ACTIVATION OF PHOSPHORYLASE PHOSPHATASE BY A NOVEL PROCEDURE: EVIDENCE FOR A REGULATORY MECHANISM INVOLVING THE RELEASE OF A CATALYTIC SUBUNIT FROM ENZYME-INHIBITOR COMPLEX(ES) OF HIGHER MOLECULAR WEIGHT.

Howard Brandt, S. Derek Killilea and Ernest Y.C. Lee
Department of Biochemistry
University of Miami
Miami, Florida 33152

Received October 10, 1974

SUMMARY. Phosphorylase phosphatase in crude tissue extracts of rat liver, skeletal and heart muscle can be markedly activated by a treatment involving precipitation with ammonium sulfate and ethanol. The activation of the enzyme in rat liver extracts is shown to occur with the concomitant conversion of the enzyme from multiple molecular weight forms to a single form of lower molecular weight (M.W.~30,000). After activation by ethanol treatment, phosphorylase phosphatase activities of rat liver, skeletal and heart muscle were shown by sucrose density ultracentrifugation to sediment in a similar manner with a sedimentation coefficient of 2.8S and M.W. 32,000.

We have recently developed a procedure for the isolation of rabbit liver phosphorylase phosphatase (1-3). During this procedure we had consistently noted a marked activation of the enzyme during the initial purification steps which involved ammonium sulfate and ethanol precipitations (1-3). In this paper we report the results of an examination of this phenomenon in tissues of the rat. These results provide evidence for the existence of inactive forms of the enzyme and their conversion by our procedure to a single species of lower molecular weight.

METHODS.

Phosphorylase phosphatase was assayed using rabbit muscle phosphorylase *a* as the substrate, one unit of activity being defined as the amount of enzyme which converted 0.2 mg phosphorylase *a* (1nmole) per minute (3). Extracts were prepared from liver, skeletal and heart muscle of male adult rats by homogenization in 3-4 volumes of 0.1M NaCl in IED buffer (50 mM imidazole, 5 mM EDTA, 0.5 mM dithiothreitol, pH 7.45), followed by centrifugation (10,000g, 20 min.). Column chromatography was performed in 0.1M NaCl in IED buffer. Sucrose density gradient ultracentrifugation was carried out by the method of

Martin and Ames (4). *Ethanol treatment:* tissue extracts were precipitated by the addition of 2 volumes of saturated ammonium sulfate. The ammonium sulfate precipitate was collected by centrifugation and resuspended in 1 volume of IED buffer. Five volumes of room temperature 95% ethanol were then added. The precipitate was immediately collected by centrifugation (5,000g, 10 min.), extracted with 1 volume of IED buffer, and dialysed overnight against the same buffer. All operations were carried out at 4°C, except where otherwise indicated.

RESULTS AND DISCUSSION.

The effects of the combined precipitation with ammonium sulfate and ethanol on tissue extracts of rat liver, heart and skeletal muscle are shown in Table I. Very marked increases in activity were observed in all cases, as we had noted before for rabbit liver extracts (13). It seemed unlikely that the activation was due to the removal of known dialysable inhibitors such as adenine nucleotides (1,5,6), since the extracts were dialysed prior to assay and ethanol treatment. In other studies we have shown the exist-

TABLE I. ACTIVATION OF PHOSPHORYLASE PHOSPHATASE IN RAT TISSUE EXTRACTS BY PRECIPITATION WITH AMMONIUM SULFATE AND ETHANOL

TISSUE	PHOSPHATASE ACTIVITY (units/gm)		ACTIVATION (fold)
	CRUDE EXTRACT	AFTER PRECIPITATION	
LIVER	1.9	56	30
	1.4	48	34
	1.5	48	43
SKELETAL MUSCLE	6.6	26	4
HEART MUSCLE	2.8	38	14
	1.9	37	19

Tissue extracts were prepared as described in Methods; extracts were dialysed overnight in 0.1M NaCl in IED buffer, and then assayed for phosphorylase phosphatase activity. The same dialysed extracts were treated with ammonium sulfate and ethanol as described in Methods, dialysed, and assayed.

ence of a heat-stable, non-dialysable proteinaceous inhibitor of phosphorylase phosphatase in rabbit liver (3). This might explain the effects observed if the treatment with room-temperature ethanol resulted in the selective removal of a protein inhibitor.

