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EVIDENCE FOR THE NON-IDENTITY OF PROTEINS HAVING SYNTHASE PHOSPHATASE, PHOSPHORYLASE PHOSPHATASE AND HISTONE PHOSPHATASE ACTIVITY IN RAT LIVER

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Summary

Synthase phosphatase, phosphorylase phosphatase and histone phosphatase in rat liver were measured using as substrates purified liver synthase D, phosphorylase *a* and ^{32}P-labelled phosphorylated f1 histone, respectively. The three phosphatase enzymes had different sedimentation characteristics. Both synthase phosphatase and phosphorylase phosphatase were found to sediment with the microsomal fraction under our experimental conditions. Only 10% of histone phosphatase was in this fraction; the majority was in the cytosol. No change in histone phosphatase was observed in the adrenalectomized fasted rat whereas synthase phosphatase and phosphorylase phosphatase activities were decreased 5—10-fold. Fractionation of liver extract with ethanol produced a dissociation of the three phosphatase activities. When a partially purified fraction was put on a DEAE-cellulose column, synthase phosphatase and phosphorylase phosphatase both exhibited broad elution profiles but their activity peaks did not coincide. Histone phosphatase eluted as a single discrete peak. When the supernatant of $CaCl_2$-treated microsomal fraction was put on a Sepharose 4B column, the majority of synthase phosphatase was found to elute with the larger molecular weight proteins whereas the majority of phosphorylase phosphatase eluted with the smaller species. Histone phosphatase migrated as a single peak and was of intermediate size. Synthase phosphatase was inhibited by phosphorylase *a* ($K_i < 1$ unit/ml) and phosphorylase phosphatase by synthase D ($K_i \approx 2$ units/ml). The inhibition of synthase phosphatase by phosphorylase *a* was kinetically non-competitive with substrate. Histone phosphatase activity was not inhibited by synthase D or by phosphorylase *a*. The above results suggest that different proteins are involved in the dephosphorylation of synthase D, phosphorylase *a* and histone in the cell.

Introduction

Glycogen synthase and phosphorylase exist as active and inactive forms interconvertible by kinase and phosphatase enzymes. A synthase phosphatase ([UDP glucose—glycogen glucosyltransferase-D]phosphohydrolase, EC 3.1.3.42) has been isolated from muscle by Kato and Bishop [1] which also dephosphorylated histone and co-purified with histone phosphatase. In addition, it was shown to be active on non-activated phosphorylase kinase [2] and phosphorylase *a* [3]. This lack of substrate specificity was also exhibited by protein phosphatases purified from heart [4] and from liver [5]. A small molecular weight phosphorylase phosphatase has been obtained from liver and was reported to have synthase phosphatase activity as well [6]. These studies led to the concept that a single non-specific protein carried out the dephosphorylation of multiple phosphoproteins in the cell.

The lack of specificity of the phosphatase, however, is not a universal finding. Fisher and coworkers [7] purified phosphorylase phosphatase (phosphorylase *a* phosphohydrolase, EC 3.1.3.17) from muscle and reported that phosphorylase *a*, phosphopeptides derived from this molecule and the inhibitor component of muscle troponin were the only substrates for the enzyme. It did not act on other phosphoproteins such as histone, protamines, phosphorylase kinase, casein and phosvitin. Goris et al. [8] indicated that their partially purified phosphorylase phosphatase from liver and adrenal cortex did not activate synthase D. We have observed a dissociation of synthase phosphatase activity from phosphorylase phosphatase activity when liver extracts were treated with ethanol [9].

In this report we describe physiological changes, elution profile, sedimentation and fractionation characteristics of synthase phosphatase, phosphorylase phosphatase and histone phosphatase. These results, together with kinetic data, indicate that different sets of enzymes are involved in the dephosphorylation of synthase, phosphorylase and histone in rat liver. An abstract of this work has been published previously [10].

Materials and Methods

Male Holtzman rats 180—250 g were used. Control animals were allowed to feed ad lib. Adrenalectomized animals were fasted 48 h and were maintained with 0.45% NaCl in the drinking water. Bilateral adrenalectomy was performed 5—8 days before the animals were used.

