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THE PURIFICATION AND CHARACTERIZATION OF BOVINE HEART GLYCOGEN SYNTHASE PHOSPHATASE

Iowa State University PH.D. 1980

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The purification and characterization of bovine heart glycogen synthase phosphatase

by

Barbara Gayle Ruffin Slaughter

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LIST OF ABBREVIATIONS

cAMP	Adenosine 3',5'-monophosphate
ATP	Adenosine 5'-triphosphate
UDPG	Uridine 5'-diphosphateglucose
EDTA	Ethylenediaminetetraacetate
EGTA	Ethyleneglycol-bis-(aminoethylether)-N,N,N',N'-tetraacetate
Tris	Tris (hydroxymethyl) aminomethane
DTT	Dithiothreitol
SDS	Sodium dodecyl sulfate
P_i	Orthophosphate
LDH	Lactate dehydrogenase
U	Units of enzyme
TCA	Trichloroacetic acid

DEDICATION

To Michael, for 10 years of learning and growing.

and

To my parents and grandparents, for their indefeatable spirits.

INTRODUCTION

Coordination of Glycogen Synthesis and Degradation

The discovery that glycogen phosphorylase[1] exists in two forms with different properties (1) led to the realization that the covalent modification of proteins is an important mechanism for controlling enzymatic activity and protein function. Study of the phosphorylation and dephosphorylation of glycogen metabolizing enzymes has continued to lead to the elucidation of mechanisms of regulation that are applicable to many areas of biochemistry. The effects of cAMP were first demonstrated (2,3) and the action of protein kinases (4) and phosphoprotein phosphatases (5) were initially observed while studying the regulation of glycogen metabolism. Current studies are directed toward determining which protein kinases and phosphoprotein phosphatases catalyze the in vivo interconversion of glycogen metabolizing enzymes and how the reactions are influenced by activators and inhibitors.

Each of the rate-limiting enzymes in glycogen synthesis and degradation is modified by phosphorylation. Glycogen synthase[2], which catalyzes the transfer of glucose moieties from UDP-glucose to a glycogen primer (6), is inactivated by phosphorylation (7). Phosphorylase, which catalyzes the sequential removal of glucose units from glycogen (8), is activated by phosphorylation (9) at a single site, serine 14 (10). Elevations in cAMP increase phosphorylation of both glycogen synthase (11) and phosphorylase

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[1] α-1,4-Glucan:orthophosphate glycosyltransferase, EC.4.1.1.
[2] UDP-glycose:glycogen -1,4-glycosyltransferase, EC2.4.1.11.

(12,13) resulting in a concerted decrease in synthesis and increase in degradation of glycogen. Phosphorylase kinase[3], which phosphorylates phosphorylase b (9), is activated by Ca^{+2} (14). Recent evidence that phosphorylase kinase utilizes glycogen synthase as a substrate *in vitro* (15,16,17, 18) indicates that another mechanism may exist for coordinating glycogen synthesis and degradation.

Phosphorylation of Glycogen Synthase

Leloir *et al.* (19) found that glucose-6-phosphate stimulated glycogen synthesis in muscle extracts. The effect of glucose-6-phosphate on glycogen synthase activity was decreased by insulin (20), leading Villar-Palasi and Larner to propose that there were two interconvertable forms of synthase. The enzyme forms were designated glycogen synthase D, because optimum activity was dependent upon millimolar concentrations of glucose-6-phosphate, and glycogen synthase I, since activity was independent of glucose-6-phosphate (21,22). Conversion of glycogen synthase D to glycogen synthase I was shown to be accompanied by release of ^{32}P from radioactively labeled glycogen synthase D (23), confirming that the interconversion of the enzyme forms involved a phosphorylation-dephosphorylation mechanism. The phosphorylation state of glycogen synthase has traditionally been expressed as an activity ratio obtained by dividing the activity of synthase measured in the absence of glucose-6-phosphate to that in the presence of 10 mM glucose-6-phosphate.

A number of studies have focused on determining which kinases phosphorylate glycogen synthase. Belocopitow (11) demonstrated that glycogen

[3] ATP:phosphorylase phosphotransferase, EC2.7.1.38.

synthase was converted to an inactive form in epinephrine-treated tissues or as a consequence of adding cAMP to broken-cell preparations. Larner's laboratory then demonstrated that the cAMP stimulation of conversion of glycogen synthase I to D in skeletal muscle was not catalyzed by phosphorylase kinase (24,25,26). Several laboratories provided evidence that cAMP-dependent protein kinase could phosphorylate glycogen synthase with conversion from the I to the D form (27,28,29). It soon became apparent that phosphorylation of glycogen synthase is complex.

In contrast to the situation for phosphorylase in which a single phosphorylation site exists, glycogen synthase is phosphorylated in multiple sites. From one to twelve (30) phosphates have been incorporated per glycogen synthase subunit depending on the synthase preparation used, the kinases present, and the reaction conditions. Soderling (31) found that both protein kinase and ATP concentration affected the number of phosphates incorporated into each rabbit skeletal muscle glycogen synthase molecule. At 0.23 mM ATP and low protein kinase concentration, there was a rapid incorporation of one phosphate per subunit. A slower phosphorylation was enhanced by elevating the ATP concentration to 3.5 mM. At high protein kinase concentrations, as many as 5 phosphates could be incorporated per subunit, however, only the first 2 or 3 affected the activity ratio. The first phosphate was incorporated into a region of glycogen synthase that was sensitive to trypsin cleavage (32). This phosphorylation did not completely convert glycogen synthase I to D. The second phosphate was incorporated at a slower rate into a trypsin insensitive region of glycogen synthase and had more effect on the glucose-6-phosphate dependency of the enzyme. Using a purified rabbit skeletal muscle synthase, Huang et

al. (33) also noted only a partial conversion of synthase when 2 phosphates per subunit were incorporated by cAMP-dependent protein kinase. Soderling (31) found that the slower phase of phosphorylation was not inhibited by cAMP-dependent protein kinase inhibitor (34), suggesting that a kinase distinct from cAMP-dependent protein kinase could phosphorylate glycogen synthase.

Varying numbers of phosphates incorporated into glycogen synthase cannot be attributed to the action of cAMP-dependent protein kinase (27,31). Kinases have been isolated that are distinct from cAMP-dependent protein kinase, but can also phosphorylate glycogen synthase. Schlender et al. (35) demonstrated that a casein kinase isolated from rabbit kidney medulla converted glycogen synthase to an activity ratio of 0.50. A casein kinase purified by Brown et al. (36) from rabbit skeletal muscle added one phosphate to glycogen synthase with a decrease in the activity ratio to 0.02. A phosvitin kinase (37) catalyzed the incorporation of as many as 4 phosphates per synthase subunit, but incorporation of only the first 2 phosphates were necessary to decrease the activity ratio to 0.10. A rabbit skeletal muscle kinase isolated using glycogen synthase as a substrate (38), associated with the protein-glycogen complex. It incorporated one phosphate into each glycogen synthase subunit. When synthase was phosphorylated in the presence of this cyclic nucleotide-independent kinase and cAMP-dependent protein kinase, 2 phosphates were incorporated per synthase subunit resulting in an activity ratio of 0.01. Using bovine heart glycogen synthase that was not contaminated by kinase activity, Mitchell (39) demonstrated that 2 phosphates per subunit could be incorporated into synthase by the catalytic subunit of cAMP-dependent protein kinase. One

phosphate was incorporated rapidly at only 10 μM ATP. Incorporation of the second phosphate required higher concentrations of ATP or ITP, indicating an allosteric effect may control phosphorylation of that site. Phosphorylation of the second site was substantially slower than that of the first site. Glycogen synthase kinase was partially purified from bovine heart. It was free of histone kinase activity and was not stimulated by cAMP. This kinase incorporated one phosphate per glycogen synthase subunit with virtually complete conversion of the activity to a glucose-6-phosphate-dependent form. The site specificity of the kinases were analyzed by SDS polyacrylamide electrophoresis of the cyanogen bromide peptides obtained from glycogen synthases phosphorylated under different conditions. One phosphorylated peptide was produced by the action of cAMP-dependent protein kinase at 10 μM ATP. Two phosphorylated peptides (one of which migrated with the same R_f as the one formed at 10 μM ATP) were produced by cAMP-dependent protein kinase at 0.5 mM ATP. A different phosphopeptide was obtained from the glycogen synthase phosphorylated by the cAMP-independent protein kinase. All three phosphopeptides were found in glycogen synthase phosphorylated by both kinases at 1 mM ATP.

These reports support the concept that phosphorylation of glycogen synthase is catalyzed by at least two distinct kinases, a cAMP-dependent protein kinase and a cAMP-independent protein kinase, and involves at least three sites that influence the activity ratio. Very recently, a number of reports have appeared that indicate that phosphorylase kinase can phosphorylate glycogen synthase (15,16,17,18). The site phosphorylated by this kinase is serine 7 (17) and is distinct from those phosphorylated by other kinases studied (40,41).

Although glycogen synthase was described some years ago as synthase I or D in reference to the activity ratio determined in the absence or presence of glucose-6-phosphate, it has become obvious that this designation is not adequate to specify the phosphorylation states of the enzyme. The activity ratio itself is not descriptive since synthase phosphorylated by different kinases may have similar activity ratios, but different kinetic properties. Dopere et al. (42) isolated three different synthase fractions from dog liver. The K_m for UDP-glucose increased from 0.27 to 2.4 as the activity ratio decreased from 0.05 to 0.02. Mitchell and Thomas (43) found that bovine heart synthase, phosphorylated in two sites by cAMP-dependent protein kinase, had an $A_{1/2}$ for glucose-6-phosphate of 0.44 mM. Synthase with a similar activity ratio was produced when one phosphate per subunit was introduced by a cAMP-independent protein kinase, but the $A_{1/2}$ for glucose-6-phosphate was 2.0 mM.

Accurate description of the phosphorylation state of glycogen synthase requires sequencing and locating the phosphorylated site(s) within the synthase molecule. Since the primary structure of glycogen synthase has not been determined, an exact location of the phosphorylated sites is not possible, except for serine 7 which is phosphorylated by phosphorylase kinase (17). It is possible to determine the average number of sites phosphorylated per synthase subunit and to specify which kinase(s) catalyzed the phosphorylation reactions. The kinetic constants of the phosphorylated glycogen synthase can also be determined. Both the K_m for UDP-glucose and the $A_{1/2}$ for glucose-6-phosphate reflect the phosphorylation state of the enzyme (43).

The physiological significance of phosphorylation of different sites

on glycogen synthase remains to be determined. McCullough and Walsh (44) have recently reported the isolation of glycogen synthase phosphorylated by [γ-^{32}P]ATP *in vivo*. Two major cyanogen bromide ^{32}P-peptides were detected. The phosphorylation of one peptide was influenced by alterations in the level of cAMP. The existence of multiple forms of phosphorylated glycogen synthase *in vivo* is also supported by kinetic studies. Kaslow *et al.* (45) have demonstrated alterations in the $A_{1/2}$ for glucose-6-phosphate for adipose tissue glycogen synthase following treatment of fat pads by epinephrine or insulin. The $A_{1/2}$ for glucose-6-phosphate in epinephrine-treated fat pads is two-fold higher than in controls, and lower in extracts from pads treated first with epinephrine and then insulin than in controls. Glucose starvation of choriocarcinoma cells (46) results in a partial dephosphorylation of synthase that lowers the $A_{1/2}$ for glucose-6-phosphate. These data are consistent with *in vitro* data that support the concept that increasing the number of phosphates incorporated into glycogen synthase increases the $A_{1/2}$ for glucose-6-phosphate (35,37). Phosphorylation of glycogen synthase would therefore decrease the activity of the enzyme at a given glucose-6-phosphate concentration, thus inhibiting glycogen synthesis.

Phosphoprotein Phosphatases

The dephosphorylation of glycogen synthase was observed before the phosphorylation was known (21). Studies of the dephosphorylation reaction have been complicated by several factors. It has been extremely difficult to purify enzymes that dephosphorylate glycogen synthase. Multiple phosphatase activities have been isolated by many of the purification procedures. The phosphatases have frequently been unstable to the isolation

conditions resulting in low yields of active enzyme. Some of the phosphoprotein phosphatases require divalent cations to detect their activity (47,48). Progress has also been hampered by the availability of phosphatase substrates. Large quantities of phosphorylated glycogen synthase, or alternate substrates, are needed to develop purification procedures for glycogen synthase phosphatase(s). Preparation of such quantities of phosphatase-free glycogen synthase is a formidable task. Consequently, several substrates have been used to detect phosphoprotein phosphatase activity, including phosphohistone (49), casein (50), and phosphorylase a (51). There are five histone fractions (52), each of which can be phosphorylated and there are several types of casein, which can be phosphorylated at multiple sites (53). The problems encountered in specifying the phosphorylation state of glycogen synthase have already been discussed. It is difficult to interpret data or even compare studies that use the same phosphoprotein as substrate, since the same sites may not be phosphorylated. Even comparing the sizes of phosphatases is difficult because the asymmetric nature of many of the phosphatases requires that both a Stokes' radius and sedimentation coefficient be determined in order to adequately describe the size of the enzymes. Unfortunately, many of the molecular sizes reported have been calculated from comparing the elution volume of the phosphatase activity on gel filtration to the elution volume of proteins of known molecular weight that are spherical. Instead of reporting the size of the phosphatase as a Stokes' radius, molecular weights have been reported that do not consider the shape of the enzyme. Any molecular size derived from gel filtration data will be referred to in the thesis as an apparent M_r. The designation M_r will be assigned to a

molecular size calculated from both a Stokes' radius and a sedimentation coefficient according to Siegel and Monty (54).

Substrate specificity of phosphoprotein phosphatases

In 1972, Kato and Bishop demonstrated that a glycogen synthase phosphatase preparation could also dephosphorylate ^{32}P-histone (49). Since their preparation was not homogenous, it was not certain that only one phosphatase was present. Nakai and Thomas (55) attempted unsuccessfully to separate bovine heart phosphohistone phosphatase, glycogen synthase phosphatase and phosphorylase phosphatase activities by several methods including DEAE-cellulose chromatography, molecular sieve chromatography, isoelectric focusing, gel electrophoresis, and heat denaturation. The phosphatase preparation studied in that report was obtained by pooling only part of the active fractions eluting from a DEAE-cellulose column. While their data suggested that a bovine heart enzyme exhibited a broad substrate specificity, it did not exclude the possibility that other substrate specific phosphatases were present.

The possible existence of a glycogen synthase-specific phosphatase was suggested by experiments in which hormonal and nutritional treatments altered glycogen synthase phosphatase activity with no changes in phosphorylase phosphatase (56) or phosphohistone phosphatase activity (57). These data can be explained by hypotheses that do not propose that a specific glycogen synthase phosphatase exists. The rate of dephosphorylation of glycogen synthase could be influenced by changes in the phosphorylated form of synthase present (58). Alterations in the sensitivity of synthase phosphatase to glycogen inhibition have been suggested as a possible hormonally

responsive control mechanism (59). The levels of phosphatase inhibitor activity that influence substrate specificity might also be altered in response to hormones (60,61).