We therefore examined the possibility that the activation of the enzyme in tissue extracts might be due to the disruption of an enzyme-inhibitor complex. A rat liver extract was chromatographed on a Sephadex G-75 column. Assay of the column fractions revealed that all the activity was eluted in the void volume (Fig. 1). Each fraction was then subjected to the ethanol treat-

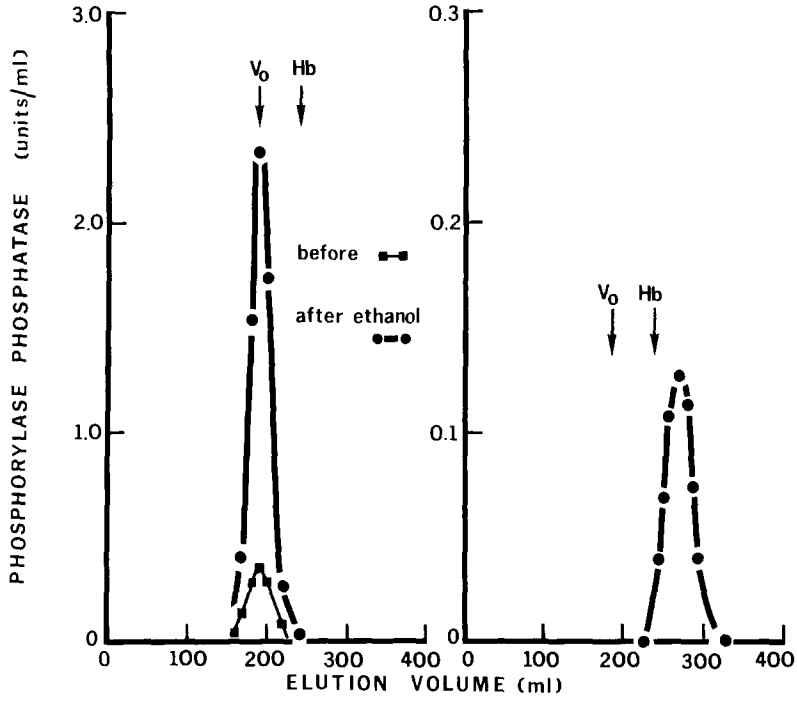

Figure 1. Behaviour of Rat Liver Phosphorylase Phosphatase on Sephadex G-75 Chromatography, Before and After Treatment with Ethanol.
A rat liver extract (5 ml) was chromatographed on a Sephadex G-75 column (2.5 X 90 cm). Fraction volumes of about 8 ml were collected and assayed for phosphorylase phosphatase activity (■—■, left panel). Each fraction was then treated by ammonium sulfate and ethanol precipitations as described in Methods, and then assayed for phosphorylase phosphatase activity (● — ●, left panel). Samples (2 ml) of the fractions containing activity were pooled, and then treated with ammonium sulfate and ethanol precipitations as before. The treated enzyme was then re-chromatographed on the same column, and assayed for phosphorylase phosphatase activity (● — ●, right panel). Recovery of applied activities was in all cases greater than 85%. Vo and Hb refer to the void volume and the elution volume for hemoglobin, respectively.

ment, and a marked activation of the enzyme (8-fold) was observed (Fig. 1). This further confirmed that the effect was not due to the presence of dialysable inhibitors. When pooled samples of the void volume fractions were treated with ethanol, and re-chromatographed on Sephadex G-75, the activity was now retained on the column and was eluted as a single peak of lower molecular weight (Fig. 1). Further experiments (not shown) indicated that this ethanol-activated enzyme had a M.W. of about 35,000 by Sephadex G-75 chromatography.

In other experiments we examined the behaviour of the enzyme activity in rat liver extracts during column chromatography on 10% agarose (Biogel 0.5m, exclusion limit M.W. 500,000). The enzyme activity exhibited multiple molecular weight forms, ranging from about 140,000 - 300,000 (Fig. 2). Each of