Preparation of substrates for the phosphatases

Synthase D was purified from rabbit liver using the method of Lin and Segal [11]. The specific activity was between 4—10 units/mg protein and the enzyme was free of glycogen, phosphorylase *a*, phosphorylase *b*, synthase phosphatase and phosphorylase phosphatase.

Phosphorylase *a* was purified from livers of glucagon-treated rabbits using the method of Maddaiah and Madsen [12]. The specific activity was between 30—50 units/mg protein. The preparation was free of glycogen, synthase D, synthase I, phosphorylase phosphatase and synthase phosphatase.

^{32}P-Labelled f1 phosphohistone was obtained by phosphorylation of f1 histone with cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP essentially as described by Meisler and Langan [13]. The f1 histone was isolated from calf thymus histone (Sigma type II A) by the method of Johns [14]. Phosphorylation increased the alkaline labile phosphates on f1 histone from 10 to 45 μmol/g.

Conditions for phosphatase assays

The synthase phosphatase incubation mixture contained 50 mM glycylglycine, pH 7.4, 10 mM Na_2SO_4, 1.5 units/ml of purified synthase D and the phosphatase enzyme in a final volume of 100 μl. At different times of incubation at 25°C, 20 μl were removed and diluted 1 : 6 with 100 mM KF/10 mM Na_2SO_4/10 mM EDTA, pH 7.0. The diluted samples were assayed for synthase activity at pH 8.8 with and without glucose 6-phosphate using the method of Thomas et al. [15].

The phosphorylase phosphatase incubation mixture contained 50 mM glycylglycine, pH 7.4, 20 units/ml of purified phosphorylase *a* and the phosphatase enzyme in a final volume of 100 μl. At different times of incubation at 25°C, 20 μl were diluted 1 : 10 with 50 mM KF/50 mM Mes (2[*N*-morpholino] ethanesulfonate), pH 6.3. The diluted samples were assayed for phosphorylase activity with low and high substrates as described by Tan and Nuttall [16].

Details of the above phosphatase assays were discussed elsewhere [9].

The histone phosphatase incubation mixture contained 50 mM glycylglycine, pH 7.4, 200 mM NaCl, 1.2 mg/ml of f1 [^{32}P]phosphohistone and the phosphatase enzyme in a final volume of 100 μl. At different times of incubation at 25°C, 15 μl was added to 40 μl of 0.1 M silicotungstic acid in 0.1 M H_2SO_4. To ensure complete precipitation of the labelled protein substrate in the sample, 30 μl of bovine serum albumin (10 mg/ml) was added. After removal of the precipitate by centrifugation, 50 μl of the supernatant containing the released inorganic [^{32}P]phosphate was counted.

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 (for synthase phosphatase and phosphorylase phosphatase) or 1 nmol of inorganic phosphate released (for histone phosphatase) per min at 25°C under the conditions described.

Modified phosphatase assays for fractions from columns

To increase sensitivity and to conserve purified substrates, the above assays were modified so that a large number of samples could be assayed. Synthase phosphatase was determined in the presence of 0.4% glycogen and 5 mM glucose 6-phosphate; phosphorylase phosphatase in the presence of 0.4% glycogen and 25 mM glucose. The total volume of the phosphatase assay for these samples was 20 μl. A single time-point was used, either 5, 10 or 20 min, depending on the activity of the sample added to the column.

Results

Sedimentation characteristics of synthase phosphatase, phosphorylase phosphatase and histone phosphatase in rat liver

The amount of phosphatase enzymes in the various fractions obtained from

TABLE I

SEDIMENTATION CHARACTERISTICS OF SYNTHASE PHOSPHATASE, PHOSPHORYLASE PHOSPHATASE AND HISTONE PHOSPHATASE ACTIVITIES IN RAT LIVER

Liver from a normal fed rat was homogenized 1 : 5 in 50 mM glycylglycine/50 mM mercaptoethanol/1% glycogen (Sigma Type III), pH 7.2. All samples were preincubated 20 min at 25°C before assay. Results are averages from a number of experiments (in parentheses).