Proving that there is a glycogen synthase-specific phosphatase requires the isolation and purification of such an enzyme. There have been relatively few attempts to isolate phosphatase by assaying the dephosphorylation of glycogen synthase due to the large amounts of synthase needed to develop purification schemes. Most laboratories have assayed phosphoprotein phosphatase by dephosphorylation of phosphorylase a (51), phosphohistone (48), or casein (50). Actual glycogen synthase phosphatase activity has usually not been determined for all of the fractions eluting from column chromatography steps. Although it has been expedient to use substrates other than glycogen synthase to isolate phosphatase, several recent observations have emphasized the importance of using the appropriate phosphoprotein substrate. A peak of rat liver synthase phosphatase activity was separated from the phosphorylase a phosphatase by DEAE-cellulose chromatography (62). No other phosphorylated proteins were used as substrates in that study. The synthase phosphatase was highly unstable and was not purified. Synthase phosphatase and phosphorylase phosphatase activities from crude mouse liver extracts were also separated by DEAE-cellulose chromatography (63). The synthase phosphatase peak which had low phosphorylase phosphatase activity coeluted with a heat-stable protein that inhibited phosphorylase phosphatase but not synthase phosphatase, thereby complicating the interpretation that a glycogen synthase-specific phosphatase activity had been observed. A low molecular weight phosphatase isolated from dog heart (64) dephosphorylated glycogen synthase and casein,

but did not act on phosphohistone, H_1. While some phosphatases dephosphorylate a variety of phosphoproteins, these studies indicate that glycogen synthase phosphatase may not always be detected by assaying with other substrates. Assays using phosphorylase, particularly in impure fractions, may fail to detect glycogen synthase phosphatase activity. Phosphohistone phosphatase activity, except for activity on H_1, does appear to correlate with glycogen synthase phosphatase activity.

There have been some reports of phosphoprotein phosphatase preparations that did not dephosphorylate synthase. Gratecos et al. (65) obtained a highly purified phosphatase, isolated by assaying dephosphorylation of phosphorylase a, that did not convert glycogen synthase. The assays were done in the absence of divalent metal ions which are required for activity for some phosphatases (49). Antoniw et al. (66) separated phosphatase fractions on Sephadex G200 and found two peaks of phosphatase that dephosphorylated glycogen synthase, phosphorylase a, and phosphohistones, in addition to a high molecular weight enzyme that was predominantly active on histones H1 and H2B and an enzyme that specifically dephosphorylated phosphorylase kinase phosphorylated in the α-subunit.

Accurate assessment of phosphatase substrate specificity will require the determination of kinetic parameters using well characterized substrates. Recently procedures have been developed for preparing kinase-free glycogen synthase from bovine cardiac muscle (39) and rabbit skeletal muscle (67). It is now feasible to prepare glycogen synthase phosphorylated in specific sites. Determining the K_ms for each type of phosphorylated glycogen synthase and the relative velocity of dephosphorylation of the synthases and other substrates will allow better characterization of substrate specificity of different enzymes.

Molecular sizes of phosphoprotein phosphatases

Multiple phosphoprotein phosphatase activities have been observed both in crude extracts and in partially purified enzyme preparations. As early as 1974, Thomas et al. (68) demonstrated that bovine heart contained multiple molecular weight phosphoprotein phosphatases that differed in their substrate specificity. Previously, several molecular weight phosphatases had been observed in bovine adrenal cortex and liver by Goris et al. (69) and Kalala et al. (70) and in skeletal muscle by Chelala and Torres (71).

A myriad of phosphatase activities that differ in charge or molecular weight have been partially purified. The size of phosphatases with activity on glycogen synthase range from $M_r = 300 \times 10^3$ (72) to $M_r = 35 \times 10^3$ (73) as determined by gel filtration. It is still not established which, if any, of these forms are active in vivo. Several types of mechanisms have been shown to alter molecular weights of phosphatases.

Lee's laboratory has studied the effects of various tissue homogenization techniques on the elution profile of phosphorylase phosphatase from molecular sieve columns (74). A complex elution profile was observed when the liver extract was prepared by vigorous homogenization. At least five molecular size species were detected. When the extract was prepared in a Dounce homogenizer, a single species with a Stokes' radius of 54 Å and a sedimentation coefficient of 7.9S was found. Vigorous homogenization itself may not just physically disrupt oligomeric phosphatases. Imaoka and Ishida (75) homogenized porcine heart tissue for three minutes in a Waring blender. The resulting phosphatase activity eluted from a molecular sieve column as a single peak with an apparent $M_r = 260 \times 10^3$.

One explanation proposed for the generation of multiple peaks of phosphatase is that they arise as a result of proteolysis. Mellgren et al. (76) have shown that a single phosphorylase phosphatase activity is observed in fresh extracts from rabbit skeletal muscle. Storing the extract at 5° for 20 hours in the absence of EGTA resulted in some decrease in the activity with an apparent $M_r = 260 \times 10^3$ and the appearance of an activity with an apparent $M_r = 70 \times 10^3$. Two Ca^{+2}-dependent proteases were partially purified from rabbit skeletal muscle. Addition of one protease to the phosphorylase phosphatase (apparent $M_r = 260 \times 10^3$) resulted in the detection of activity only at an apparent $M_r = 70 \times 10^3$. Only activity at an apparent $M_r = 35 \times 10^3$ could be found after incubation in the presence of the other protease and 2.5 mM $CaCl_2$. Including 5 mM EGTA in the phosphatase preparation inhibited protease activity. The activity of either protease in the presence of Mg^{+2} or Mn^{+2} was only a fraction of that observed in the presence of Ca^{+2}. However, the inclusion of any of these divalent cations in buffers used in the preparation of phosphatases may stimulate proteolytic degradation of the native enzyme(s). Many of the studies to date have been done in the absence of EDTA and EGTA or in the presence of Mg^{+2}, Mn^{+2}, or Ca^{+2}. Phosphatases purified under these conditions may be proteolytically degraded species.

Phosphatase preparations have also been observed to aggregate upon storage (65). The sizes of other preparations have decreased following isolation, possibly due to dissociation or proteolysis (48). Several peaks of activity have been observed upon ion-exchange chromatography of phosphatase preparations (48). Multiple peaks of activity are usually encountered when DEAE-cellulose chromatography is used. Interpretation of elution pro-

files from either ion-exchange chromatography or molecular sieve chromatography is complicated by the presence of endogenous substrates and inhibitors (63).

Purification of phosphoprotein phosphatases

Purification of phosphoprotein phosphatase has been extremely difficult, primarily due to enzyme instability and the generation of multiple phosphatase activities. Enormous effort has been invested in attempting to purify high molecular weight phosphatases. None of the purifications published produces homogenous high molecular weight phosphatase in reasonable quantities. Rabbit skeletal muscle phosphorylase phosphatase has been purified 3000-fold by isolation of the muscle protein-glycogen complex (77). But the phosphatase activity eluted from molecular sieve columns with an apparent $M_r = 125 \times 10^3$ or variably as a mixture of two peaks with apparent $M_r = 80 \times 10^3$ or 48×10^3. Lee et al. (78) purified a phosphorylase phosphatase 750-fold from rat liver. There was evidence that the $M_r = 225 \times 10^3$ species was a proteolytic product of a larger native form of enzyme from which a $M_r = 35 \times 10^3$ subunit had been released. Using DEAE-cellulose chromatography, a 7.1S phosphatase with a Stokes' radius of 55 Å (apparent $M_r = 260 \times 10^3$) was purified 10-fold (79). This phosphatase was only one of four peaks eluting from the ion-exchange column. A porcine heart phosphoprotein phosphatase with an apparent $M_r = 250 \times 10^3$ has been purified 50-fold (75). A spherical 7.5S phosphatase with activity on phosphohistone and phosphorylase has been highly purified from rat liver (80). The phosphohistone phosphatase activity was purified 600-fold in a 0.2% yield, while the phosphorylase phosphatase was purified 1800-fold with

a 0.6% yield. The discrepancy between purification of activity assayed on the different substrates was attributed to the removal of protein that specifically inhibited dephosphorylation of phosphorylase a. A 3.8S, 39 Å Mn^{+2}-dependent phosphoprotein phosphatase was purified 1400-fold from canine heart (81) with a 3% yield. The preparation involved an 80% ethanol precipitation step that also produced low molecular weight phosphatases.

The first phosphoprotein phosphatase purified to homogeneity was a low molecular weight nonspecific phosphatase. Lee's laboratory (73) found that precipitation of impure rabbit liver phosphorylase phosphatase preparations with ammonium sulfate followed by treatment with 80% ethanol led to the isolation of a $M_r = 35 \times 10^3$ phosphatase. The 25,000-fold purified enzyme (82) was shown to be homogenous by SDS gel electrophoresis and dephosphorylated phosphohistone, phosphorylase, phosphorylase kinase, casein, and glycogen synthase. Phosphatases have been highly purified from rabbit skeletal muscle (82), bovine heart (83,84) and canine heart (50). In each of these cases a low molecular weight ($M_r = 30\text{-}35 \times 10^3$) phosphatase capable of acting on several substrates has been obtained. Khandelwal *et al.* (85) purified phosphatase without the ethanol precipitation step by using histone-Sepharose chromatography and obtained two bands on SDS gel electrophoresis that correspond to subunit molecular weights of 31 and 35×10^3 daltons. Gratecos *et al.* (65) purified liver phosphorylase phosphatase several thousand-fold, but the purity could not be satisfactorily established because of a complex pattern on gel electrophoresis, possibly due to aggregation.

Subunit structure of phosphoprotein phosphatases

Decreases in the molecular weight of the active phosphatase following freezing in the presence of mercaptoethanol (48) or treatment with Mn^{+2}, urea, or SDS (68) lead to the hypothesis that some of the active phosphatases observed might contain smaller catalytic subunits. Lee's group found that treatment of various forms of partially purified phosphatase or impure rat liver preparations, containing more than one form of activity, with 80% ethanol, trypsin, or urea (86) resulted in the recovery of only a single $M_r = 35 \times 10^3$ active species. This low molecular weight phosphatase complexed with other proteins in a crude extract. These data supported the hypothesis that multiple forms of phosphoprotein phosphatase contain a common catalytic subunit and regulatory subunits that confer specific properties on the individual forms of phosphatase. It has been difficult to obtain conclusive evidence that the 35×10^3 dalton phosphatase is a catalytic component of an active high molecular weight phosphatase due to the problems of preparing homogenous high molecular weight phosphatase. Protein bands with subunit molecular weights of 35, 55, and 65×10^3 were observed on SDS gel electrophoresis of 750-fold purified rat liver phosphorylase phosphatase (78). Freezing and thawing the preparation in 0.2 M mercaptoethanol produced a single active species of $M_r = 35 \times 10^3$. Another rat liver preparation (80) that migrated as a single protein band on non-denaturing gel electrophoresis contained subunits with molecular weights of 35 and 69×10^3. A major protein band with a subunit molecular weight of 35×10^3 daltons was observed on SDS gel electrophoresis of 3000-fold purified rabbit skeletal muscle phosphatase (apparent $M_r = 125 \times 10^3$), but no data were presented to indicate that the protein band was actually

derived from an active phosphatase (77). These studies indicate that phosphatases of different sizes from different tissues may contain a subunit of similar size.

Regulation of phosphoprotein phosphatase activity

The regulation of phosphatase activity has been studied by three approaches. Substrates have been dephosphorylated by partially purified phosphatases. The phosphatase activity has been determined in crude extracts of tissues. The activity ratio of the endogenous substrate itself has also been used as an indication of phosphatase activity. Several potential types of regulation have been identified by a combination of these approaches.

A number of ionic species influence phosphatase activity. Fluoride is a potent inhibitor as are inorganic phosphate and pyrophosphate (51). Inhibitory effects of ATP have been observed in glycogen-pellets (87), and in crude extracts from rat liver (88) and in partially purified preparations from rabbit skeletal muscle (72) and canine heart (89). The inhibition by ATP and pyrophosphate has been reversed by the addition of Mn^{+2} or Co^{+2}, leading to the proposal that multivalent anions may inhibit phosphatase by chelating an essential metal ion (88,90,91). A number of phosphatases are stimulated by divalent metal cations (48), particularly Mg^{+2} and Mn^{+2}, or are inhibited by EDTA (51). While it has been proposed that phosphatases are metalloproteins (88,90,91), there are no metal ion analyses to indicate that any of the phosphatases contain a metal.

Laloux and Hers (92) contend that the effects of ATP and EDTA may not involve metal ion chelation, but rather may be anionic-charge substrate-

directed effects. The inhibition of phosphatase by ATP in crude mouse liver extracts was observed only when phosphorylase a was present. They propose that ATP and EDTA inhibit conversion of phosphorylase a to b by interacting with phosphorylase. It has been well established that phosphorylase a inhibits the dephosphorylation of partially purified glycogen synthase (51). Studies in crude extracts and glycogen particles indicate that glycogen synthase is not dephosphorylated until the level of phosphorylase a reaches a minimum (93). AMP, glucose, caffeine, and nicotinamide, which stimulate phosphorylase a to b conversion, shorten the lag in glycogen synthase inactivation (94). A number of other phosphorylated proteins can inhibit glycogen synthase dephosphorylation, including histone and phosphorylase kinase (51).

Phosphatase activity can also be modulated by inhibitory proteins. Two low molecular weight protein inhibitors have been purified from rabbit skeletal muscle (95). One of the inhibitors is active only when it is phosphorylated by cAMP-dependent protein kinase (96). Recent evidence indicates that phosphorylation of this inhibitor is induced in vivo in rabbit skeletal muscle by epinephrine (97).

The substrates of phosphoprotein phosphatases are complex molecules themselves, which can be affected by a number of modulators. Changes in the conformation of the phosphoprotein can influence the dephosphorylation reaction. These substrate-directed effects can be mediated by modulators independently of effects directly on the phosphatase. Adenosine and uridine nucleotides inhibit the dephosphorylation of glycogen synthase or phosphorylase a, but not phosphohistone (51). Glycogen specifically inhibits the dephosphorylation of glycogen synthase (51).

The activity ratio of glycogen synthase is altered by hormonal and nutritional treatments. Insulin increases synthase phosphatase activity in dog liver (98), rat adipocytes (99), rat heart (59) and rat liver (100). Decreased glycogen synthase phosphatase activity has been observed in rat heart (59) and dog liver (98) of diabetic animals and in hearts from fasted rats (59). Whether the decreased phosphatase activity is due to alterations in the phosphatase, synthase, or to an effector of synthase dephosphorylation has not been established. The increased activity of glycogen synthase phosphatase(s) in response to insulin in fasted or experimentally induced diabetic animals does not appear to be due to a small molecule (101). Protein synthesis is required for insulin to increase cardiac phosphatase activity in diabetic rats (102). Increases in a heat-stable phosphoprotein phosphatase inhibitor have been observed (103). Glycogen inhibition of synthase dephosphorylation is decreased by feeding and by insulin treatment (59). Bovine heart contains a factor that affects glycogen inhibition of synthase dephosphorylation (104).

The presence of phosphate at one site on a protein may influence the dephosphorylation of another site. Dephosphorylation of the β-subunit of phosphorylase kinase is enhanced by phosphorylation of the α-subunit (105). Hutson et al. (57) have suggested that phosphorylation of a trypsin-sensitive site in rabbit skeletal muscle glycogen synthase may increase the rate of dephosphorylation of the trypsin-insensitive region which regulates glucose-6-phosphate dependency of synthase. Recent evidence from Stalmans' laboratory (42) indicates that the different sites on rat liver glycogen synthase may even be dephosphorylated by distinct enzymes.

When this project began, a procedure had been developed for extensive-

ly purifying bovine heart glycogen synthase (106). A phosphoprotein phosphatase had been purified 150-fold from bovine cardiac tissue (51). The kinetics and regulation of dephosphorylation of synthase by this phosphatase were studied (51,107). However, the enzyme represented only a portion of the activity eluted from DEAE-cellulose chromatography. The elution of the phosphatase from Sephadex G-100 chromatography indicated that the enzyme was smaller than the active species observed in crude extracts of bovine heart (68). The goal of this study was to develop a method of purifying a high molecular weight phosphoprotein phosphatase observed in crude extracts of bovine heart that could be used for studying the dephosphorylation of glycogen synthase. The sizes of the phosphatases present following each purification step were determined during development of the procedures to detect the generation of different molecular weight species.