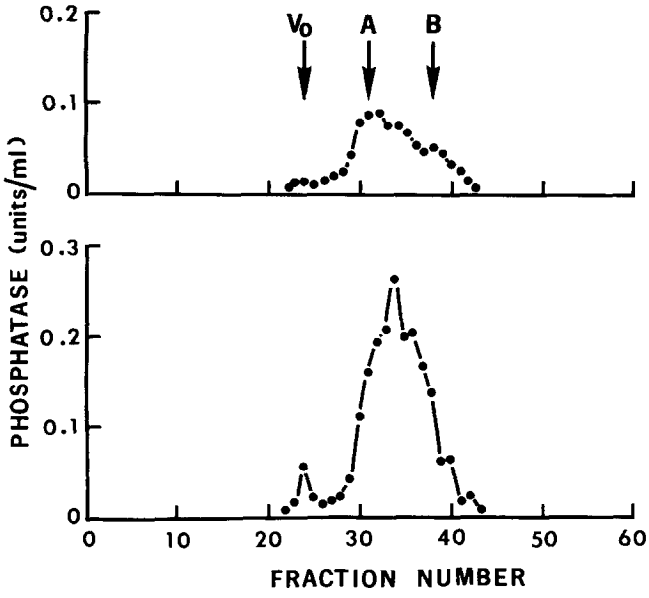

Figure 2. Behaviour of Rat Liver Phosphorylase Phosphatase on Biogel 0.5m Chromatography, Before and After Treatment with Ethanol. A rat liver extract (1 ml) was chromatographed on a Biogel 0.5m column (1.6 X 60 cm). Fraction volumes of about 2 ml were collected and assayed for phosphorylase phosphatase activity (upper panel). Samples (1 ml) of each fraction were treated with ammonium sulfate and ethanol precipitations as described in Methods, and then assayed for activity. Vo refers to the void volume; A is the elution volume of R-phycoerythrin (M.W. 290,000) and B is the elution volume of R-phycocyanin (M.W. 138,000, ref. 16).

the active fractions could be activated by ethanol treatment, although the activation was only about 2-fold (Fig. 2). When the pooled activated enzyme was re-chromatographed, it again behaved as a single low molecular weight species as in the previous experiment (not shown).

Ethanol-treated extracts of rat liver, skeletal and heart muscle were examined by sucrose density ultracentrifugation (Fig.3). Phosphorylase phosphatase activity in all three extracts sedimented at the same rate, within experimental error. For the skeletal muscle extract, human erythrocyte carbonic anhydrase C (7) was used a standard. On this basis (4), the ethanol-

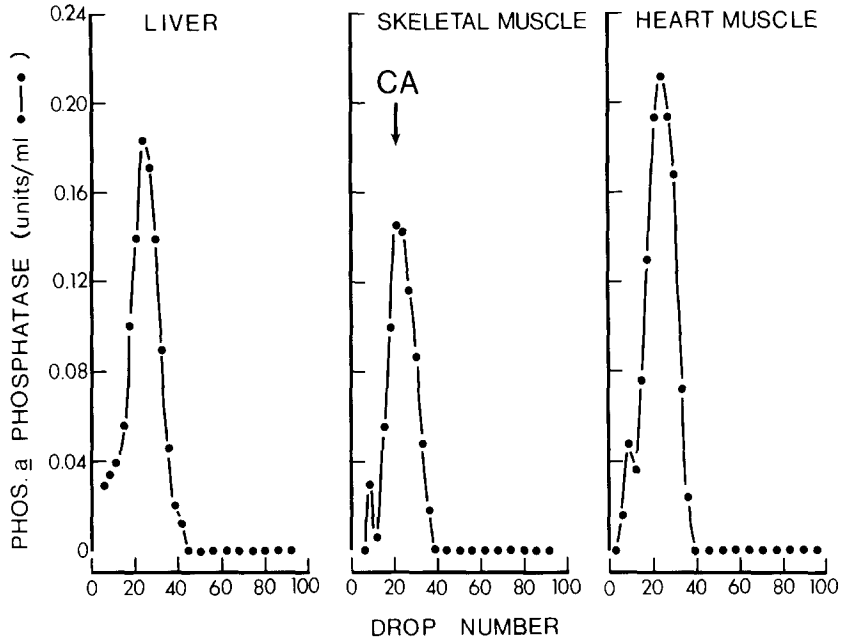

Figure 3. Sedimentation Behaviour of Rat Liver, Skeletal Muscle and Heart Muscle Phosphorylase Phosphatase after Activation by Ethanol Treatment.
Extracts of rat liver, skeletal muscle and heart muscle were activated by treatment with ammonium sulfate and ethanol precipitations as described in Methods. Samples of the activated enzymes (0.15 ml) were layered onto sucrose gradients (5-20% sucrose in IED buffer) and centrifuged in a Spinco Model L centrifuge in a S.W. 50L rotor at 39,000 rpm for 16 hours at 4°C. Fractions of 3 drops each were collected form the bottom of the tubes and assayed for phosphorylase phosphatase activity. The recovery of applied activities was in all cases greater than 80%. The direction of sedimentation is from left to right. Human erythrocyte carbonic anhydrase C was used as a marker (M.W. 27,900, 2.65S, ref. 15) in the tube containing the skeletal muscle enzyme.

treated enzymes had a molecular weight of 32,000 and a sedimentation coefficient of 2.8 S. These results for the ethanol-treated enzymes of rat tissues are consistent with our studies of homogeneous rabbit liver phosphorylase phosphatase, which consists of a single polypeptide chain of M.W. 34,000 as determined by Sephadex G-75 chromatography and SDS polyacrylamide disc gel electrophoresis (3).

