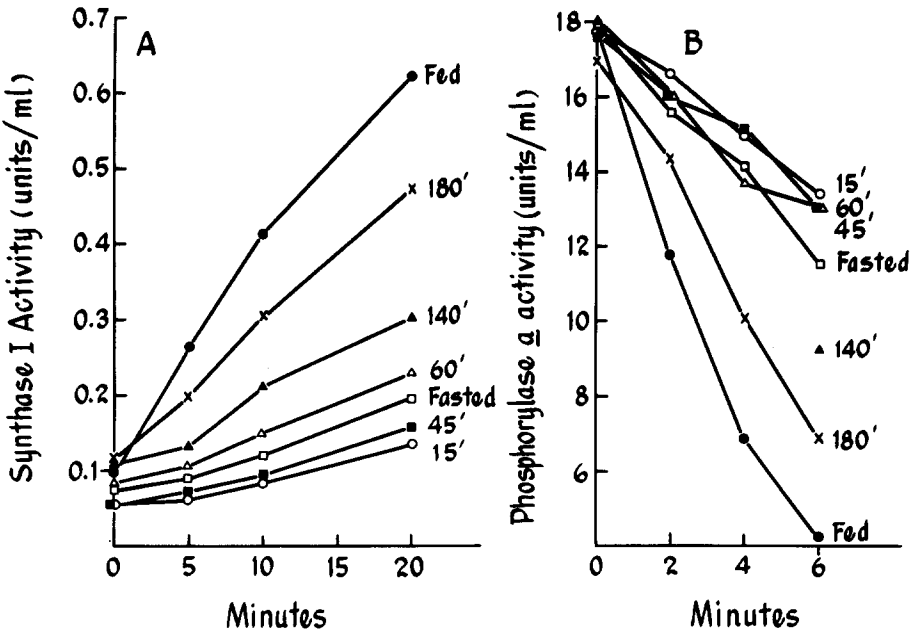

Fig. 2. Synthase phosphatase and phosphorylase phosphatase activities on exogenous substrates in adrenalectomized fasted rats gavaged with glucose (4 mg/kg). They were killed at the times indicated and synthase phosphatase and phosphorylase phosphatase activities in liver were measured as described in Materials and Methods. Results from adrenalectomized fasted and adrenalectomized fed rats gavaged with saline are given for comparison. A Synthase phosphatase activity. B. Phosphorylase phosphatase activity. Earlier phosphatase time points for the 140 min sample were not available due to a technical error.

fasted rat, the rate of activation was approximately that obtained when the extract from a normal fed rat was similarly diluted with buffer, indicating the absence of an inhibitor in adrenalectomized-fasted rat liver extract (Fig. 3A). However not all of the available substrate was converted as was the case in the extract of the normal fed rat. In order to differentiate more clearly whether the synthase D from the adrenalectomized fasted rat could be converted to synthase I by the phosphatase, an extract from a normal fed rat was first preincubated 60 min to allow complete conversion of the endogenous synthase D to synthase I. The synthase phosphatase in the preincubated extract readily utilized added purified synthase D (Fig. 3B). When the same preincubated extract was mixed with extract from an adrenalectomized fasted rat, however, no conversion of synthase D to I was seen even though a large amount of synthase D was available. An inhibitor was not generated during the preincubation since conversion of synthase D occurred when preincubated normal extract was mixed with non-incubated normal extract.

After prolonged incubation of a mixture of extracts from normal fed and adrenalectomized fasted rats (Fig. 3A), slightly more synthase I was obtained than could be accounted for from normal extract. This could perhaps be explained by the presence of a labile activator in the extract from normal rat which was absent in the extract of adrenalectomized fasted rat. The activator could have changed synthase D to a more suitable form for the phosphatase.