The practicality of preparing a glycogen synthase phosphatase by copurifying the phosphatase and its substrate by sedimentation in the presence of glycogen was investigated. Many of the glycogen metabolizing enzymes from skeletal muscle can be isolated in a protein-glycogen particle (108). Bovine heart routinely contains low glycogen levels. Only a small fraction of bovine heart synthase sediments in a centrifugation step that would pellet endogenous glycogen, but most of the glycogen synthase can be sedimented following the addition of exogenous glycogen (106). Preparations of glycogen synthase are frequently contaminated by phosphatase activity (68). The activities and sizes of the phosphatases that were isolated with glycogen synthase were determined.

A procedure was developed for purifying a Mn^{+2}-dependent 74 x 10^3 dalton phosphatase approximately 250-fold. Enzyme activities were assayed

on both phosphohistone and phosphorylated glycogen synthase in order to detect phosphatases with different substrate specificities. Some of the molecular size parameters of the partially purified phosphatases were compared to those of enzymes observed in crude extracts.

MATERIALS AND METHODS

Materials

Chemicals and biochemicals

UDPG, glucose-6-phosphate, ATP, cAMP, diaminobutane, dithiothreitol, mercaptoethanol, Tris base, rabbit liver glycogen (Type III), electrophoresis grade sodium lauryl sulfate, Coomassie Brilliant Blue G, Sepharose 4B-200, Sephacryl S-200 and hydroxylapatite were purchased from Sigma Chemical Company. Rabbit liver glycogen was treated with Amberlite MB-3 from Mallinkrodt. Oyster glycogen was obtained from J.T. Baker Chemical Company. Canal Industrial Company was the source of electrophoresis quality bromophenol blue, analine blue black, and ammonium persulfate. Acrylamide, N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylenediamine were purchased from Canal Industrial Company and Biorad. Ultra-pure RNAase-free sucrose was acquired from Schwartz/Mann. Whatman Et31 paper used for glycogen synthase, protein kinase, and ^{32}P-protein phosphatase assays was from Reeve Angel Company. Cyanogen bromide was a product of Aldrich Chemical Company. Protosol, uniformly labeled [U^{14}C]glucose, and carrier free ^{32}P-phosphoric acid were purchased from New England Nuclear. DE23 was from Whatman.

Enzymes and proteins

Calf thymus histone (Type II-A), rabbit muscle phosphorylase a, *E. coli* grade IV β-galactosidase, beef liver catalase, beef blood hemoglobin (Type I), horse heart cytochrome c (Type III) and cAMP-dependent protein kinase inhibitor were obtained from Sigma Chemical Company. Rabbit muscle

lactate dehydrogenase was purchased from Worthington. Miles Laboratories was the supplier of bovine serum albumin.

Methods

Biochemical preparations

UDP-[U^{14}C]glucose was prepared according to Thomas et al. (109). [γ-^{32}P]ATP was made by the procedure of Glynn and Chappell (110). Diaminobutane Sepharose was prepared as described by Mellgren (106). The preparations were aged at 4° for one week to one month depending on the enzyme purification for which they were to be used.

Enzyme preparations

cAMP-dependent protein kinase catalytic subunit: The catalytic subunit of bovine heart cAMP-dependent protein kinase was prepared according to Schlender and Reimann (35) with modifications. Cardiac tissue was homogenized in 2 volumes of 10 mM potassium phosphate buffer, pH 6.8 containing 1 mM EDTA and 0.1 mM DTT. The homogenate was centrifuged at 10,000 x g for 45 minutes. The supernatant was clarified with little loss of activity by a second centrifugation. The supernatant from the second centrifugation was applied to a 400 ml DE23 column. The column was washed with 50 mM potassium phosphate, pH 6.8, containing 1 mM EDTA and 0.1 mM DTT (PED buffer). The catalytic subunit of the protein kinase was dissociated by adding cAMP to the eluting buffer at a final concentration of 10 μM. The enzyme was applied directly to a hydroxylapatite column equilibrated in PED buffer, eluted by a linear 0-0.5 M KCl gradient in PED buffer, and dialyzed overnight against PED buffer. The preparation was applied to a 15 ml hydroxylapatite column equilibrated in PED buffer and eluted with 0.35 M potassium phosphate, pH 6.8, 1 mM EDTA, 0.1 mM DTT. The active fractions

were dialyzed against PED buffer containing 60% glycerol. The final volume of the preparation was 10 ml, with a specific activity of 500 nmole ^{32}P transferred/min/mg protein. The activity was stable for at least 12 months when stored at -20°. The phosphorylation of histone was not stimulated by 10 μM cAMP. Addition of protein kinase inhibitor completely inhibited histone phosphorylation.

Bovine heart glycogen synthase: Glycogen synthase I was prepared by one of two methods depending upon the type of phosphorylated synthase to be produced from it. The procedure described by Mellgren (106) was followed when the synthase was to be phosphorylated by endogenous kinases. The modification of that purification scheme described by Mitchell (39) was used to prepare glycogen synthase free of endogenous kinase. The synthase preparations were phosphorylated as described in the section concerning the preparation of phosphoprotein substrates.

Purification of phosphoprotein phosphatases

Purification of glycogen-pelleted phosphoprotein phosphatases: One kg of frozen bovine cardiac muscle (stored at -70° for 2 days to 2 months) was broken into small pieces with a mallet. The pieces were thawed and homogenized in a Waring Blender with two 20-second bursts in 2 ml of buffer/g tissue. The buffer contained 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, pH 7.8 (TEE buffer). The homogenate was centrifuged at 10,000 x g for 45 minutes and the supernatant was strained through glass wool. Ethanol (95%) was added to the supernatant until the final ethanol concentration reached 30%. The ethanol temperature was -20°, and the temperature of the enzyme preparation was maintained at 1° until enough ethanol had been

added to reach a concentration of 7% ethanol. The solution was then cooled to -1° and the rest of the ethanol added while keeping the temperature between -1° and 0°. The preparation was then cooled to -5° and centrifuged at 10,000 x g for 45 minutes at -10°. The supernatant was decanted and the pellets inverted to remove the ethanol-containing fluid. The 30% ethanol pellet was homogenized in a motor-driven Potter Elvejhem Teflon glass homogenizer, surrounded by ice, in 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 50 mM mercaptoethanol, pH 7.8 (TEEM buffer). The 270 ml resuspended pellet was centrifuged at 78,000 x g for 5 hours. The supernatant was filtered through glass wool and oyster glycogen (80 mg/ml) was added to the fraction to a final concentration of 5 mg/ml. The preparation was allowed to sit for 6 hours and then it was centrifuged at 78,000 x g for 6 hours. The pellet was resuspended in approximately 20 ml of 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 50 mM mercaptoethanol, 25% glycerol, pH 7.8 (TEEMG-25 buffer). Glycogen was digested by adding α-amylase to a final concentration of 10 μg/ml and then dialyzing the preparation for 3 hours at room temperature against 1 liter of TEEMG-25. The enzyme was then applied to a 40 ml diaminobutane-Sepharose column equilibrated in TEEMG-25. The resin was prepared one week prior to use and stored at 4° in the presence of 50 mM Tris-HCl, pH 7.8, 0.02% sodium azide. The enzyme was applied to the column at a flow rate of 0.5 ml/min. and the column subsequently washed at 1 ml/min with TEEMG-25 until the OD_{280} of the column effluent reached the baseline. The column was then eluted with a linear gradient (500 ml) of 0-0.5 M NaCl in TEEMG-25. The fractions were cooled and stored at 4° immediately after elution and then assayed for glycogen synthase phosphatase activity and/or phosphohistone phosphatase activity.

The active fractions were pooled and dialyzed for 12 hours against 1 liter of TEEM containing 60% glycerol (TEEMG-60). The phosphatase preparation was then stored at -20°.

Preparation of soluble phosphoprotein phosphatase: The initial stages of the purification were identical to the scheme just described for preparing glycogen-pelleted phosphatases, except that the starting material was 500 g of frozen bovine cardiac muscle. The resuspended 30% ethanol-pellet was centrifuged at 161,000 x g for 2.5 hours. Glycerol, cooled to 4°, was added to the supernatant with gentle stirring to a final concentration of 25%. A solution containing 500 mM Tris-HCl, pH 7.8, 50 mM EDTA, 50 mM EGTA and 500 mM mercaptoethanol was added to maintain the final concentrations at 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA and 50 mM mercaptoethanol. The preparation was allowed to warm to room temperature slowly (approximately 30 minutes) while gently stirring. The phosphatase preparation (200 ml) was then loaded onto a 60 ml diaminobutane-Sepharose 4B column (1.7 x 27 cm), equilibrated in TEEMG-25, at a flow rate of 1 ml/min. The column was washed with approximately 100 ml of TEEMG-25 until the OD_{280} returned to the baseline level and then eluted with a 300 ml linear gradient of 0-0.4 M NaCl in TEEMG-25. Fractions were immediately transferred to 4° and then assayed for phosphatase activity. The active fractions were pooled and dialyzed for 8 hours against TEEMG-60. The phosphatase preparation (100 ml) was concentrated by applying it to a 20 ml diaminobutane-Sepharose column which was washed with 50 mM NaCl in TEEM and eluted with 300 mM NaCl in TEEM at a flow rate of 0.3 ml/min. The active fractions were pooled and dialyzed against TEEMG-60 for 8 hours, then against TEEM for 1.5 hours. The fraction (approximately 1.5 ml) was chromatographed on

a 170 ml Sephacryl S-200 column (1.6 x 85 cm) at a flow rate of 0.75 ml/min. The active phosphatase was pooled and dialyzed for 12 hours against TEEMG-60 and stored at -20°. The enzyme was stable for several months when stored under these conditions.

Preparation of phosphoprotein substrates

Phosphohistone: Phosphohistone was prepared by incubating 35 mg/ml Sigma Type II-A calf thymus histone, 25 mM MES, 6.25 mM $MgCl_2$, pH 6.5 and 5 U/ml catalytic subunit of cAMP-dependent protein kinase with 1 mM ATP or 1 mM [γ-^{32}P]labeled ATP (1-5 x 10^5 cpm/nmole) for 3 hours at 30° then 12 hours at 4°. The protein was precipitated by adding cold 100% TCA to a final concentration of 20%. After sitting on ice for 20 minutes, the supernatant was removed by centrifuging at 10,000 x g for 10 minutes. The precipitated phosphohistone was resuspended in 0.36 N H_2SO_4 and dialyzed for 12 hours against 1 liter of the acid at 4°, then for 12 hours against 5 mM Tris, pH 7.5, and for 12 hours against 0.5 mM Tris, pH 7.5. The substrate was stored at -20°.

Phosphorylated glycogen synthase: Glycogen synthase purified according to Mellgren (106) contains contaminating kinase activity. This activity has been shown to be due to both cAMP-dependent protein kinase and cyclic nucleotide-independent protein kinase(s) (39). Glycogen synthase I (5-10 U/ml) was phosphorylated by incubation for 15 minutes with DTT and cAMP at pH 7.8, then $MgCl_2$ and ATP or [γ-^{32}P]ATP (2-7 x 10^5 cpm/nmole) added to give a final ATP-Mg concentration of 1 mM and 5 mM Mg^{++}. The final concentration of DTT was 5 mM while that of cAMP was 10 µM. After 5 minutes at 30°, 1 M KF was added to a concentration of 50 mM. The synthase phosphorylation reaction was incubated at 30° for 2 hours then

incubated at 4° until the activity ratio had decreased to less than 5% I. The phosphorylation usually required 48 to 72 hours. The phosphorylated glycogen synthase was then purified as described by Mellgren (106). The final specific activity of the enzyme preparations ranged from 15 to 35 U/mg protein. Only preparations that were used as substrates were not contaminated by glycogen synthase phosphatase.

Glycogen synthase, phosphorylated by the catalytic subunit of cAMP-dependent protein kinase at 1 mM ATP or 1 mM [γ-^{32}P]ATP (1.5-5 x 10^5 cpm/nmole) was prepared and purified according to Mitchell (39). The phosphorylated synthase had an activity ratio of 26% I and a specific activity of 20 U/mg protein and was free of contaminating glycogen synthase phosphatase.

The ^{32}P-labeled glycogen synthase used as a substrate contained less than 5% TCA soluble ^{32}P. The phosphorylated synthase was stored in a liquid nitrogen freezer.

Enzyme assays

Glycogen synthase: The activity of glycogen synthase was assayed by following the incorporation of [U-^{14}C]glucose from UDP-[U-^{14}C]glucose (4 x 10^4 cpm/µmole) into glycogen according to Thomas et al. (109). One unit of glycogen synthase activity is defined as the amount of enzyme catalyzing the incorporation of 1 µmole of glucose from UDP-glucose into glycogen per minute at 30°. Total glycogen synthase activity was measured in the presence of 10 mM glucose-6-phosphate while glycogen synthase I was determined in the absence of glucose-6-phosphate and the presence of 14 mM Na_2SO_4 (111). The ratio of activity in the presence of Na_2SO_4 to that in the presence of glucose-6-phosphate multiplied by 100 is defined as percent glycogen synthase I.

cAMP-dependent protein kinase: cAMP-dependent protein kinase catalytic subunit activity was assayed using Sigma Type II-A histone as a substrate (35). The amount of enzyme catalyzing the transfer of 1 mole of ^{32}P from [γ -^{32}P]ATP (1 x 10^5 cpm/nmole) to histone per minute at 30° is defined as 1 unit of kinase activity.

Glycogen synthase phosphatase: Glycogen synthase phosphatase activity was measured by conversion of the enzyme from the forms that were dependent on glucose-6-phosphate to the form that did not require glucose-6-phosphate for activity. Glycogen synthase was preincubated at 30° for 15 minutes at a concentration of 1.5 U/ml in 5 mM DTT, 50 mM Tris-HCl at pH 7.5. Immediately before starting the phosphatase reaction, $MnCl_2$ was added to a final concentration of 5 mM free Mn^{+2}. The assay was started by the addition of phosphatase and incubated for 10 or 20 minutes at 30°, then terminated by a 10- to 20-fold dilution in 50 mM Tris-HCl, 20 mM DTT, 1 mg/ml rabbit liver glycogen, 5 mM EDTA, 50 mM KF, pH 7.8 at 4°. Aliquots were assayed for synthase I and total synthase activity. Glycogen synthase phosphatase activity was calculated as units of glycogen synthase converted from the D form to the I form per minute. Total synthase activity was constant during the incubation period. A control reaction containing no added phosphatase was run along with each set of assays to correct for any change in the activity ratio of the synthase during the incubation period. Usually less than 2% variation was found in the percent synthase I determined at the beginning of the incubation compared to that found after the control reaction was terminated.

Phosphoprotein phosphatase: Phosphoprotein phosphatase could also be measured as the release of ^{32}P from radioactively labeled substrates.

Assays on all substrates were measured in the presence of 5 mM DTT and 50 mM Tris-HCl at pH 7.5 and in the presence of 5 mM free Mn^{+2} added as $MnCl_2$ or with no added Mn^{+2}. Phosphohistone phosphatase was assayed at 1 or 2 mg of phosphohistone/ml in the presence of 0.1 M NaCl. Assays were stopped by adding 20 µl of 80 mM silicotungstic acid in 80 mM H_2SO_4 to the 50 µl reaction volume. Release of ^{32}P from ^{32}P-glycogen synthase was quantitated by assaying the phosphatase as described for the non-radioactive substrate. The reaction was terminated by adding 10 µl of 100% TCA to a 25 µl assay followed by 20 µl of H_2O and 5 µl of 10 mg/ml bovine serum albumin. Assays for both substrates were allowed to sit on ice for 20 minutes, and centrifuged for 5 minutes at 10,000 x g. Fifty µl aliquots were spotted on 2 x 2 cm Whatman ET31 filter papers, dried, and counted for radioactivity. Control reactions containing no phosphatase were run along with each set of assays to correct for nonprecipitable ^{32}P-protein counts.