	Synthase phosphatase (3)		Phosphorylase phosphatase (4)		Histone phosphatase (4)	
	Total units	% recovery	Total units	% recovery	Total units	% recovery
8 000 × *g* 10 min supernatant	6.8	100	410	100	882	100
44 000 × *g* 40 min supernatant	5.4	79	425	103	776	88
44 000 × *g* 40 min precipitate	0.4	6	4	1	26	3
105 000 × *g* 60 min supernatant	0.6	9	7	2	679	77
105 000 × *g* 60 min precipitate	4.7	69	247	60	123	14

differential centrifugation was measured and results are shown in Table I. Samples were preincubated before assay. This served the dual purpose of converting endogenous substrates to products and degrading to low levels metabolites such as ATP and AMP, known modifiers of synthase phosphatase [17,18] and phosphorylase phosphatase [19] respectively. Also the concentration of phosphorylase *a* which was inhibitory to synthase phosphatase [20] was decreased to insignificant levels. Sephadex G-25 column treatment was found to be unnecessary after this preincubation step. Synthase phosphatase and phosphorylase phosphatase had similar distribution patterns. Both were found to sediment with the microsomal fraction (105 000 × *g* precipitate). Only a small amount of activity was present in the glycogen pellet (44 000 × *g* precipitate) and in the cytosol (105 000 × *g* supernatant). The majority of histone phosphatase was found in the cytosol as has been reported previously [13]. Only a small fraction was present in the glycogen pellet. About 10—15% of activity was measurable in the microsomal fraction.

Changes in phosphatase activities after adrenalectomy and fasting

We have previously reported that both glucocorticoid and insulin had a role in the control of synthase phosphatase and phosphorylase phosphatase [9]. Both enzymes were reduced to very low levels when rats were adrenalectomized and fasted for 48 h. These studies were extended to include determination of histone phosphatase activity in these animals (Table II). Under conditions in which there was 5—10-fold decrease in synthase phosphatase and phosphorylase phosphatase, no change in histone phosphatase was observed either in the extract or the microsomal fraction. It is apparent that histone phosphatase is not under the same hormonal and metabolic control as the other two phosphatases.

Effect of ethanol fractionation on phosphatase activities

Enzymes associated with glycogen can be purified by careful precipitation with ethanol. Using a more severe ethanol treatment, Brandt et al. noted a large

TABLE II

SYNTHASE PHOSPHATASE, PHOSPHORYLASE PHOSPHATASE AND HISTONE PHOSPHATASE ACTIVITIES IN NORMAL FED AND ADRENALECTOMIZED FASTED RATS

Extracts were made in 50 mM glycylglycine/10 mM Na_2SO_4/1% glycogen, pH 7.2, and aliquots were preincubated 40 min at 25°C and assayed for phosphatase activities. Part of the extract was centrifuged at 105 000 × *g* for 60 min. The microsomal layer, above the glycogen pellet, was resuspended in homogenizing buffer and assayed for phosphatase activities. Numbers of animals used are in parentheses.

	Treatment	Synthase phosphatase (units/g)	Phosphorylase phosphatase (units/g)	Histone phosphatase (units/g)
Extract	Normal fed	0.88 ± 0.04 (4)	62 ± 7 (4)	104 ± 6 (6)
	Adrenalectomized fasted	0.06 ± 0.01 (4)	15 ± 3 (4)	116 ± 4 (6)
Microsomes	Normal fed	0.60 ± 0.03 (2)	49 ± 2 (2)	9 ± 1 (6)
	Adrenalectomized	0.02 ± 0.01 (2)	10 ± 2 (2)	9 ± 0.2 (6)

activation of phosphorylase phosphatase activity [21]. The effect of ethanol fractionation on synthase phosphatase, phosphorylase phosphatase and histone phosphatase was determined and the results are shown in Table III. Precipitation of proteins in a liver extract with 30% ethanol at 0°C produced a fraction which contained 75—85% of synthase phosphatase and phosphorylase phosphatase but only 30% of histone phosphatase activity. Using the conditions of Brandt et al. [21], i.e. ammonium sulfate precipitation followed by 80% ethanol at room temperature, a large amount of protein was irreversibly denatured.