Mixing experiments of extracts from normal fed and diabetic fed animals

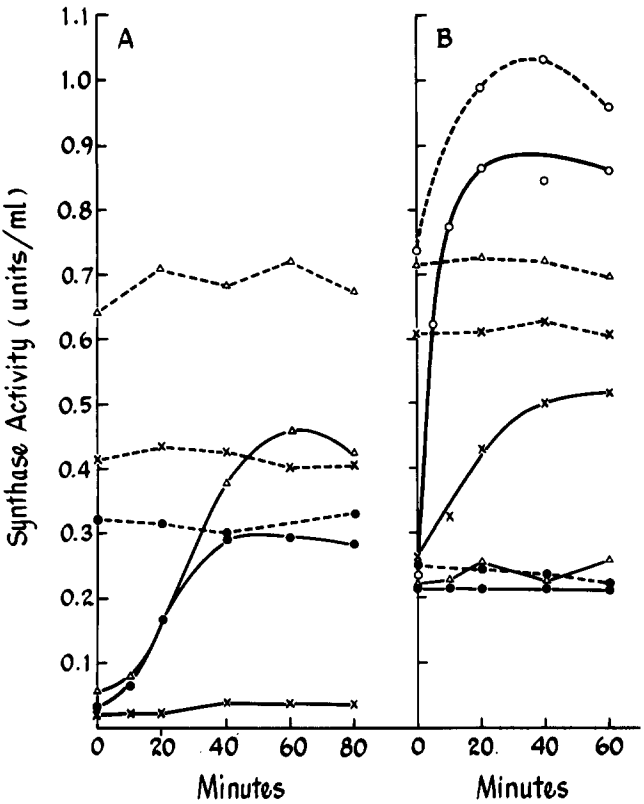

Fig. 3. Incubation of liver extract from normal fed rats with extract from adrenalectomized-fasted rats. Livers from normal fed rat (Nfd) and adrenalectomized rat fasted for 48 h (Aft) were homogenized 1 : 4 in 50 mM glycylglycine, 10 mM Na_2SO_4, 1% glycogen (pH 7.4) and centrifuged at 8000 × *g* for 10 min. Extracts were incubated for different times at 25°C. A •, 90 µl Nfd extract + 90 µl buffer (same as homogenizing bufer); ×, 90 µl Aft extract + 90 µl buffer; △, 90 µl Nfd extract + 90 µl Aft extract. B. A portion of 1 : 4 extract from normal fed liver was first preincubated at 25°C for 60 minutes. To 60 µl of this preincubated Nfd extract was added the following and incubation was continued for 60 minutes: •, 120 µl buffer; △, 120 µl Aft extract; × 120 µl Nfd extract; ○, 0.1 units of purified synthase D in 20 µl + 100 µl buffer. Aliquots were taken at different time points and diluted 1 : 4 with 100 mM KF 10 mM EDTA 10 mM Na_2SO_4 and assayed for synthase activity: solid lines without and dotted lines with glucose 6-phosphate.

gave qualitatively the same results, again suggesting the presence of a synthase D which had been modified such that it was a poor substrate for the phosphatase.

In vivo response of adrenalectomized fed and fasted rats to acute glucose administration

In intact and adrenalectomized fed rats, an intravenous dose of glucose brought about an increase in liver synthase I in less than 10 min (Table II). In other experiments using intact fasted rats, an increase was also observed. Synthase I went from 20% of total activity to 45% of total activity. However in adrenalectomized fasted rats, there was only a slight and not statistically significant increase in synthase I. The low synthase phosphatase activity measured in vitro and the presence of modified synthase D therefore could explain

TABLE II

ACUTE GLUCOSE RESPONSE OF SYNTHASE AND PHOSPHORYLASE ACTIVITIES IN ADRENALECTOMIZED FED AND FASTED ANIMALS

Animals were anesthetized with seconal (40 mg/kg) and were injected with either saline or glucose (500 mg/kg) via a tail vein. 10 min later they were killed and blood drained for glucose determination. One lobe of liver was immediately homogenized 1 : 13 in 100 mM KF, 10 mM EDTA, pH 7.0. After centrifugation at 8000 × *g*, 10 min, the extract obtained was assayed directly for synthase activity. Part of the extract was diluted further 1 : 3 in 50 mM MES/50 mM KF, pH 6.3 and assayed for phosphorylase activity. Number of animals used follows Treatment in parentheses. Values represent means ± S.E.

Condition	Treatment	Blood glucose (mg/100 ml)	Synthase		Phosphorylase	
			Total units/g	% I	Total units/g	% *a*
Normal fed	Saline (7)	95 ± 7	1.56 ± 0.16	2.0 ± 0.2	31.9 ± 2.5	51.5 ± 3.7
	Glucose (8)	153 ± 5	1.46 ± 0.11	12.9* ± 2.0	31.3 ± 1.6	40.9* ± 2.7
Adrenalectomized fed	Saline (6)	57 ± 4	1.30 ± 0.17	4.2 ± 0.7	32.8 ± 1.8	58.9 ± 1.7
	Glucose (5)	164 ± 5	1.39 ± 0.22	18.2* ± 2.7	30.6 ± 1.9	47.2* ± 2.9
Adrenalectomized fasted	Saline (6)	41 ± 3	1.16 ± 0.06	1.8 ± 0.2	22.3 ± 0.7	59.9 ± 1.1
	Glucose (6)	157 ± 8	1.15 ± 0.05	2.5 ± 0.3	23.7 ± 1.3	51.0** ± 4.2

* $P < 0.01$, compared to the saline controls.
** $P < 0.05$, compared to the saline controls.

this observation in vivo. On glucose administration the percentage of phosphorylase *a* decreased in all three groups. A response in the phosphorylase system was observed in the adrenalectomized fasted rat despite greatly reduced phosphorylase phosphatase activity measured.