Fractions from the initial stages of purification contain endogenous phosphoprotein phosphatase substrates and inhibitors. The phosphoprotein phosphatase activity was proportional to enzyme concentration, but only within a narrow concentration range. Fractions were diluted 1:10 and 1:20 or 1:20 and 1:40. Fractions were assayed at the two concentrations that produced the highest constant specific activity.

Electrophoretic procedures

Electrophoresis of histones: Histone fractions were separated by the urea-acetic acid system described by Panyim and Chalkley (112). Twenty µg ^{32}P-histone prepared according to Methods were applied to a 10 cm 15% polyacrylamide gel and electrophoresed for 4 hours at 2 milliamps/gel at

room temperature. The gels were stained with 0.1% analine blue black and destained in acetic acid:methanol:H_2O (7:20:73).

SDS polyacrylamide gel electrophoresis: Samples were electrophoresed in the SDS-polyacrylamide gel system described by Weber and Osborn (113). The polyacrylamide concentration of the gels in a particular experiment was either 6.0 or 6.5% polyacrylamide. Protein samples (4-50 µg) were incubated in 4 M urea, 1% SDS, 1% mercaptoethanol, 0.01 M sodium phosphate, pH 7.0 for 2 hours at 37° or boiled for 10 minutes. Three µl of 0.05% bromophenol blue were added to each sample immediately before applying to gels. The 0.6 x 9 cm gels were electrophoresed in a Shandon analytical polyacrylamide electrophoresis apparatus at 8 milliamps/gel. The running buffer consisted of 0.1 M sodium phosphate, 0.1% SDS, pH 7.2. Electrophoresis was terminated after the bromophenol blue tracker dye migrated approximately 8 cm. β-galactosidase, phosphorylase a, bovine serum albumin, lactate dehydrogenase, and cytochrome c were run simultaneously in separate gel tubes. The gels were washed for 12 hours in 1 liter of 3.5% perchloric aid to fix the protein and to remove SDS. The acid solution was changed once during the washing procedure. The gels were stained for 12 hours in a 0.04% Coomassie Brilliant Blue G-250 solution containing 3.5% perchloric acid and destained in 5% acetic acid, 20% ethanol. The molecular weights of the protein bands observed on the gels were calculated from comparison to standard curves of mobility, relative to bromophenol blue, as a function of the log of the molecular weight.

Selected stained gels were scanned for protein at 595 nm in a Gilford spectrophometer, provided by Dr. Stan Cox. Relative protein concentrations were determined by measuring the area under the triangulated peaks.

The radioactivity present in electrophoresed ^{32}P-proteins was detected by incubating 1.5 mm gel slices in 10 ml of 3% Protosol/scintillation fluid at 37° for 20 hours. Liquid scintillation counting of the solubilized gel slices yielded a 34% counting efficiency.

Nondenaturing polyacrylamide gel electrophoresis: A Tris-glycine-mercaptoethanol gel system was used for electrophoresing phosphatase samples while maintaining activity (114). Six cm gels were formed containing 7.5% w/v polyacrylamide. After polymerization, the gels were cooled to 4°, pre-electrophoresed for 1.5 hours in 25 mM Tris, 192 mM glycine, 10 mM mercaptoethanol, pH 8.3 and allowed to equilibrate overnight. Ten µg of phosphatase in 50 mM Tris, 5 mM EDTA, 5 mM EGTA, 50 mM mercaptoethanol, pH 7.8 and 2 µl of 0.5% bromophenol blue were applied to replicate gels and electrophoresed for 4 hours at 2 milliamps/gel at 4°. The gels were either stained for protein or sliced into 1.5 mm segments for activity determinations. Protein was visualized by immersing gels in 12.5% TCA for 30 minutes, then staining for 60 minutes in 0.05% Coomassie Brilliant Blue G-250 in 12.5% TCA and destaining in 12.5% TCA. The gel slices used for activity determinations were crushed and incubated in 0.20 ml of assay solution containing 150 µg of ^{32}P-histone (3×10^4 cpm/nmole), 0.10 M NaCl, 20 mM DTT, 5 mM $MnCl_2$, and 50 mM Tris, pH 7.5 at 30° for 3 hours, then for 12 hours at 4°. Fifty µl aliquots were removed at 2 and 15 hours and precipitated by 20 µl of 80 mM silicotungstic acid. ^{32}P acid-soluble counts were determined as described for phosphatase assays.

Sucrose gradient electrophoresis: Two-tenths ml phosphatase was layered on a 10 ml 5-30% linear sucrose gradient in 50 mM Tris, 5 mM EDTA, 5 mM EGTA, 5 mM DTT, pH 7.8 and run in an ISCO model 212 sucrose gradient

electrophoresis apparatus at 4°C at 400 volts and 4-5 milliamps for 4 hours. The $OD_{280\ nm}$ was monitored during collection of the 0.3 ml fractions.

Molecular size determinations

Sucrose gradient centrifugation: Five ml 5-25% linear sucrose gradients in 50 mM Tris, 5 mM EDTA, 5 mM EGTA, 5 mM DTT, pH 7.8 were made with an ISCO 570 gradient former. Two-tenths ml of sample were layered on each gradient and the gradients were run according to Martin and Ames (115) for either 20 hours at 40,000 rpm or 17 hours at 50,000 rpm in an SW 50.1 rotor in a Beckman L-4 or L-5-75 centrifuge at 4°. Gradient fractions were collected using an ISCO model 184 density gradient fractionator. Hemoglobin and phosphorylase a were used as molecular size markers. The gradients were determined to be linear with respect to sucrose concentration and isokinetic with respect to phosphorylase migration. Sedimentation coefficients of enzyme peaks were determined by their relative mobility to the marker proteins (115).

Molecular sieve chromatography: Phosphatase samples were chromatographed on Sephacryl S-200, or Biogel A columns. Catalase, bovine serum albumin, lactate dehydrogenase, hemoglobin, and cytochrome c were run as standards. Blue dextran and ATP were used to determine the void volume and the totally included column volume respectively. The Stokes' radius of each phosphatase peak was determined according to Siegel and Monty (54).

The sizes of the protein standards used to calculate the molecular parameters of the phosphatases are listed in Table 1.

Other analytical methods: Sucrose concentrations were determined by measuring the refractive index with a N-1 hand refractometer from Atago Company, Limited.

Table 1. Molecular size parameters of standard proteins

Protein	Stokes radius (Å)	$s_{20,w} \times 10^{13}$ (S)	Molecular weight (daltons)	Subunit molecular weight (daltons)	Reference
Catalase	52	11.3	250×10^3		54
Lactate dehdyrogenase	42		114×10^3	36.0×10^3	54
β-Galactosidase				130×10^3	113
Phosphorylase a		8.4		97.4×10^3	116,117
Bovine serum albumin	35	4.3		65.0×10^3	54
Hemoglobin		4.3	66×10^3		54
Cytochrome c	17	1.9		12.4×10^3	54

Conductivity was measured at ambient temperature using a type CDM2e Radiometer conductivity meter. NaCl concentrations in column fractions were determined by comparing the conductivity to that of standard solutions prepared in the buffer used to elute the column.

Protein concentrations were determined according to Lowry, et al. (118). Crystalline bovine serum albumin was used as the standard.

Glycogen concentrations were assayed by precipitating the high molecular weight carbohydrate by adding ethanol to 66% (119) and using the phenolsulphuric acid test to detemine total carbohydrate (120).

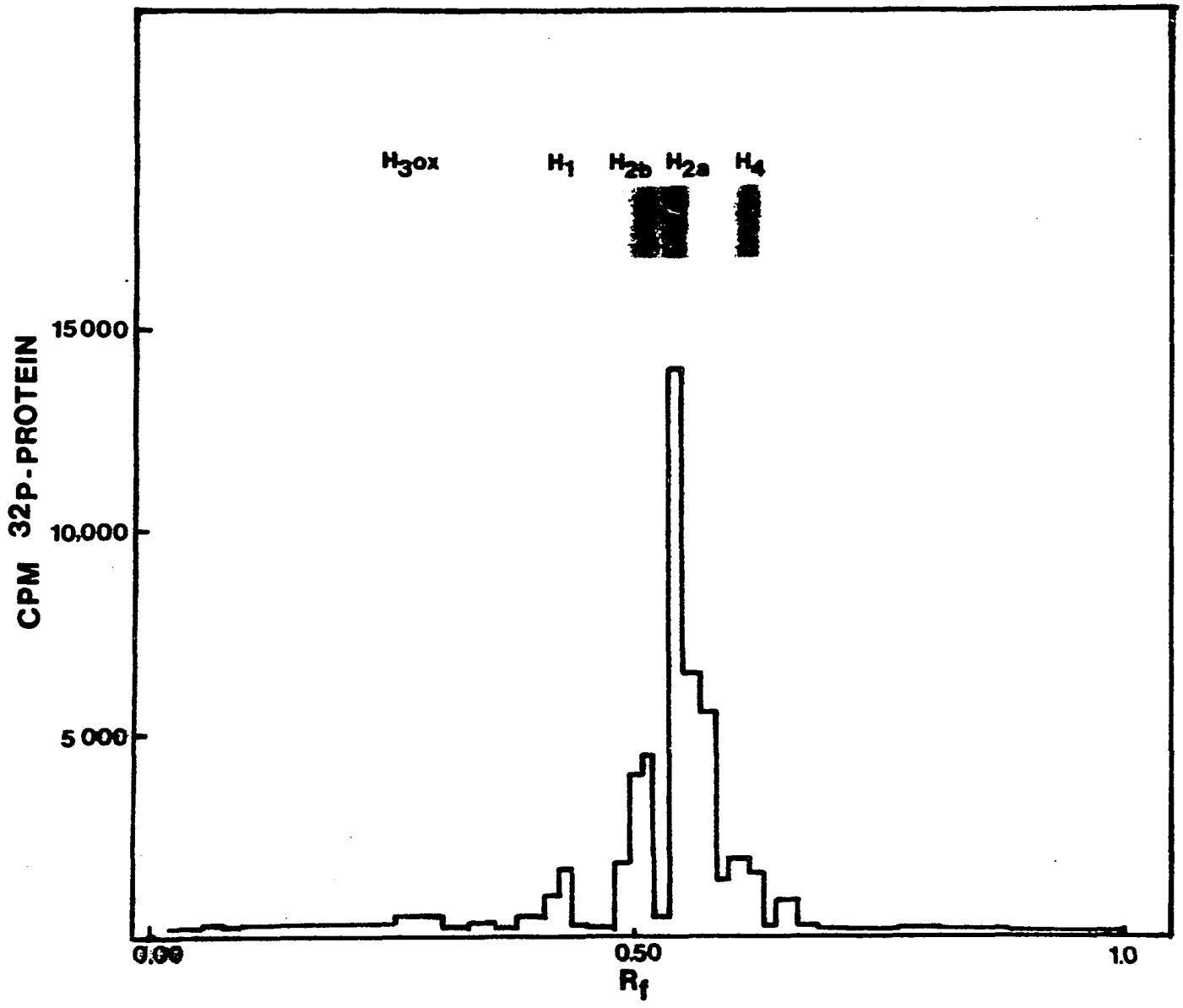
RESULTS

The lack of available purified phosphoprotein phosphatases has hindered studying the dephosphorylation of glycogen metabolizing enzymes. The highly purified phosphatases studied to date have been smaller species than those observed in crude extracts. One of the goals of this research was to develop a purification scheme for a high molecular weight bovine heart phosphoprotein phosphatase that dephosphorylated glycogen synthase.

Preparation of Phosphorylated Substrates

The development of an enzyme purification requires a sufficient quantity of substrate for determining enzyme activities. Phosphorylated histone was used throughout much of this study to detect phosphoprotein phosphatase activity. Phosphohistone was selected as a substrate for several reasons. Histone fractions are commercially available as highly purified preparations that are free of other phosphorylatable proteins. Cyclic AMP-dependent protein kinase, which is used to phosphorylate histones, can be prepared in large quantities and is stable for long periods of time. The amounts of cyclic AMP-dependent protein kinase needed to phosphorylate histone are small enough that extensive separation of the kinase from the phosphorylated histone is not necessary. Phosphohistones are precipitated well by silicotungstic acid, therefore, intereference with the determination of ^{32}P released from the P-histone substrate during phosphohistone phosphatase assays is minimal, allowing low activity to be detected. The release of less than 2% of the ^{32}P in the substrate is easily and accurately quantitated. The rapid assay of ^{32}P release from ^{32}P-histone allows

Figure 1: Twenty μg ^{32}P-histone were mixed 1:1 (v/v) with 20% sucrose, 0.90 M acetic acid and electrophoresed on 15% polyacrylamide gels for 4 hours at 8 milliamps/gel as described in Methods. Replicate gels were immersed for 12 hours in 0.01% analine blue black staining solution, then destained in 5% acetic acid, 20% methanol or sliced into 1.5 mm segments and incubated for 20 hours in 10 ml 3% Protosol-Beckman scintillation fluid and counted in a scintillation counter.


H_{3ox}
H_1
H_{2b}
H_{2a}
H_4
15000
10,000
5 000
CPM ^{32}P-PROTEIN
0.00
0.50
1.0
R_f

active fractions of phosphatase to be quickly located and further processed. Since the phosphatases are not stable at dilute concentrations encountered during some purification steps, a rapid reliable phosphatase assay is crucial to obtaining good recovery of activity.

The amount of phosphate incorporated into histone was in the range of 6-13 nmoles phosphate/mg protein. This represents approximately one to two phosphates per molecule of histone, based on an average molecular weight of 15 x 10^3 daltons for histone. Sigma Type II-A histone contains all 5 of the major subfractions of calf thymus histones. The major classes of histones are identified as fraction H1, H3, H2B, H2A or H4 based on electrophoretic mobility (112). Each of these fractions can be phosphorylated by cAMP-dependent protein kinase. Figure 1 shows that all of the ^{32}P in a phosphohistone preparation could be localized in protein bands that could be attributed to histone fractions. The silicotungstic acid-soluble ^{32}P represented 0.1-0.5% of the total radioactivity.

Phosphatase activity was also assayed on bovine heart glycogen synthase. The purity of the phosphorylated glycogen synthase is shown in Figure 2. The molecular weight of the major protein band on SDS polyacrylamide gels was calculated to be 86 x 10^3 daltons which concurs with the subunit molecular weight of bovine heart glycogen synthase (106). The specific activities of the synthase preparations used in this study ranged from 15-35 U/mg protein. The highest specific activity reported for the bovine enzyme is 35 U/mg protein (106). The high specific activity and the observation of a single major protein band on SDS polyacrylamide gels indicate that the glycogen synthase used as a substrate for the phosphoprotein phosphatase was highly purified.

Figure 2: Correlation of ^{32}P-protein and with glycogen synthase. Twenty µg of ^{32}P-labeled glycogen synthase (phosphorylated by endogenous kinases) were electrophoresed in 6.5% SDS-polyacrylamide gels according to Methods. Replicate gels were either stained for protein with Coomaissie Brilliant Blue G250 and the molecular weights of the bands calculated from the standard curves or sliced into segments for location of ^{32}P-protein as described in Methods.