TABLE III

SYNTHASE PHOSPHATASE, PHOSPHORYLASE PHOSPHATASE AND HISTONE PHOSPHATASE ACTIVITIES FOLLOWING ETHANOL FRACTIONATION

Extract and microsomal fraction from a normal fed rat were prepared as described in Table II. An aliquot of the extract was precipitated with 30% ethanol at 0°C and another aliquot with 80% ethanol at room temperature. A portion of the extract and of the microsomal fraction were treated with $(NH_4)_2SO_4$ and 80% ethanol at room temperature as described [21]. All ethanol precipitates were extracted with 50 mM glycylglycine/50 mM mercaptoethanol/10 mM Na_2SO_4, pH 7.2, centrifuged and dialyzed against the same buffer for 3 h.

Treatment		Total activity (units/g)			Activity ratios		
		Synthase phosphatase (SP)	Phosphorylase phosphatase (PP)	Histone phosphatase (HP)	$\frac{PP}{SP}$	$\frac{PP}{HP}$	$\frac{HP}{SP}$
Extract	None	0.92	48	112	50	0.4	120
	30% ethanol	0.76	36	35	50	1	50
	80% ethanol	0.37	110	16	300	7	40
	$(NH_4)_2SO_4$ + 80% ethanol	0.32	303	21	900	14	60
Microsomes	None	0.44	39	27	90	1	60
	$(NH_4)_2SO_4$ + 80% ethanol	0.03	120	6	≈4000	20	≈200

The fraction obtained from extracting the precipitate contained 30% of the synthase phosphatase and 14% of the histone phosphatase activity, but the phosphorylase phosphatase activity had increased six-fold. If the ammonium sulfate step was omitted, the activation of phosphorylase phosphatase was slightly less but the reduction in synthase phosphatase and histone phosphatase still occurred. An increase in phosphorylase phosphatase activity also was obtained with the microsomal fraction. A comparison of the ratio of activities indicated that synthase phosphatase and phosphorylase phosphatase were precipitated together at the 30% ethanol step but activities were dissociated with more severe ethanol treatment. It is possible that a single protein obtained after 80% ethanol has both synthase phosphatase and histone phosphatase activities. Apart from this, the activity ratio was quite different between each two of the three phosphatases.

Elution profile on DEAE-cellulose

When a microsomal fraction was passed through a DEAE-cellulose column, the three phosphatases were retained and could be separated from the unabsorbed proteins (Fig. 1). Both phosphorylase phosphatase and synthase phosphatase activities were dispersed compared with the sharp peaks of phosphorylase *b* and synthase D. There was considerable overlap in the synthase phosphatase and phosphorylase phosphatase elution profile but the activity peaks