Our results provide good evidence that phosphorylase phosphatase exists in an active and an inactive form. On the basis of this work, we propose the hypothesis that the inactive form consists of an enzyme-inhibitor complex. In this view, a single catalytic subunit would be involved, this being the enzyme of M.W. 34,000 which we have studied (3). The inhibitor moiety would be a protein subunit(s). The multiple forms observed may be explained if the inhibitor subunit(s) existed in several molecular weight forms, or if the enzyme-inhibitor complex has a multiple stoichiometry and undergoes partial dissociation. The effects which we have noted may be explained if the ethanol treatment leads to the selective denaturation of the inhibitor protein(s). This proposal for the basis of the active and inactive forms would implicate a regulatory mechanism for phosphorylase phosphatase which has a parallel in the known regulation of cAMP-dependent protein kinase (8). The physiological regulation of the phosphatase would require that the dissociation or activation of the proposed enzyme-inhibitor complex be mediated by a hormone signal, perhaps a second messenger. While a physiologically relevant procedure for the activation of phosphorylase phosphatase has yet to be demonstrated, the procedure which we describe may provide a useful tool for the study of the effects of physiological stress on the activation of the enzyme *in vivo*.

Our findings that multiple forms of phosphorylase phosphatase in liver may be activated and concomitantly converted to a single smaller species of M.W. about 30,000, have some bearing on other studies of phosphoprotein phosphatases. With the exception of the work of Fischer *et al.* (9),

studies of partially purified preparations of phosphoprotein phosphatases from a variety of tissues have revealed multiple molecular weight forms varying from 70,000 - 510,000 (10-13), and which exhibit multiple activities toward glycogen synthase, phosphorylase and phosphohistone (10-12). A high molecular weight enzyme fraction from rabbit skeletal muscle has been reported to be converted from M.W. 300,000 to M.W. 150,000 by freezing (10), while an enzyme from bovine heart (M.W. 150,000) has been reported to be converted to lower molecular weight by treatment with cations, urea or SDS (11). We have also obtained evidence that rabbit liver phosphorylase phosphatase (M.W. 34,000) is also the enzyme which dephosphorylates glycogen synthase (14), and we suggest that the higher molecular weight forms of phosphoprotein phosphatase reported by others will also be found to be converted to a catalytic subunit of M.W. 34,000 by our procedure via the removal of the putative inhibitor subunit(s).

ACKNOWLEDGEMENTS.

The authors thank Dr W.J. Whelan for useful discussions of this work. Support for this work was provided by NIH grant AM 12532 and a grant from the Upjohn Co. E.Y.C. Lee is an Established Investigator of the American Heart Association. Human erythrocyte carbonic anhydrase C was a gift of Dr P.L. Whitney.

1. Lee, E.Y.C., Capulong, Z.L., and Martin, S. (1973) *Fed. Proc. 32,* 554.
2. Lee, E.Y.C., Brandt, H.,Capulong, Z.L., and Seber, J. (1973) Abstr. Biol. 226, 166th ACS Meeting, Chicago.
3. Brandt, H., Capulong, Z.L., and Lee, E.Y.C. In preparation.
4. Martin, R.G., and Ames, B.N. (1961) *J. Biol. Chem. 236,* 1372-1379.
5. Bailey, J.M., and Whelan, W.J. (1972) *Biochem. Biophys. Res. Commun. 46,* 191-197.
6. De Barsy, T., Stalmans, W., Laloux, M., De Wulf, H., and Hers, H.G. (1972) *Biochem. Biophys. Res. Commun. 46,* 183-190.
7. Whitney, P.L. (1974) *Anal. Biochem. 57,* 467-476.
8. Walsh, D.A., and Krebs, E.G. (1973) *In* The Enzymes, 3rd Edition, vol. 8, pp. 555-581, Academic Press, New York.
9. Gratecos, D., Detwiler, T., and Fischer, E.H. (1974) *Fed. Proc. 32,* 575.
10. Kato, K., and Sato, S. (1974) *Biochim. Biophys. Acta 358,* 299-307.
11. Thomas, J.A., Young, T., and Mellgren, R. (1974) *Fed. Proc. 33,* 1315.
12. Bishop, J.S., Kato, K., DeSain, C.V., Hedlund, B., and Kobayashi, M. (1974) *Fed. Proc. 33,* 1430.
13. Abe, N., and Tsuiki, S. (1974) *Biochim. Biophys. Acta 350,* 383-391.
14. Killilea, S.D., Brandt, H., Lee, E.Y.C., and Whelan, W.J. (1974) *Fed. Proc. 33,* 1431.
15. Armstrong, J. McD., Myers, D.V., Verpoorte, J.A., and Edsall, J.T. (1966) *J. Biol. Chem. 241,* 5137-5149.
16. Nolan, D.N., and O'hEocha, C. (1967) *Biochem. J. 103,* 39p.