Synthase phosphatase and phosphorylase phosphatase activities in liver extracts of normal fed and adrenalectomized fasted rats after treatment with ethanol

It has been reported that ethanol precipitation of proteins from an extract of liver from normal rats results in a 30 fold increase in phosphorylase phosphatase activity [26]. Since phosphorylase phosphatase was considerably reduced in an extract from adrenalectomized fasted animals we decided to determine if ethanol precipitation would also increase the activity in these animals. In liver extracts prepared in imidazole and EDTA buffer as used by Brandt et al. [26] much lower activity was found prior to ethanol precipitation in both normal fed and adrenalectomized fasted rats than when extracts were prepared as described in the present report (Table III). The difference can be explained, at least in part, by the absence in the extraction buffer of glycogen and the presence of EDTA, which we found to be inhibitory. Also there was some loss of synthase phosphatase and phosphorylase phosphatase activity in the dialysis step used. Following ethanol precipitation phosphorylase phosphatase activity was considerably increased and reached the same high level in both the normal fed and adrenalectomized fasted animals. Synthase phosphatase activity in extracts from normal fed rats on the other hand was not activated after ethanol precipitation. It was very low in adrenalectomized fasted rats and increased slightly after ethanol precipitation. Thus ethanol produced a striking dissociation in phosphorylase phosphatase and synthase phosphatase activity.

TABLE III

SYNTHASE PHOSPHATASE AND PHOSPHORYLASE PHOSPHATASE ACTIVITIES IN LIVER EXTRACTS OF NORMAL FED AND ADRENALECTOMIZED FASTED RATS AFTER TREATMENT WITH ETHANOL

From each liver, one lobe was homogenized in 50 mM glycylglycine/1% glycogen/10 mM Na_2SO_4 pH 7.4, (glycogen buffer) and assayed as described in Materials and Methods; another lobe was homogenized in 50 mM imidazole/5 mM EDTA/0.5 mM dithiothreitol pH 7.45, (IED buffer) plus 0.1 M NaCl and treated with ammonium sulfate and ethanol as described [26]. The ethanol precipitate was extracted three times with IED buffer by continuous stirring for 30 min. The extracts in IED buffer and the combined supernatants from the ethanol precipitates were dialysed for 4 h at 4°C against IED buffer. Samples were assayed the same day for synthase phosphatase and phosphorylase phosphatase activity as described in Methods. Values represent mean ± S.E.

	Normal fed (4)		Adrenalectomized fasted (4)	
	Phosphorylase phosphatase (units/g)	Synthase phosphatase (units/g)	Phosphorylase phosphatase (units/g)	Synthase phosphatase (units/g)
Extract (glycogen)	62 ± 7	0.88 ± 0.04	15 ± 3	0.06 ± 0.01
Extract (IED)	17 ± 1	0.50 ± 0.02	15 ± 2	0.06 ± 0.01
Ethanol ppt.	410 ± 32	0.20 ± 0.01	452 ± 37	0.24 ± 0.02

Discussion

Assay conditions have been established capable of detecting small changes in synthase phosphatase and phosphorylase phosphatase activity. We used as index of phosphatase activity a change in the activity characteristics of the substrates. The use of ^{32}P release from labeled substrate could be misleading since over-phosphorylation of protein substrates has been reported and under certain conditions a lack of correlation has been found between activity characteristics and state of phosphorylation of the substrate enzymes [27]. Investigators interested in liver phosphatase often have used muscle phosphorylase *a* [28,29] and muscle synthase D as substrate [25]. Even though there is cross-reactivity between the muscle and liver enzymes, only liver substrates were used since subtle differences in substrates might influence the results obtained.

In extracts from adrenalectomized fasted rats, phosphorylase phosphatase activity on endogenous phosphorylase *a* was only moderately decreased in contrast to the complete loss of synthase phosphatase activity on endogenous synthase D. This was initially considered to be indicative of two separate phosphatase enzymes. However, using exogenous substrates, both phosphatase activities were measurable even though they were greatly reduced. Under different physiological conditions changes in phosphorylase phosphatase were qualitatively similar but the amount of decrease was often not parallel to the decrease in synthase phosphatase. It was therefore not possible with these studies to distinguish whether they were the same or different enzymes. However, the almost identical time course of recovery of both phosphatases after oral glucose administration to adrenalectomized fasted rats suggested an integrated regulatory mechanism for these enzymes.