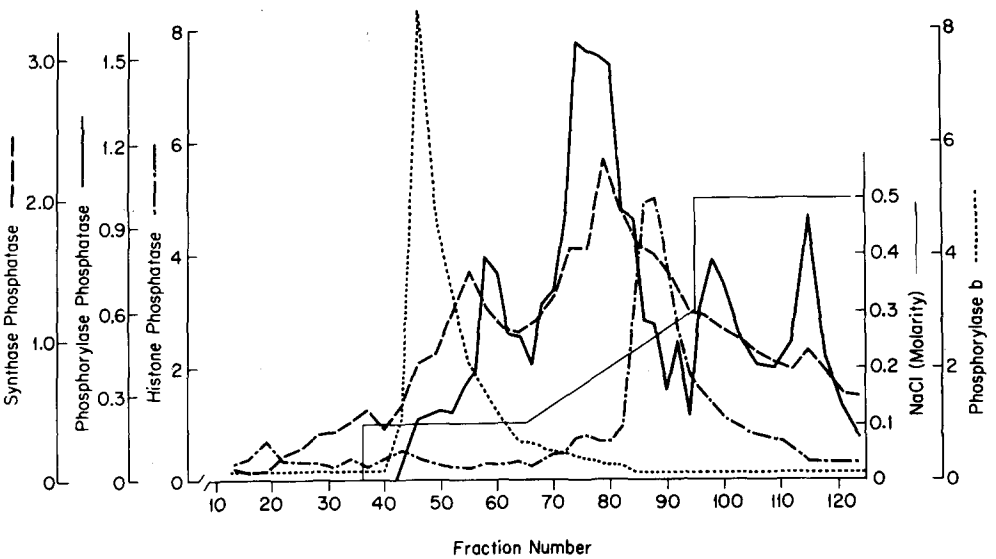

Fig. 1. Elution Profile on DEAE-cellulose. Extract from livers of 3 normal fed rats was prepared in 50 mM glycylglycine/50 mM mercaptoethanol/1% glycogen, pH 7.2. It was centrifuged at 105 000 × *g* for 60 min. The microsomal fraction, above the glycogen pellet, was resuspended in glycerol buffer (20 mM Tris · HCl/50 mM mercaptoethanol/25% glycerol, pH 7.2). The sample was put on a DEAE-cellulose column (2.5 × 35 cm) equilibrated with the glycerol buffer and eluted with glycerol buffer containing NaCl as shown. A continuous dialyzer (Bio-Med Instrument Model D-1) was used to decrease the salt concentration of the effluent before collection into fractions. Not shown in this figure was the elution of synthase D which occurred as a narrow band with peak at fraction No. 82. Phosphatase activities are expressed as Δ cpm × 10^{-3} after 20 min of incubation and phosphorylase *b* activity as cpm × 10^{-3}.

did not coincide. Histone phosphatase activity present in the microsomal fraction eluted as a discrete peak at the later part of the synthase phosphatase and phosphorylase phosphatase activity regions.

Elution profile on Sepharose 4B

A microsomal fraction was first treated with 8 mM $CaCl_2$ [22] and insoluble material removed. The supernatant was found to contain over 50% of the synthase phosphatase and phosphorylase phosphatase activities. When this fraction was concentrated and put on Sephadex G200 column, most of the synthase phosphatase and phosphorylase phosphatase eluted just behind the void volume. However small molecular weight species containing phosphorylase phosphatase activity were identified which had no synthase phosphatase activity. On a Sepharose 4B column a clear dissociation of the two enzymes was observed (Fig. 2). The majority of synthase phosphatase was associated with the larger molecular weight proteins and the majority of the phosphorylase phosphatase with the smaller species. Histone phosphatase migrated as a single peak on this column and was of intermediate size. Due to rapid loss of activity upon concentration, no further studies were done on these peaks.

Substrate competition studies

Synthase phosphatase activity measured in the microsomal fraction was strongly inhibited by the addition of purified phosphorylase *a* (Fig. 3A). Activity was reduced 50% when less than 0.7 unit/ml phosphorylase *a* was added. This was much below the K_m value (60 units/ml) of phosphorylase phosphatase

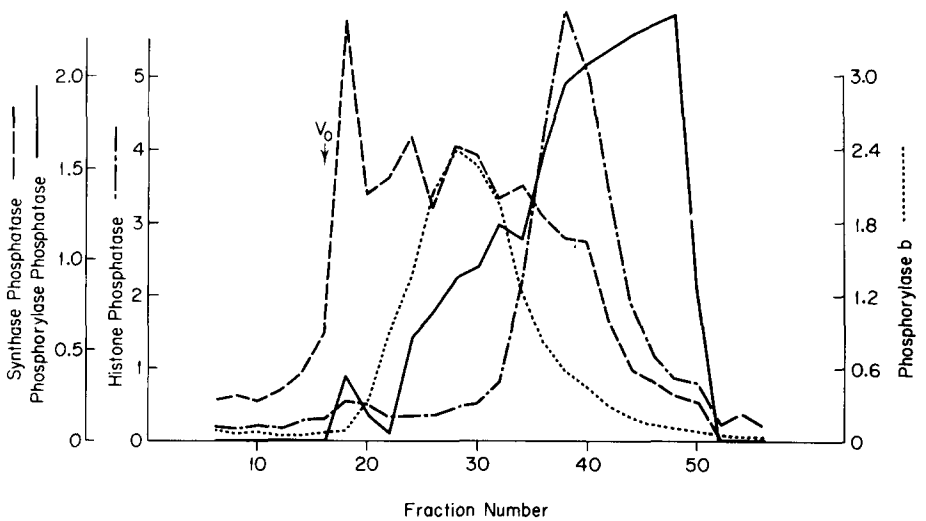

Fig. 2. Elution Profile on Sepharose 4B. Preparation of sample was as described for Fig. 1 except the microsomal fraction was resuspended in 8 mM $CaCl_2$/1 mM imidazole/50 mM mercaptoethanol, pH 7.0. Insoluble material was removed by centrifugation and the sample was concentrated by precipitation with two volumes of ammonium sulfate before addition to Sepharose 4B column (2.5 X 60 cm). Phosphatase activities are expressed as Δ cpm X 10^{-3} after 20 min of incubation and phosphorylase *b* activity as cpm X 10^{-3}.