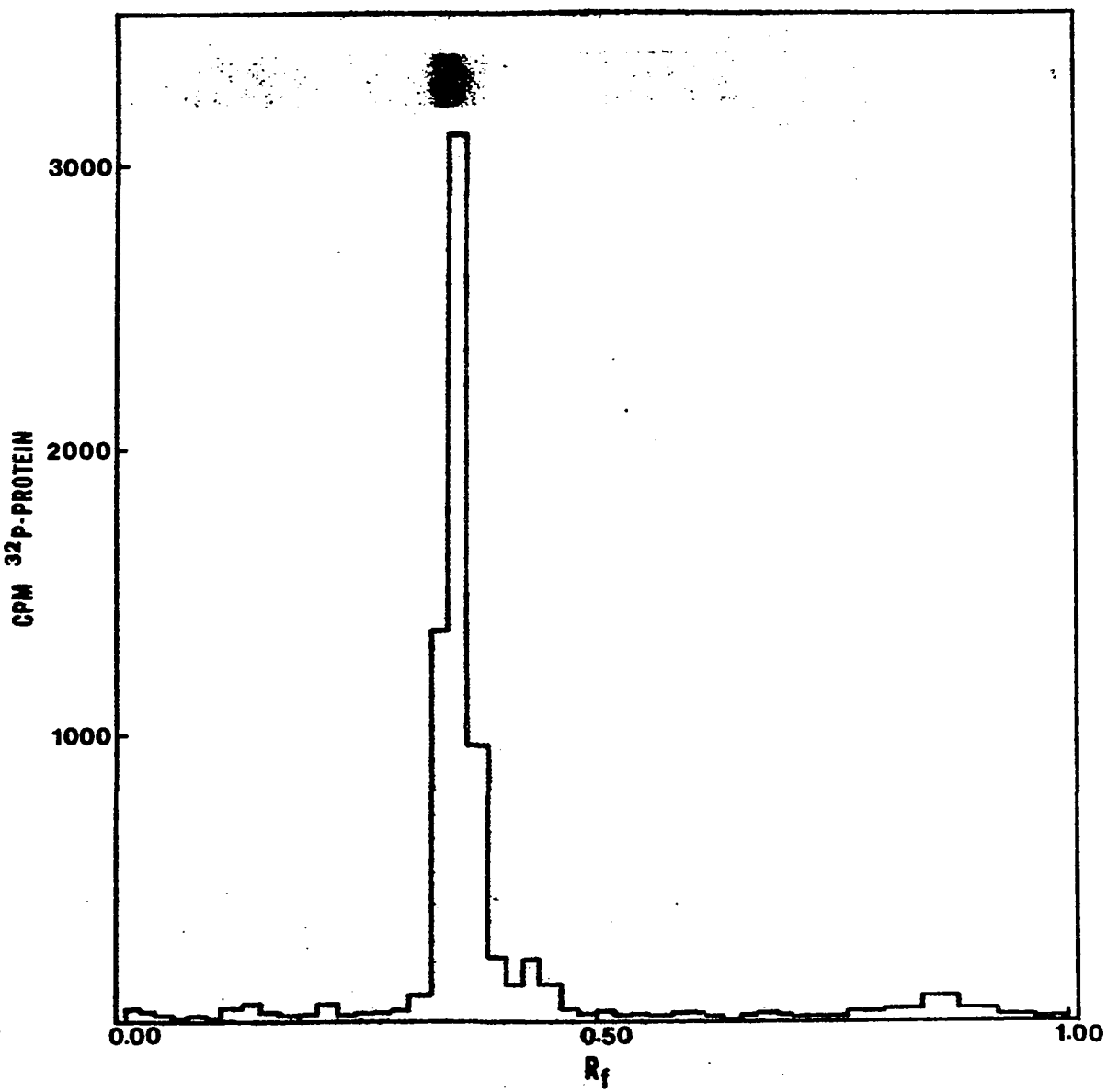

3000
2000
1000
CPM ^{32}P-PROTEIN
0.00
0.50
1.00
R_f

Figure 2 shows that the ^{32}P-protein present in the glycogen synthase substrate preparation migrated as glycogen synthase. The absence of ^{32}P-protein contaminants in the ^{32}P-glycogen synthase preparations permitted phosphatase activity to be determined by quantitating the release of ^{32}P from the radioactively labeled substrate.

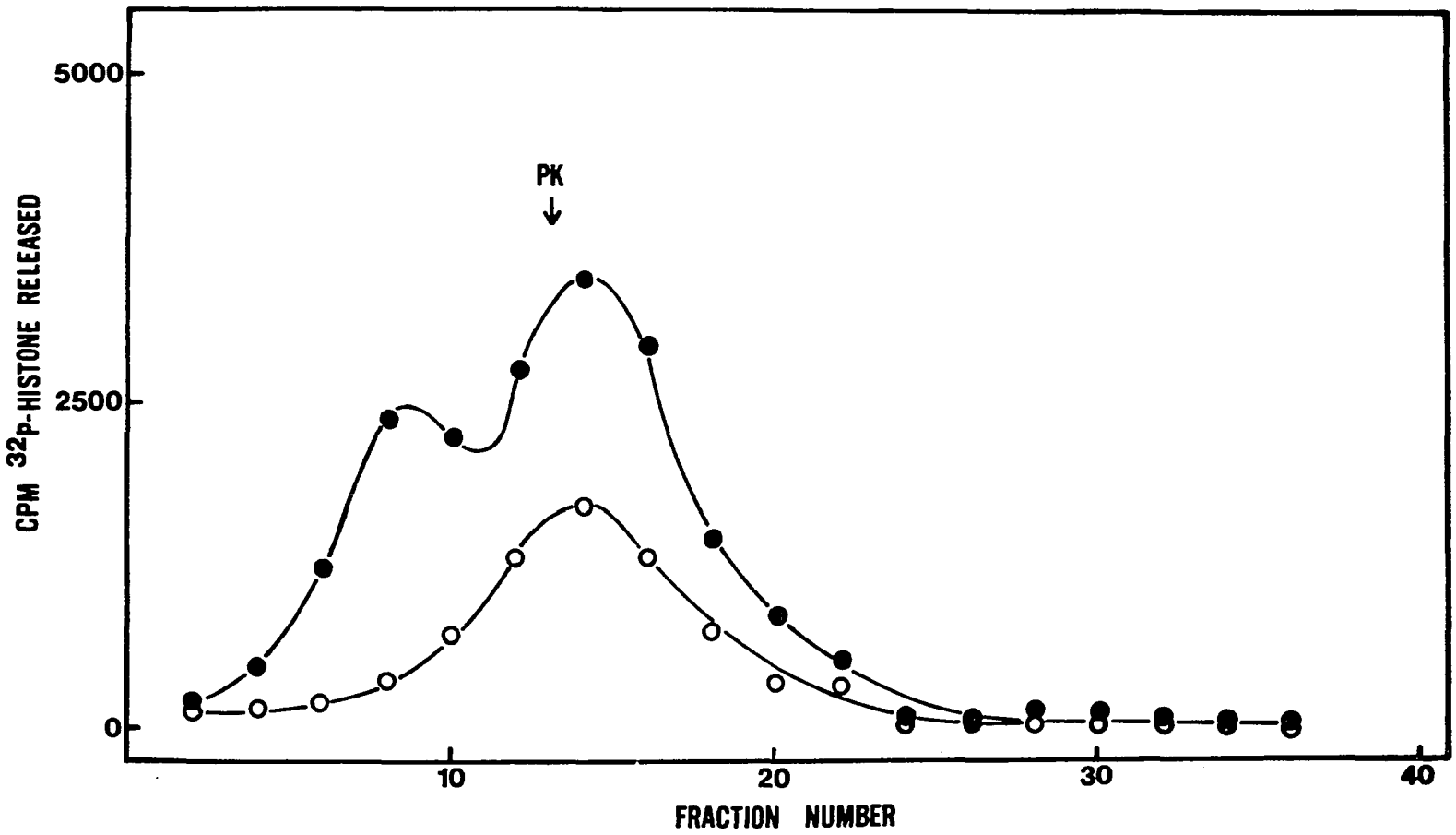
Phosphoprotein Phosphatase Activities in Bovine Heart

Phosphoprotein phosphatases from crude extracts of a number of tissues have been observed to have multiple molecular weights. It was of interest to determine the size of phosphoprotein phosphatases present in bovine heart. Bovine heart was homogenized in a Potter Elvejhem homogenizer and the crude extract was centrifuged at low speed to remove debris. The sizes of the phosphatases present in this crude extract supernatant were analyzed by sucrose gradient centrifugation. A single peak of phosphatase activity with a $s_{20,w} = 7.0S$ was detected when the fractions were assayed in the absence of divalent cation (Fig. 3). When the fractions were assayed in the presence of 5 mM Mn^{+2}, the $s_{20,w} = 7.0S$ phosphatase activity was stimulated approximately two-fold, and another peak of phosphatase activity was observed migrating at a position corresponding to $s_{20,w} = 4.3S$. Since the lower molecular weight phosphatase activity was highly Mn^{+2}-dependent, 5 mM Mn^{+2} was included in assays of total phosphatase activity.

Association of Phosphoprotein Phosphatase Activity with Glycogen and Glycogen Synthase

Phosphorylase, phosphorylase kinase, phosphorylase phosphatase, glycogen synthase, and glycolytic enzymes can be sedimented with glycogen in extracts from rabbit skeletal muscle (108). This enzyme-carbohydrate

Figure 3: Sucrose gradient centrifugation of phosphoprotein phophatases present in a bovine heart homogenate. Eighteen g of frozen bovine heart were thawed and homogenized in a motor-driven Potter Elvejhem Teflon glass homogenizer in 2 volumes of 50 mM Tris-HCl, 5 mM EDTA, 50 mM mercaptoethanol, pH 7.8. All steps were carried out at 4°. The extract was centrifuged at 10,000 x g for 45 minutes and the supernatant filtered through glass wool. The supernatant (0.2 ml) was layered on a sucrose gradient and centrifuged and collected as described in Methods, except that the gradients were centrifuged for 16 hours at 2° and 37,000 rpm. The gradient fractions were assayed for phosphohistone phosphatase activity in the absence of cation (o - o) or in the presence of 5 mM free Mn^{+2} (● - ●). Cyclic AMP-dependent protein kinase was assayed as described in Methods. The peak of protein kinase activity used as an internal reference ($s_{20,w}$ = 6.5S), is denoted by an arrow.

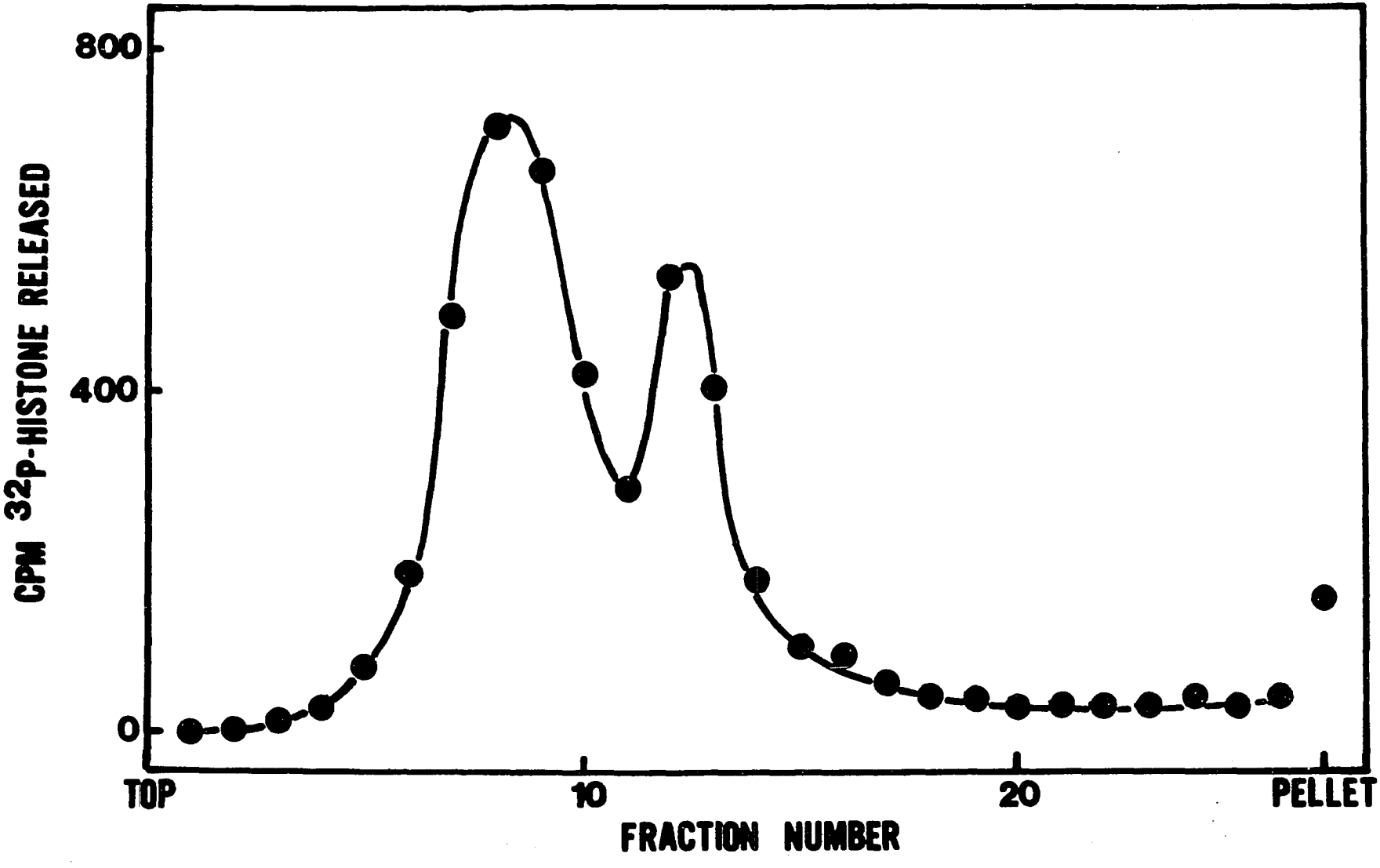

5000
2500
0
PK
CPM 32P-HISTONE RELEASED
10
20
30
40
FRACTION NUMBER

complex has been called a glycogen particle. These glycogen particles can be isolated by ultracentrifugation. Electron micrographs of the isolated particles are similar to micrographs of glycogen in cells (121). Compartmentalization of glycogen and its metabolizing enzymes provides an efficient regulatory system. If a specific glycogen synthase phosphatase exists, one might expect the activity to be associated with its substrate. Glycogen synthase phosphatase activity is routinely present in glycogen synthase preparations (68). In fact, only very highly purified preparations of glycogen synthase are free of phosphatase activity. The feasibility of preparing glycogen synthase phosphatase by isolating glycogen synthase and the phosphatase activity that copurifies was investigated.

The procedure developed to isolate bovine heart glycogen synthase was used to purify phosphoprotein phosphatase, as described in Methods (p. 23). EGTA was included throughout the purification to minimize Ca^{+2}-stimulated protease activity. Such proteases have been shown to decrease the size of phosphoprotein phosphatase (76). The sedimentation coefficients of the phosphatase activities were monitored at each step of the preparation to detect procedures that altered the sizes of the dephosphorylating enzymes.

The sedimentation profile in a sucrose gradient of the phosphatase activity precipitated from a crude extract by 30% ethanol (30% ethanol pellet) is shown in Figure 4. The phosphatases in the 30% ethanol pellet migrated with $s_{20,w}$ = 4.8S and 6.8S. The distribution of activity was very similar to the profile of activity in a crude extract as shown in Figure 3. No low molecular weight phosphatases were observed. A small amount of activity pelleted to the bottom of the gradient. A 5-fold purification was obtained in the ethanol precipitation step, with no loss of activity.

Figure 4: Sucrose gradient centrifugation of phosphoprotein phosphatases present in a 30% ethanol-pellet. A bovine heart extract was prepared and treated to yield a 30% ethanol-pellet as described in Methods. The homogenized pellet was layered on a 5 ml 5-25% sucrose gradient and centrifuged for 16 hours at 2° and 37,000 rpm. Fractions were collected and assayed for ^{32}P-histone phosphatase activity in the presence of 5 mM Mn^{+2} (o - o). Cytochrome c, hemoglobin and phosphorylase a, used as standard marker proteins, were centrifuged in a parallel gradient and detected as described in Methods.