Our results indicated that synthase D in extracts from adrenalectomized fasted and diabetic fed rats was a poor substrate for synthase phosphatase. Synthase D purified from livers of adrenalectomized fasted rats by the procedure of Sanada and Segal [30], although catalytically active itself, also was not a substrate for synthase phosphatase (unpublished observations). In certain preparations of synthase D from normal rat liver, the enzyme obtained was also not a good substrate for the phosphatase. This suggests that modification of protein conformation such as occurs during purification may affect its substrate characteristics more readily than its catalytic characteristics. Alteration in protein phosphatase activity by changes in substrate protein has also been suggested by other workers [31—33].

Synthase phosphatase activity was reduced in normal fasted, adrenalectomized fed and alloxan-diabetic fed and fasted animals but in no case was the reduction as great as that seen in the adrenalectomized fasted animals. These are all situations where a low circulating insulin concentration is expected but it should be lowest in the diabetic animals. It has been considered that the effects of glucocorticoid on glycogen synthesis and the glycogen synthase system are mediated indirectly through an effect on circulating insulin concentration [35]. The present studies, however, suggest a more direct role of glucocorticoids in the maintenance of synthase phosphatase activity.

Alloxan-diabetic rats fasted overnight generally had a lower blood sugar and higher synthase phosphatase activities than diabetic fed rats. This was a consistent finding but the reason for this difference is not clear. These results are slightly different from those reported by Gold [15] who described a loss of the synthase activating system in diabetic animals, either fed or fasted. However, neither was he able to demonstrate activation of synthase in livers of normal fasted rats. Others have reported reduced but significant activation of synthase in livers of alloxan-diabetic fed rats [35] and pancreatomized fasted dogs [25].

Little or no increase in synthase I was found when adrenalectomized fasted rats were given an acute dose of glucose in vivo. Stimulation of synthase D to synthase I conversion has been reported to be reduced [34] or completely absent [3] when livers from adrenalectomized fasted rats were perfused with glucose. Nichols and Goldberg [35] observed a three-fold decrease in liver synthase phosphatase activity in crude extracts from adrenalectomized fasted rats but a rapid increase in synthase I still occurred after glucose administration. The discrepancies could perhaps be explained by the length of fasting used. In the latter study and in the perfusion experiments of Miller et al. [34], adrenalectomized animals were fasted only 12—14 hours. In the studies by Glinsmann et al. [3] and in the present report a 48—hour period of fasting was used. In view of the reduced synthase phosphatase activity and the reduced concentration of synthase D in substrate form found in diabetic fed animals, it is interesting that the synthase system has been reported not to respond to glucose when livers from these diabetic animals were perfused in vitro [34].

Glucose stimulation of phosphorylase *a* — phosphorylase *b* conversion in vivo was little changed in the adrenalectomized fasted animals when compared to the normal fed controls even though phosphorylase phosphatase activity was greatly reduced. The concentration of phosphorylase phosphatase was therefore not limiting under this physiological condition. In the perfused liver,

a lack of response of phosphorylase to glucose was reported [34]. The implication of this is not clear.

Despite the lower amount of synthase phosphatase in normal fasted and adrenalectomized fed rat, the response to glucose in vivo was normal. This indicates that glycogen synthesis generally is not controlled by the total amount of synthase phosphatase present. Only in the case of the adrenalectomized fasted rat was synthase phosphatase found limiting. Several potentially important physiological regulatory mechanisms of phosphatase activities by glucose have already been described. Glucose may decrease phosphorylase phosphatase activity by direct interaction with its substrate phosphorylase *a* [36,37]. Glucose and other stimuli may activate the synthase phosphatase system by either decreasing the effective concentration of the inhibitor phosphorylase *a* [20] or reversing the inhibition of ATP [6].

The activation of phosphorylase phosphatase in extracts from adrenalectomized fasted by ethanol precipitation to the level seen in normal fed animals was unexpected. In normal fed animals, Brandt et al. [38] have attributed the increase in phosphorylase phosphatase activity to a dissociation of an inhibitor protein from a catalytic subunit. If this interpretation is correct it would imply that in the adrenalectomized fasted rat, a modification of the inhibitory rather than the catalytic portion of the protein has occurred. After ethanol precipitation synthase phosphatase activity was not activated. This is at variance with the conclusion of the above investigators [11]. Since their data and experimental details regarding synthase phosphatase activity have not been published it is difficult to compare the results with those in the present study.

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