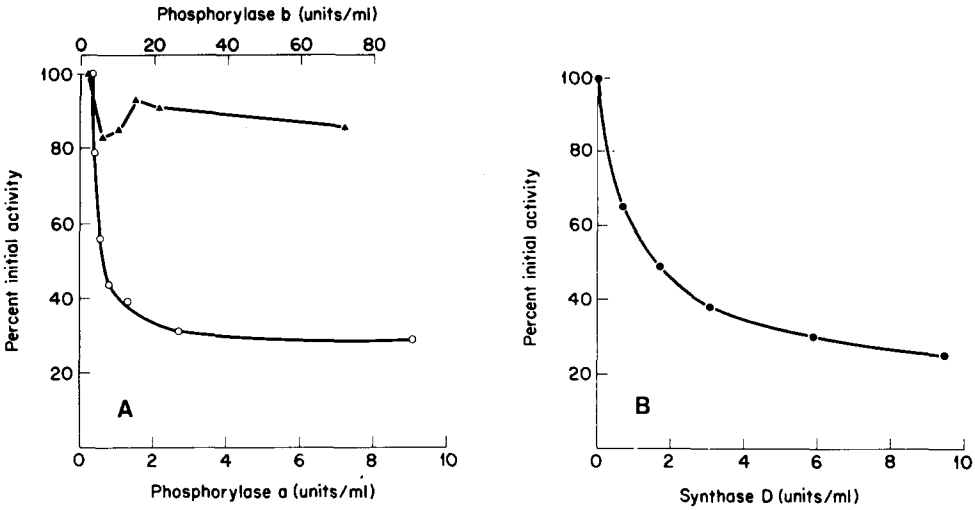

Fig. 3. Inhibition of synthase phosphatase by phosphorylase *a* (A) and phosphorylase phosphatase by synthase D (B). Extract was prepared in 50 mM Tris · HCl/1% glycogen/50 mM mercaptoethanol, pH 7.2. It was centrifuged at 105 000 × *g* for 60 min and the pellet was resuspended in the homogenizing buffer and preincubated at 25°C for 20 min before assay. Phosphorylase *a*, 40 units/mg and phosphorylase *b*, 23 units/mg were present in 20 mM Mes/1.5 mM EDTA/5 mM mercaptoethanol, pH 6.7. Synthase D, 4 units/mg was in 0.25 M sucrose/50 mM glycylglycine/3 mM dithiothreitol/4 mM glucose 6-phosphate, pH 7.2. Volume of additions (enzymes and/or respective buffers) totalled 45 μl in the synthase phosphatase and 65 μl in the phosphorylase phosphatase assays. The final assay volume was 100 μl. Symbols: phosphorylase *a*, ○; phosphorylase *b*, ▲; synthase D, ●.

for phosphorylase *a* [9]. Phosphorylase *b* on the other hand was not inhibitory up to 70 units/ml. Phosphorylase phosphatase was also inhibited by the addition of purified synthase D (Fig. 3B). Synthase D at a concentration of 2 units/ml decreased the activity 50%. However histone phosphatase activity was not

TABLE IV

HISTONE PHOSPHATASE ACTIVITY IN THE PRESENCE OF ADDED PHOSPHORYLASE *a* AND SYNTHASE D

Liver extract was prepared in 50 mM imidazole/2 mM dithiothreitol, pH 7.4. It was assayed for histone phosphatase activity at the various substrate concentrations as described in Methods. Phosphorylase *a*, 70 units/mg, was present in 20 mM Mes/10 mM mercaptoethanol/1.5 mM EDTA, pH 6.7. Synthase D, 5.2 units/mg, was in 0.25 M sucrose/50 mM glycylglycine/3 mM dithiothreitol/4 mM glucose 6-phosphate, pH 7.2. Volume of additions (phosphorylase *a*, synthase D and/or respective buffers) totalled 15 μl in a final assay volume of 100 μl.