800
400
0
CPM 32P-HISTONE RELEASED
TOP
10
20
PELLET
FRACTION NUMBER

The sedimentation of the phosphatase with glycogen was studied by determining the amount of phosphatase activity that pelleted at 78,000 x g in the absence and presence of exogenous glycogen. Oyster glycogen was added to the post-microsomal supernatant which was centrifuged for 6 hours at 78,000 x g. The pellet from this centrifugation step was resuspended as described in Methods and is called the glycogen-pellet. The soluble fraction is referred to as the post-glycogen supernatant. Glycogen synthase phosphorylated by endogenous kinases and phosphohistone were used to assay the phosphatase activity present in each fraction. The phosphatase activity was stable during the centrifugation step. As Table 2 indicates, a low percentage of the phosphatase activity pelleted with the exogenous glycogen. Adding glycogen to the post-microsomal supernatant increased the percentage of phosphatase activity in the pellet by two- to three-fold. Only 16% of the phosphohistone phosphatase activity was recovered in the glycogen-pellet (average of 7 experiments). The low percentage of phosphatase sedimenting was not due to incomplete glycogen precipitation since 94% of the glycogen was recovered in the pellet. Eighty-seven percent of the glycogen synthase activity present in the post-microsomal supernatant was recovered in the pellet. Phosphatase activity that pelleted could not be attributed to simply liquid trapped in the pelleting material during centrifugation. The pellet occupied 4-5% of the total volume, while 16% of the phosphatase activity was routinely recovered in the pellet. The recovery of phosphohistone phosphatase and glycogen synthase phosphatase in the pellet was equal. These experiments provided no evidence that there was a specific glycogen synthase phosphatase that could be sedimented with glycogen and glycogen synthase.

Table 2: Sedimentation of enzyme activities with glycogen

Fraction	Glycogen[a]	Glycogen Synthase Activity		
	% Total	Units[b]	% Total	% Recovered
Post-microsomal supernatant		303		
Post-microsomal supernatant + glycogen		218		145
78,000 post-glycogen supernatant	6.0	88	40	
78,000 glycogen-pellet	94	131	60	
Post-microsomal supernatant (no exogenous glycogen)		150		100
78,000 x g supernatant		135	90	
78,000 x g pellet		15	10	

[a]Determined by precipitation of samples with 66% ethanol (119) and quantitation with phenol-sulphuric acid (120).

[b]1 Unit = 1 mole glucose transferred from UDP-glucose into glycogen per minute.

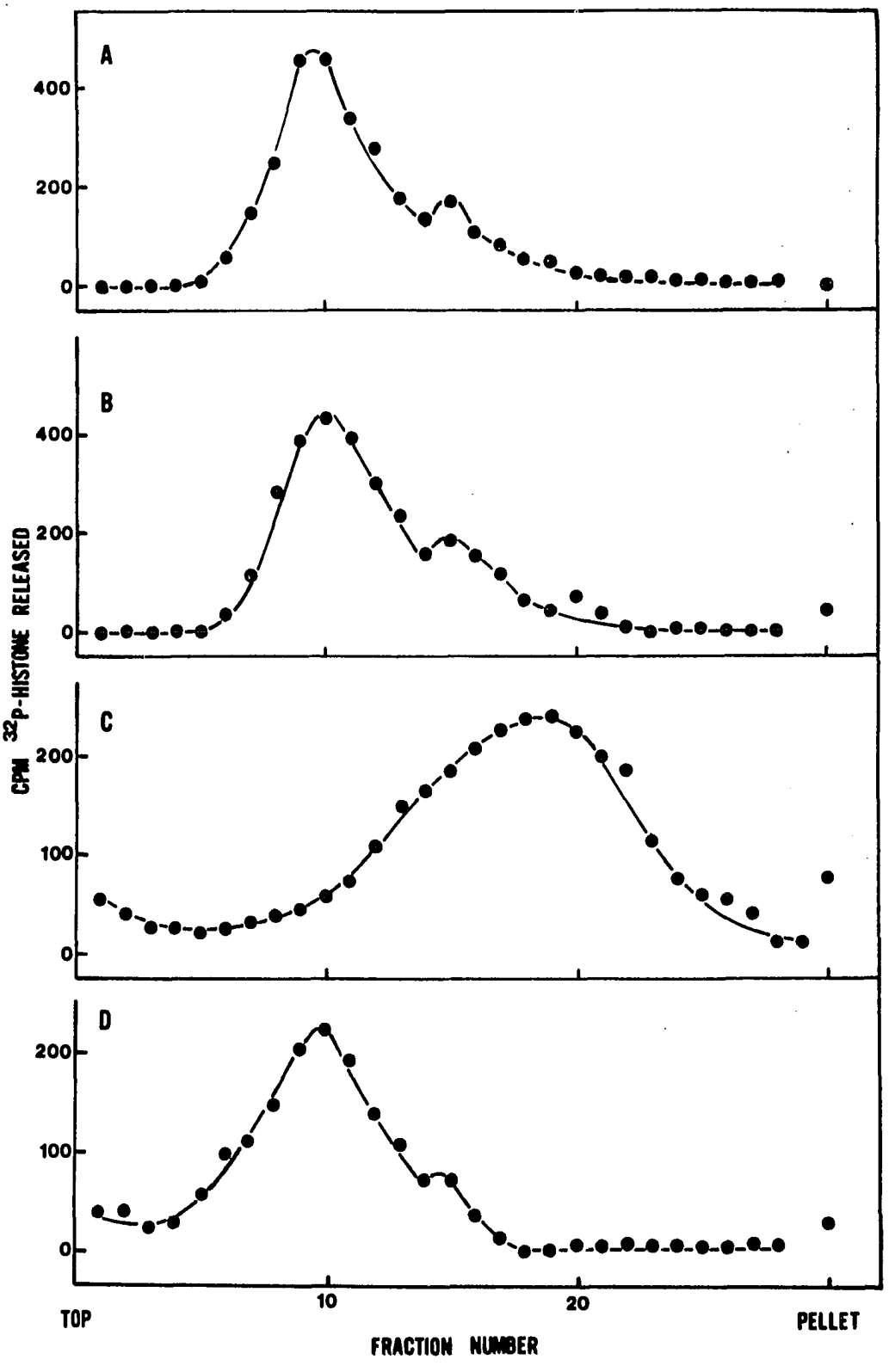
[c]1 Unit = 1 unit of glycogen synthase D converted to glycogen I per minute.

[d]1 Unit = 1 nmole P_i released from ^{32}P-histone/minute.

Glycogen Synthase Phosphatase Activity			^{32}P-histone Phosphatase Activity		
Units[c]	% Total	% Recovered	Units[d]	% Total	% Recovered
145			1072		
75		103	554		103
65	86		495	89	
10	14		58	11	
48		66	500		99
45	94		473	94	
2.7	5.7		28	5.6	

It was of interest to determine whether there was a difference in the sizes of the phosphatase activities in the glycogen-pellet and in the post-glycogen supernatant. The glycogen-pellet was prepared by adding glycogen to the post-microsomal supernatant and sedimenting at high speed. The pellet was resuspended according to Methods and aliquots were dialyzed in the absence and presence of -amylase. The phosphatase activity was stable during centrifugation. Neither the glycogen synthase phosphatase activity nor the phosphohistone phosphatase in the resuspended glycogen-pellet was altered by dialysis in the absence or presence of -amylase. Sucrose density gradients were used to determine the sizes of the phosphatases present in each of the fractions. Two forms of phosphatase activity were observed in the post-microsomal supernatant (panel A). The resuspended glycogen-pellet (panel C) that was not treated with -amylase exhibited a broad activity profile and contained some activity that sedimented to the bottom of the gradient. The phosphatase activities observed in the -amylase digested glycogen-pellet (panel D) migrated the same distance as the enzymes in the post-microsomal supernatant (panel A) and the post-glycogen supernatant (panel B). The higher mobility of phosphatase activity in the undegraded glycogen-pellet could be attributed to one of several affects. There could be phosphatase(s) that are stimulated by components in the glycogen-pellet. If high molecular weight phosphatases do exist, they must be absent or inactive in crude extracts, 30% ethanol-pellets or in post-microsomal supernatants. The presence of glycogen or other carbohydrates in the glycogen preparation could have promoted association of the phosphatases to higher molecular weight forms. There may actually have been some association of the 4.1S and 6.9S phosphatases with glycogen or

Figure 5: Molecular forms of phosphoprotein phosphatase. The fractions described in Table 2 were run on sucrose gradients. The glycogen-pelleted fractions were diluted 1/3 with TEEM immediately before layering on the gradients. The gradients were centrifuged, collected, and assayed for ^{32}P-histone phosphatase as described in Methods. Panels in the figure represent the following fractions: A post-microsomal supernatant; B. post-glycogen supernatant; C. glycogen-pellet; D. α-amylase digested glycogen-pellet.


A
B
C
D
400
200
0
400
200
0
200
100
0
200
100
0
CPM ^{32}P-HISTONE RELEASED
TOP
10
20
PELLET
FRACTION NUMBER

other carbohydrates. The protein concentration in the resuspended glycogen-pellet was 20 mg/ml compared to 13 mg/ml in the post-microscomal supernatant. However, the glycogen-pellet was diluted to 7 mg/ml protein immediately before layering on the sucrose gradient. The α-amylase digested pellet, in which the higher molecular weight form(s) of phosphatase were not observed, was diluted to the same protein concentration. Therefore, the faster sedimentation of the phosphatase activity in the undigested glycogen-pellet cannot be attributed to an effect of elevated protein concentration.

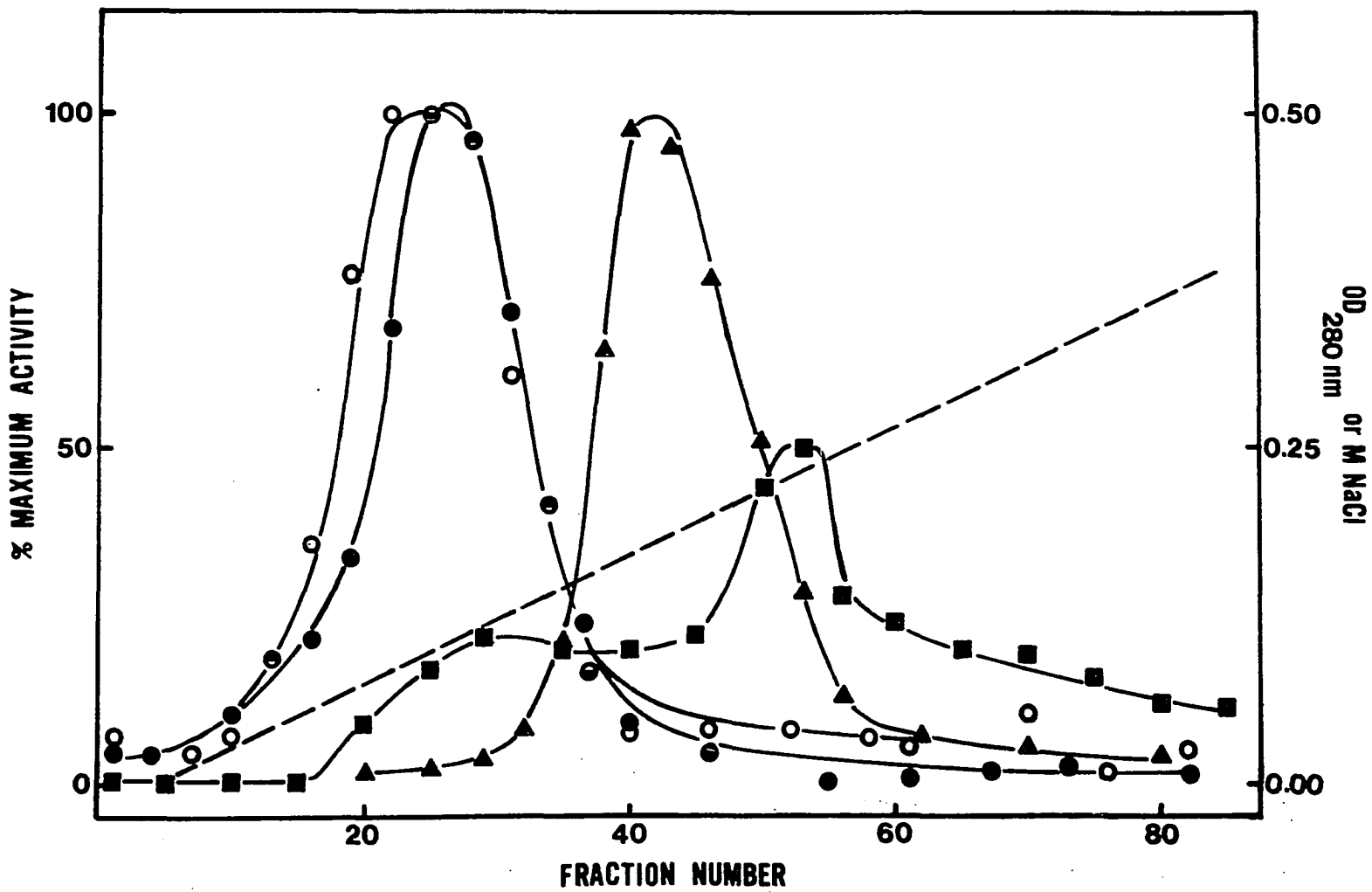
Of the possible explanations for the higher mobility of glycogen-pelleted activity, it would seem most plausible that α-amylase degradable carbohydrate was involved in a macromolecular interaction with the 4.1S and 6.9S phosphatases causing the enzymes to sediment faster in a sucrose gradient. Whether the phosphatases formed homologous or heterogenous associations, bound only carbohydrate, or interacted with protein(s) present in the glycogen-pellet was not determined. It was apparent, however, that the phosphatases present in the glycogen-pellet were converted by α-amylase digestion to the same sizes as the enzymes in the post-glycogen supernatant. There were no obvious differences between the relative activities of the 4.1S and 6.9S phosphatase in the post-microsomal and post-glycogen supernatants and the α-amylase digested glycogen-pellet.

Purification of Phosphoprotein Phosphatases

Purification of glycogen-pelleted phosphatases

In order to determine if a specific phosphatase purified with glycogen synthase, the resuspended glycogen-pellet was further purified. The phos-

Figure 6: Elution of glycogen-pelleted phosphoprotein phosphatase from diaminobutane-Sepharose chromatography. Glycogen-pelleted phosphatase was prepared and digested with α-amylase as described in Methods. The fraction was then chromatographed on a 40 ml diaminobutane-Sepharose 4B column equilibrated at room temperature in TEEMG-25. The column was eluted with a 500 ml 0 - 0.5 M NaCl linear gradient in TEEMG-25. Fractions were collected and cooled to 4°. The fractions were assayed for ^{32}P-histone phosphatase (● - ●), glycogen synthase phosphatase (○ - ○) by conversion of glycogen synthase D to I, and glycogen synthase (▲ - ▲) as described in Methods. The glycogen synthase substrate was phosphorylated by endogenous kinases. The maximum phosphatase activity represented conversion of glycogen synthase from an activity ratio (+SO_4/+G-6-P) of 0.12 to 0.81 and 673 cpm (6.7%) ^{32}P released from ^{32}P-histone. The OD_{280nm}(■ - ■) of the column effluent was continuously monitored. The NaCl concentration (— —) was determined by conductivity.


100
50
0
% MAXIMUM ACTIVITY
0.50
0.25
0.00
OD 280 nm or M NaCl
20
40
60
80
FRACTION NUMBER

phatase activity was separated from the glycogen synthase by diaminobutane-Sepharose chromatography of the α-amylase digested glycogen-pellet (Figure 6) as described in Methods. The phosphohistone and glycogen synthase phosphatase activities always coeluted. When resolution of the enzymes was optimized, only a small amount of the total phosphatase activity eluted with the glycogen synthase activity. Resolution between the phosphatase and synthase depended upon the gradient used to elute the enzymes, the age of the diaminobutane-Sepharose resin, and the flow rate of the column. A separate peak of unique phosphatase did not elute with the peak of glycogen synthase activity.