P-Histone (mg/ml)	Additions (units/ml)					
	Phosphorylase *a*			Synthase D		
	0	5	15	0	0.3	0.8
0.16	74	74	79	—	52	60
0.41	100	100	100	81	84	78
1.6	134	138	134	122	132	124

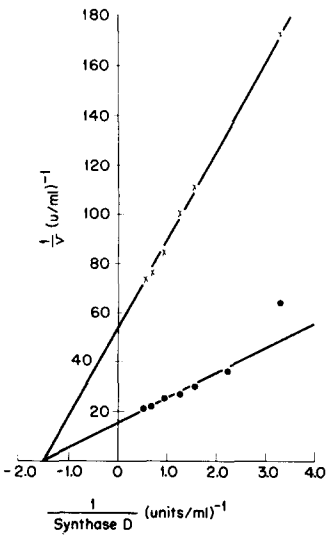

Fig. 4. Kinetics of inhibition of synthase phosphatase by phosphorylase *a*. Conditions of the experiment were the same as Fig. 3. The amount of phosphorylase a was 0.69 units/ml at zero time. Symbols: control, •; phosphorylase a, ×.

inhibited by synthase D or phosphorylase *a* either at saturating or non-saturating concentrations of phosphohistone (Table IV).

The effect of addition of a constant amount of phosphorylase *a* on the affinity of synthase phosphatase for synthase D was studied and the results are shown in Fig. 4. In the presence of 0.7 units/ml phosphorylase *a*, maximal velocity was decreased 50% but there was no change in the K_m for synthase D (0.5 unit/ml). The amount of phosphorylase *a* in the reaction mixture decreased with time and as a result the amount of inhibition also decreased. Non-competitive kinetics were obtained in each case. Similarly, the inhibition of phosphorylase phosphatase by synthase D was also of the non-competitive type (results not shown).

Discussion

We have obtained several lines of evidence which indicate that histone phosphatase in liver is distinct from synthase phosphatase and phosphorylase phosphatase. The sedimentation characteristics were different. There was no change in histone phosphatase activity in adrenalectomized-fasted rats despite a considerable decrease in synthase phosphatase and phosphorylase phosphatase. The enzyme, unlike phosphorylase phosphatase, was not activated by ethanol treatment. It eluted as a single discrete peak on column chromatography whereas synthase phosphatase and phosphorylase phosphatase exhibited multiple peaks. Finally, histone phosphatase was not inhibited by synthase D and phosphorylase *a*.

Our studies also indicate that synthase phosphatase and phosphorylase phosphatase were not activities of the same proteins. The two enzymes had different elution profiles when an untreated microsomal fraction was passed through a

DEAE column. After treatment of liver extract with ethanol and the microsomal fraction with $CaCl_2$, there was a clear dissociation of synthase phosphatase and phosphorylase phosphatase activities. Synthase phosphatase was inhibited by phosphorylase *a* and phosphorylase phosphatase by synthase D. However, non-competitive kinetics were obtained. This rules out the possibility of binding to the same substrate site. These data, together with the lack of a constant ratio of activity after various treatments, are taken as evidence that different phosphatases exist with varying substrate specificities. Preliminary data on separation of phosphatase activities with differing substrate specificity were also presented [24,25].

The separation of synthase phosphatase, phosphorylase phosphatase and histone phosphatase activities in the present study does not exclude the possibility that a single protein can be isolated which has two or more phosphatase activities [1,5,6,23]. This could be due to the existence of multiple forms of the phosphatases shown here and by various other workers [13,26—30]. A form could be chosen for purification which exhibits all three phosphatase activities in varying proportions. Since the quaternary structure of the phosphatases has been reported to be readily modified during purification [5,28,30], it also is difficult to determine if the purified protein corresponds to a native form of the enzyme.

Synthase phosphatase in liver has been reported to co-purify with phosphorylase phosphatase [5,6] and with histone phosphatase [5]. Our data are in agreement with those of Brandt et al. [31] and Killilea et al. [6] even though different conclusions were reached. After conversion to equivalent activity units, the ethanol precipitation step in both studies was found to contain the same amount of synthase phosphatase as well as a similar activity ratio of phosphorylase phosphatase to synthase phosphatase. They reported difficulty in measuring phosphatase activities in crude extract and their data showed co-purification only after the ethanol treatment step. Our phosphatase assays in crude extracts [9] were developed from studies of these enzymes in concentrated extracts using endogenous substrates [32,33]. Buffer, additives and preincubation times were carefully chosen to give maximal phosphatase activity. Using the buffer system of Brandt et al. [21] to prepare crude extracts, we also found less measurable phosphatase activity [9]. We interpret all these results to mean that ethanol treatment inactivated most of the endogenous synthase phosphatase and histone phosphatase, but produced a small and non-specific phosphatase which was active, at different rates, on many substrates. The purification procedure used by Khandelwal et al. [5] also emphasized the dissociation of larger phosphatase(s) to small molecular forms which appeared to take place without a significant change in activity characteristics. Substrates and assay conditions used were quite different and may explain part of the discrepancy with our data.

There is a close analogy of the phosphatase system with the protein kinase system. A cyclic AMP-dependent protein kinase has been obtained which has activity on synthase I as well as on many other protein substrates [34—36]. Recently, cyclic AMP-independent synthase kinases have been described with different substrate specificities, and these may have major roles in the regulation of synthase in vivo [37—39]. A protein phosphatase has been isolated

which has activity on synthase D and phosphorylase *a* as well as on other substrates [1,5,6,23]. We now report other forms of phosphatase with different substrate specificities. The question of which of the multiple forms is responsible for the regulation of glycogen metabolism in vivo remains unanswered.

In the present study, where 1% exogenous glycogen was added to the extract, the majority of synthase phosphatase and phosphorylase phosphatase activity was found to sediment with the microsomal fraction in liver. These phosphatases have been reported in the pellet after high-speed centrifugation [22,24,40] but have also been found in the supernatant [26,27,30]. The nutritional status of the animals used was not specified in every case and this could affect the results. Glycogen added during the preparation of the extract could also change the sedimentation characteristics of the enzymes. The actual subcellular distribution of the phosphatases is not clear since in the absence of glycogen, phosphatase activities were low in all fractions.

The differences in elution profile of the phosphatase enzymes could be due to protein-protein interaction of the type described by Gergeley et al. [41]. They could also be due to the differential presence of inhibitor protein(s) in the fractions. Heat-stable inhibitors of purified phosphorylase phosphatase have been reported in liver [42] and in muscle [43]. In muscle, the inhibitor was reported to alter the substrate specificity of the purified phosphatase. However, in liver the inhibitor studied was inhibitory only to the small molecular weight phosphorylase phosphatase and not inhibitory to the holoenzyme. Until the exact relationship between the regulatory protein(s) and the catalytic protein is established, we prefer to accept the simpler explanation that the different elution profiles represent different species of proteins.

Stalmans et al. reported a lag period in the activation of endogenous synthase in crude extract which could be prolonged by the addition of purified phosphorylase *a* [20]. There was no lag in our synthase phosphatase assay but we have verified the inhibition of the enzyme by phosphorylase *a*. This inhibition occurred at very low phosphorylase *a* concentrations, much below those found in normal liver. In addition, we found inhibition of phosphorylase phosphatase by synthase D, though at concentrations higher than that present in in vivo. This mutual inhibition has also been observed for purified or partially purified phosphatase from liver [5,6,44] and heart [23]. In some studies competitive inhibition and mixed-type inhibition kinetics have been obtained with the different substrates [5,23]. These data have been used as evidence that synthase phosphatase, phosphorylase phosphatase and histone phosphatase have a common identity. Our kinetic studies with crude preparations containing total phosphatase activity indicated that phosphorylase *a* was a non-competitive inhibitor of synthase phosphatase, suggesting that the majority of synthase phosphatase was not active on phosphorylase *a*. Under these conditions, competitive kinetics due to the presence of a minor non-specific form would not have been revealed. The mechanism of the inhibition is not clear but could be a protein-protein interaction. Such interactions have been described for liver phosphofructokinase with fructose diphosphatase [45], for muscle phosphorylase *b* kinase with phosphorylase *b* and with phosphorylase phosphatase [41].

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