The glycogen-pelleted phosphoprotein phosphatase activity was purified by the procedure summarized in Table 3. Sedimenting the phosphatase activity with glycogen did not increase the purity of the preparation, while only a small fraction of the phosphatase activity was recovered. Diaminobutane-Sepharose chromatography was an effective purification step, resulting in a 10-fold purification. Further purification of the phosphatase activity was not successful due to large activity losses in subsequent steps.

Molecular parameters of the phosphatases purified from the glycogen-pellet: The sizes of the glycogen-pelleted phosphatase activities purified by diaminobutane-Sepharose chromatography were determined by sucrose gradient centrifugation and gel filtration. Two peaks of phosphatase activity were observed using each method. Gel filtration on a Biogel A column indicated that the enzymes chromatographed as species with Stokes' radii of 42 Å and 52 Å as shown in Figure 7. The sedimentation coefficients of the active phosphatases were calculated to be 4.1S and 6.9S based on

Table 3: Purification of glycogen-pelleted phosphoprotein phosphatase activity

Fraction	Volume (ml)	Units[a]	Protein (mg)	Units / mg protein	Yield (%)	Purification (-fold)
10,000 x g supernatant	1520	2219	16720	0.13	100	1.0
30% ethanol-pellet	180	2466	3672	0.67	111	5.2
Post-microsomal supernatant	158	1643	2054	0.80	74	6.2
Glycogen-pellet	22	341	431	0.79	15	6.1
following α-amylase digestion	22	365	430	0.85	16	6.5
Diaminobutane-Sepharose chromatography	122	134	14.3	9.4	6.0	72

[a] 1 unit = 1 nmole P_i released from ^{32}P-histone/min.

Figure 7: Elution of glycogen-pelleted phosphoprotein phosphatase from Biogel A chromatography. Glycogen-pelleted phosphatase was prepared and purified through diaminobutane-Sepharose chromatography according to Methods. The preparation was concentrated by dialyzing for 8 hours against TEEMG-60. Five-tenths ml of the concentrated prep was applied to a 50 ml Biogel A column equilbrated with TEEM. The 0.20 ml fractions were assayed for ^{32}P-histone phosphatase (● - ●) activity as described in Methods. The elution volumes of Blue dextrane (V_O), hemoglobin (Hb), lactate dehydrogenase (LDH), and ATP determined by absorbance at the appropriate wavelengths, are indicated by arrows.

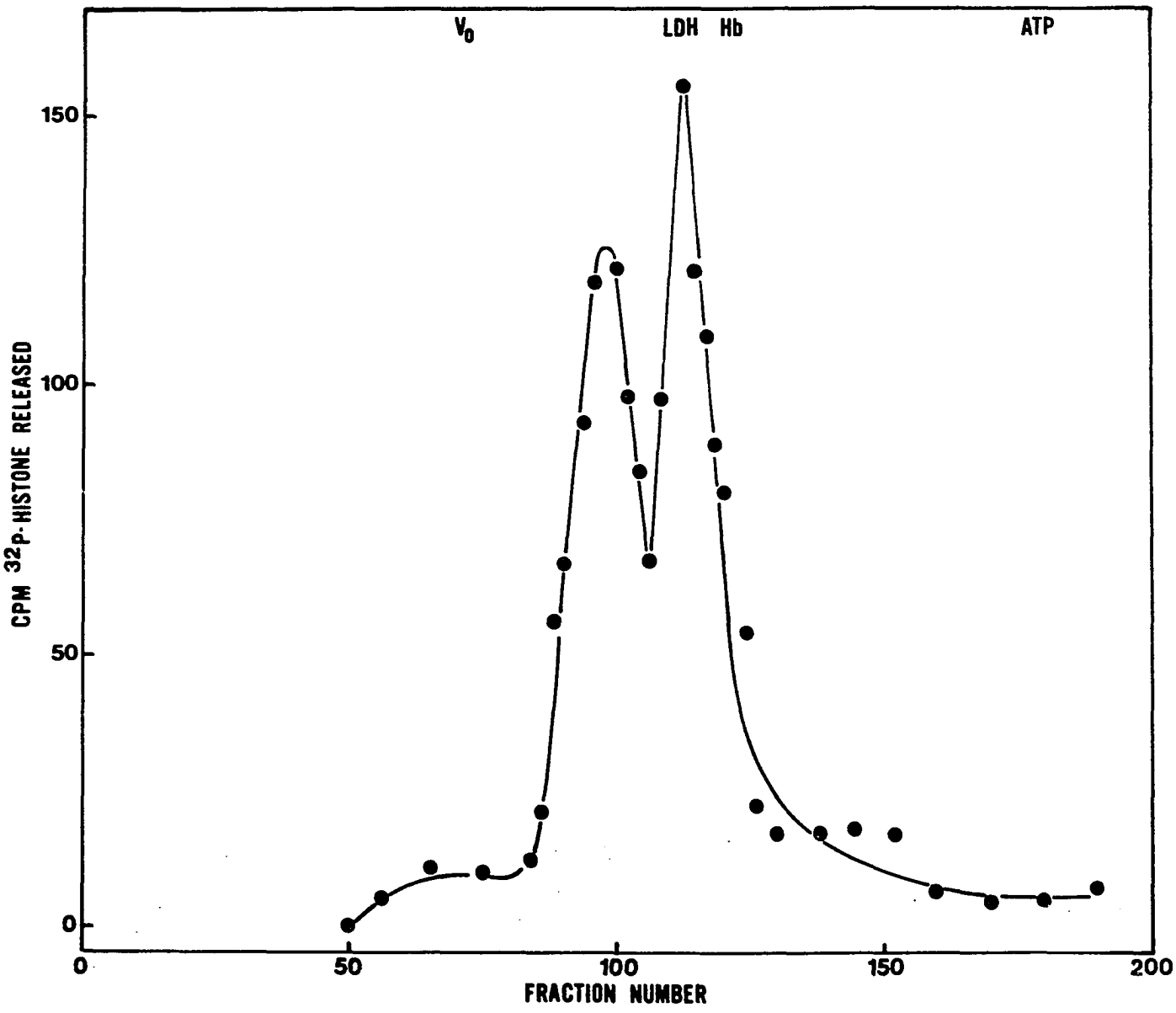
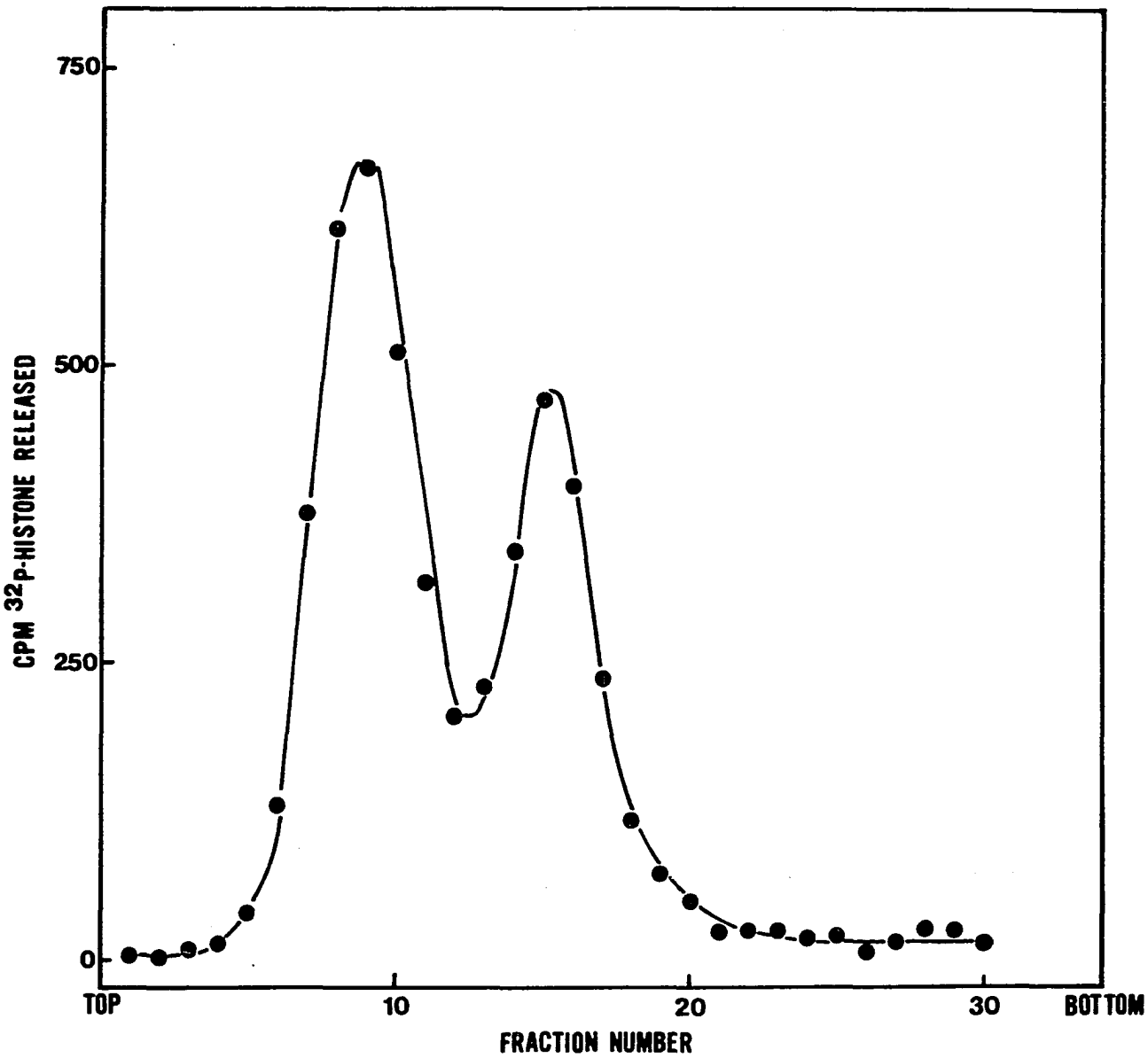

V_0
LDH
Hb
ATP
150
100
50
0
CPM ^{32}P-HISTONE RELEASED
0
50
100
150
200
FRACTION NUMBER

Figure 8: Sucrose gradient centrifugation of partially purified glycogen-pelleted phosphoprotein phosphatase activity. The phosphatase preparation described in Figure 7 was dialyzed for 3 hours against TEEM and 0.2 ml were applied to a 5-25% sucrose gradient in TEEM. The gradients were centrifuged for 14 hours 40,000 rpm in a SW50.1 rotor in a Beckman L-5-75 centrifuge at 4°. The fractions were collected and assayed for ^{32}P-histone phosphatase (● - ●) according to Methods. Phosphorylase a and hemoglobin were run in a parallel gradient as standards.


750
500
250
0
CPM 32P-HISTONE RELEASED
TOP
10
20
30
BOTTOM
FRACTION NUMBER

five determinations from three preparations (refer to Figure 8). Using these parameters the molecular weights of the phophatases were calculated to be 74 x 10^3 and 15 x 10^4 daltons.

The goal of this project was to purify a high molecular weight bovine heart phosphatase to be used to study the dephosphorylation of glycogen synthase. The first approach to developing a purification procedure was to determine if a particular phosphatase copurified with its substrate, glycogen synthase. However, only a fraction of the phosphatase activity sedimented in a centrifugtion step that almost quantitatively sedimented glycogen and glycogen synthase. This glycogen-pelleted phosphatase activity migrated much faster in a sucrose gradient than did the supernatant phosphatases. The interaction between glycogen and the phosphatase is interesting in itself, particularly if the high molecular weight phosphatase activity results from a unique form of phosphatase, or from association of individual phosphatases, or from association of other proteins with phosphatase. But purification of the glycogen-pelleted phosphatase activities altered the sedimentation pattern of the enzyme activity. The phosphatase activities purified from the α-amylase digested glycogen-pellet had the same sedimentation coefficients as the enzymes that were not pelleted with glycogen. The two phosphatases were not successfully separated due to large activity losses in subsequent purification steps. The instability, coupled with the small amount of activity in the glycogen-pellet, made further purification impractical. An alternative purification was developed to obtain a preparation containing only one of the phosphatase activities observed in the crude extract.

Purification of the soluble bovine heart phosphoprotein phosphatase

A procedure was developed for purifying a bovine heart phosphoprotein phosphatase from the post-microsomal supernatant. EGTA was included throughout the purification to inhibit Ca^{+2}-stimulated proteases. Divalent cations were omitted from buffers. DEAE-cellulose chromatography was not used as a purification step. These precautions were used to avoid producing low molecular weight phosphatases.

One of the first steps in purifying an enzyme from a large volume of material is to effectively concentrate the activity to a volume that can be practically handled. Ammonium sulfate fractionation of the crude extract could be used to precipitate the phosphatase. However, only 29% of the activity was precipitated by 70% ammonium sulfate. Less phosphatase activity was recovered at lower ammonium sulfate concentrations. Precipitation of the enzyme by ammonium sulfate also necessitated a lengthy dialysis before further purification could be continued, which was undesirable due to possible proteolysis. Several different ethanol concentrations were used to precipitate the phosphatase. Table 4 summarizes the recovery of phosphatase activity in pellets prepared by precipitation at different ethanol concentrations. The ethanol pellets were prepared as described in Methods, except that the volume of 95% ethanol added was adjusted to give the desired ethanol concentration. Only 50% of the phosphatase activity was precipitated at 17% ethanol, while 100% of the phosphatase activity was precipitated by 30% ethanol. The recovery of phosphatase (for 18 preps) was variable during the 30% ethanol step (50-175%). Higher ethanol concentrations would precipitate more phosphatase activity, indicating an activation of phosphatase, possibly due to the inactivation of inhibitors or

Table 4: Precipitation of phosphoprotein phosphatase activity by ethanol

Fraction	Ethanol Concentration	^{32}P-Histone Phosphatase		Glycogen Synthase Phosphatase	
		Units[a]	% Activity Recovered	Units[b]	% Activity Recovered
10,000 x g supernatant		300	100	65	100
Ethanol-pellet	17%	48	48	11	51
	30%	126	126	22	103
	50%	168	168	26	119

[a] 1 Unit = 1 nmole P_i released from ^{32}P-histone/minute.

[b] 1 Unit = 1 unit of glycogen synthase D converted to glycogen synthase I/minute.

the removal of competing phosphatase substrates, or to ethanol-induced changes in the phosphatases. The recoveries of phosphohistone phosphatase and glycogen synthase phosphatase were approximately equal in the 17% ethanol-pellet and in the 30% ethanol-pellet. The 50% ethanol-pellet, however, had a higher relative activity on phosphohistone than on glycogen synthase. A bovine heart phosphatase with a low K_M for phosphohistone can be precipitated by increasing the ethanol concentration of the fraction that remains soluble at 30% ethanol, to a final concentration of 50% ethanol (68). Adding ethanol to the crude extract to a final concentration of 50% might therefore precipitate a phosphohistone-specific phosphatase. The large amounts of protein precipitated at 50% ethanol made purification more difficult. The 30% ethanol fractionation was selected for use since it provided good activity recovery and no low molecular weight phosphatases were formed by precipitation at this ethanol concentration (Figure 4).

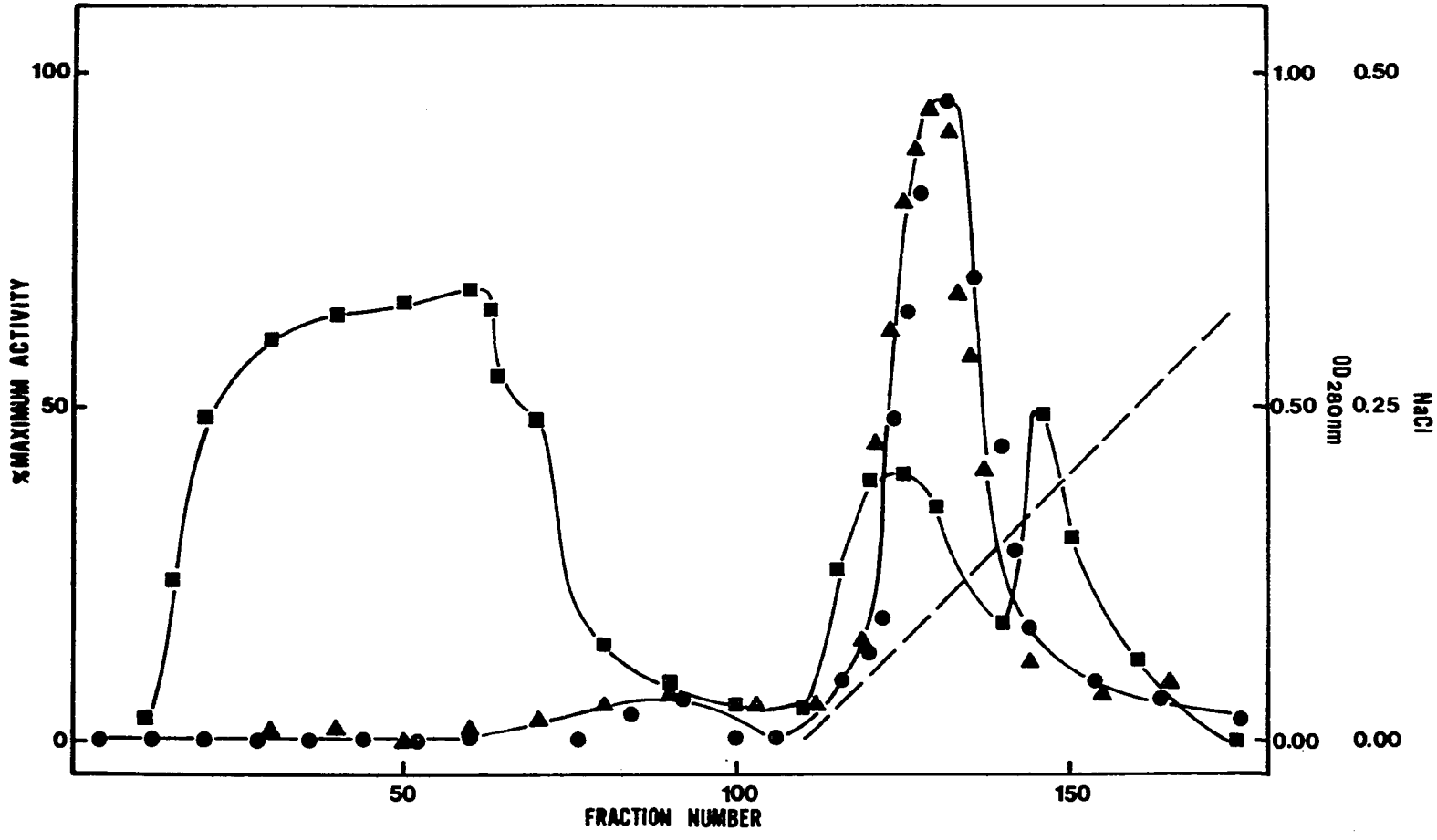
The 30% ethanol-pellet was resuspended in a Potter Elvejhem homogenizer, surrounded by ice, in 10% of the volume of the crude homogenate. The pellet was resuspended by hand, then by two or three strokes with a motor-driven pestle. The enzyme was centrifuged at 161,000 x g for 2.5 hours. The amount of phosphatase activity that pelleted at 161,000 x g varied from 0-41% for five preps. Hearts containing mainly glycogen synthase I (> 90%) were used in order to minimize the amount of phosphatase that pelleted in this step. Once the high speed supernatant was obtained, plastic equipment was used exclusively to minimize adsorption of the enzyme to glass.

The 161,000 x g supernatant (post-microsomal supernatant) was chroma-

tographed on diaminobutane-Sepharose. Poorer recoveries were obtained from diaminopentane and diaminohexane-Sepharose resins than from diaminobutane-Sepharose. The recovery of the protein phosphatase from the diaminobutane-Sepharose column ranged from 30-96% (for 5 preps). The elution profile of phosphatase activity from diaminobutane-Sepharose is shown in Figure 9. The glycogen synthase phosphatase and phosphohistone phosphatase activities coincided. No glycogen synthase phosphatase was ever observed that did not also dephosphorylate phosphohistone. The most highly purified preparations were obtained when only the latter half of the phosphatase peak was pooled and further purified. Attempts to better resolve the contaminants eluting at the front of the peak from the phosphatase by eluting with a shallower gradient did not increase the specific activity of the prep and resulted in poorer yields. The NaCl concentration needed to elute the phosphatase activity varied from 0.05 M for diaminobutane-Sepharose prepared one month before use to 0.08 M for resin prepared one week prior to use. Better purification was obtained when the resin was prepared one week before use.

One of the most difficult problems of purifying the phosphatase was concentrating the activity following elution from diaminobutane-Sepharose. Thirty percent ethanol or 70% ammonium sulfate precipitation or amicon filtration resulted in large activity losses. Samples concentrated by osmotic gradients generated by sucrose, Ficol, or Aquaside were almost completely inactivated. Dialysis of the phosphatase against TEEM buffer containing 60% glycerol concentrated the enzyme approximately 5-fold without activity loss. There was some loss of activity when the glycerol concentration was decreased to 40%.

Figure 9: Elution of soluble phosphoprotein phosphatase from diaminobutane-Sepharose chromatography. The phosphatase was prepared and eluted from diaminobutane-Sepharose as described in Methods. The column was then washed with 100 ml 0.6 M NaCl in TEEMG-25. Fractions were assayed for ^{32}P-histone phosphatase (●- ●) and glycogen synthase phosphatase (▲-▲) by ^{32}P release from ^{32}P-proteins. The OD_{280nm} (■-■) was continuously monitored. The NaCl concentration (— —) was determined by conductivity.


100
50
0
% MAXIMUM ACTIVITY
50
100
150
FRACTION NUMBER
1.00
0.50
0.00
0.50
0.25
0.00
OD 280nm
NaCl

Further concentration of the prep was necessary before purifying by gel filtration chromatography. The enzyme could be concentrated 5-fold by adsorption to a 20 ml diaminobutane-Sepharose column and elution by 0.3 M NaCl in TEEM at a slow flow rate (approximately 0.3 ml/min). Activity loss during chromatography was not reduced to less than 50%. Dialysis against 60% glycerol in TEEM further concentrated the prep. A 2-3 hour dialysis against TEEM was introduced to lower the glycerol concentration in the preparation, since higher glycerol prevented optimum resolution of contaminants by molecular sieve chromatography. The sample was applied to a Sephacryl S200 column in a volume that did not exceed 1% of the total column value. The phosphatase activity eluted from the Sephacryl column as shown in Figure 10. A symmetrical peak with a Stokes' radius of approximately 45 Å was eluted from the column in three separate preparations. Another preparation was contaminated by a minor activity at a high molecular weight (Stokes' radius = 52 Å). The 52 Å phosphatase was clearly resolved from the 45 Å phosphatase. When the sample size did not exceed 1% of the column volume a 10-fold purification of the phosphatase could be obtained by Sephacryl chromatography. More than 50% of the activity could be recovered from the Sephacryl S200 chromatography step. The rapid flow rates achievable with Sephacryl permitted much higher recovery of activity than was obtained using Biogel, Sepharose, or Sephadex resins.

The phosphatase activity was purified approximately 250-fold by the procedure summarized in Table 5. This purification scheme avoided treatments that have been shown to lead to the isolation of multiple forms of phosphatase not present in crude extracts. The recoveries and purification of phosphohistone phosphatase and glycogen synthase phosphatase were similar

Figure 10: Elution of soluble phosphoprotein phosphatase from Sephacryl S200 chromatography. Soluble phosphoprotein phosphatase was purified by diaminobutane-Sepharose chromatography and concentrated as described in Methods. The preparation was dialyzed for 2 hours against TEEM, then chromatographed on a 170 ml Sephacryl S200 column (1.6 x 85 cm) at a flow rate of 0.75 ml/min. Fractions eluting past the V_o were assayed for ^{32}P-histone phosphatase (● - ●) in the presence of 5 mM Mn^{+2} according to Methods. The OD_{280nm} (■ - ■) of the column effluent was monitored continuously.

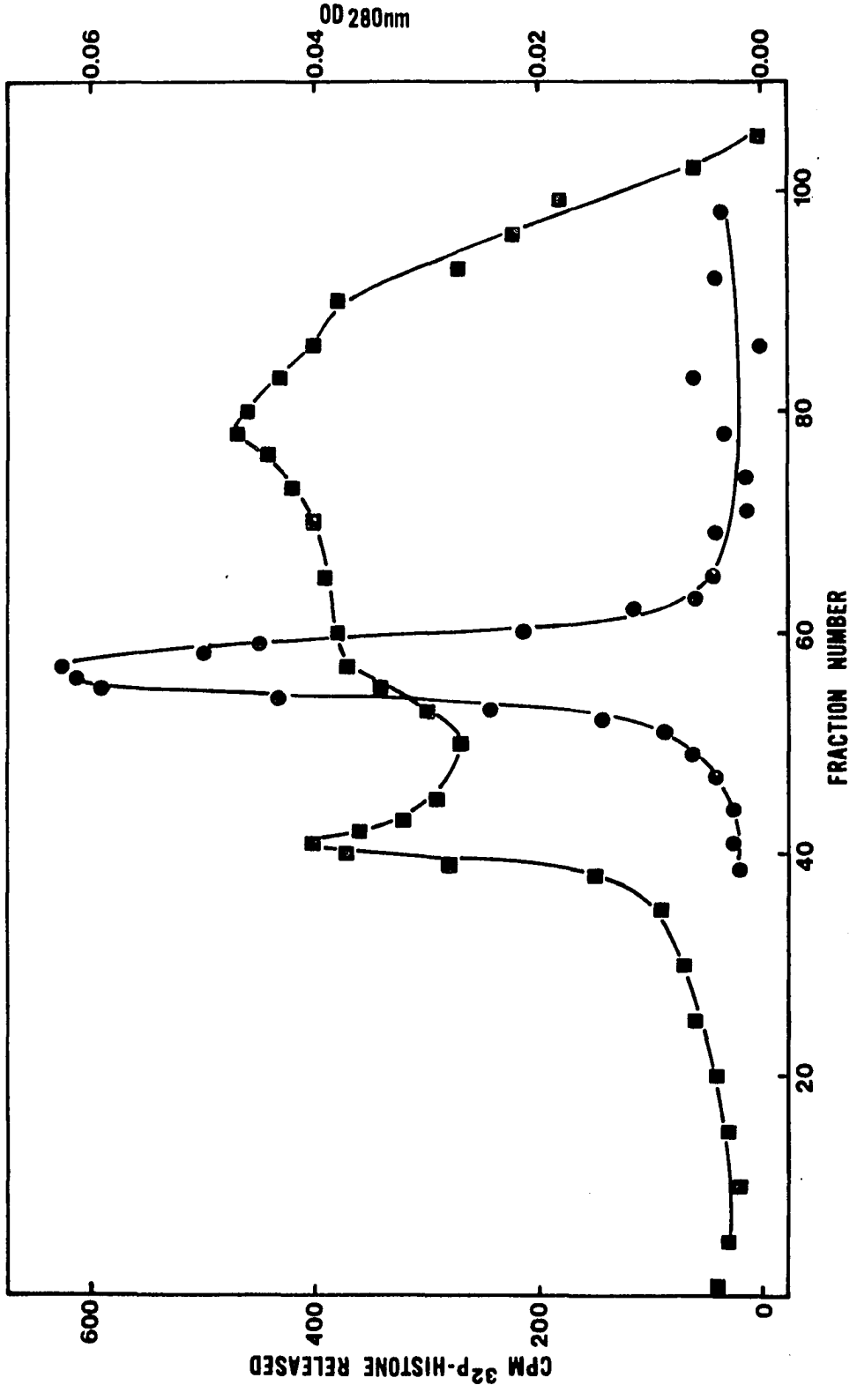

OD 280nm
0.06
0.04
0.02
0.00
600
400
200
0
CPM 32p-HISTONE RELEASED
20
40
60
80
100
FRACTION NUMBER

Table 5: Purification of phosphoprotein phosphatase[a]

Purification Step	^{32}P-Histone Phosphatase			
	Units[b]	Yield (%)	Units/mg protein	Purification (-fold)
10,000 x g supernatant	905	100	0.086	1.00
30% ethanol-pellet	482	53.3	0.199	2.31
161,000 x g supernatant	326	36.0	0.164	1.91
Diaminobutane-Sepharose[d]	34.5	3.81	2.25	26.2
Sephacryl S200	26.7	3.00	22.7	264

[a]500 g frozen bovine heart was the starting material.

[b]Phosphohistone phosphatase activity was determined as in Methods.

[c]Glycogen synthase phosphatase was determined by the conversion of glycogen synthase D to I. The glycogen synthase substrate was phosphorylated in the presence of cyclic nucleotide-dependent and -independent protein kinases according to Methods.

[d]Only the latter part of the phosphatase peak was pooled in order to obtain a higher specific activity enzyme.

Glycogen Synthase Phosphatase			
Units[c]	Yield (%)	Units/mg protein	Purification (-fold)
278	100	0.026	1.00
155	55.6	0.069	2.65
150	54.0	0.073	2.81
16.6	6.0	1.08	41.7
6.4	2.32	5.50	212

at all stages of the isolation procedure. There was no separation of phosphohistone phosphatase and glycogen synthase phosphatase during diaminobutane-Sepharose or Sephacryl chromatography. While no fractions were isolated during the course of this investigation that exhibited exclusive specificity for either phosphohistone or glycogen synthase, the assessment of substrate specificity must involve kinetic analysis using substrates with defined phosphorylation states.

Purity of the 4.1S phosphoprotein phosphatase: Samples of the fractions from each step of the preparation were electrophoresed on SDS polyacrylamide gels as shown in Figure 11. Subunits migrating with molecular weights of 125, 90, 66, 35 and 31 x 10^3 daltons were observed in the most highly purified phosphatase preparations. Several of the protein bands were of a molecular weight that could correspond to the phosphatase. The band that migrated at approximately 70 x 10^3 daltons could represent phosphatase, while one of the smaller bands could be a subunit. Several phosphatases may be dissociated to a subunit with a molecular weight of 30-35 x 10^3 daltons (79,80,86), a subunit size range observed in the partially purified preparations.

It was of interest to determine whether the phosphatase activity could be attributed to an observable protein band. It was found that phosphatase activity could be maintained during polyacrylamide gel electrophoresis using the Tris-glycine-mercaptoethanol system described in Methods. Figure 12 shows the correlation between protein staining and phosphatase activity for the most highly purified phosphatase preparation. A single protein band coincided with the activity at an R_f = 0.30 - 0.37, implying that the phosphatase activity electrophoresed with a protein band that

Figure 11: SDS polyacrylamide gel electrophoresis of fractions from the purification of soluble phosphoprotein phosphatase. Samples from each step in the phosphoprotein phosphatase prep were subjected to SDS polyacrylamide gel electrophoresis as described in Methods. The gels were stained for protein with Coomassie Brilliant Blue G250. The samples and amounts of protein applied to each gel are designated as follows: (a) 10,000 x g supernatant, 50 μg; (b) 30% ethanol-pellet, 50 μg; (c) 161,000 x g supernatant, 50 μg; (d) diaminobutane-Sepharose purified phosphatase, 20 μg; (3) phosphatase concentrated on diaminobutane-Sepharose, 20 μg; and (f) Sephacryl S200 purified phosphatase, 10 μg. The subunit molecular weights were calculated according to Methods.

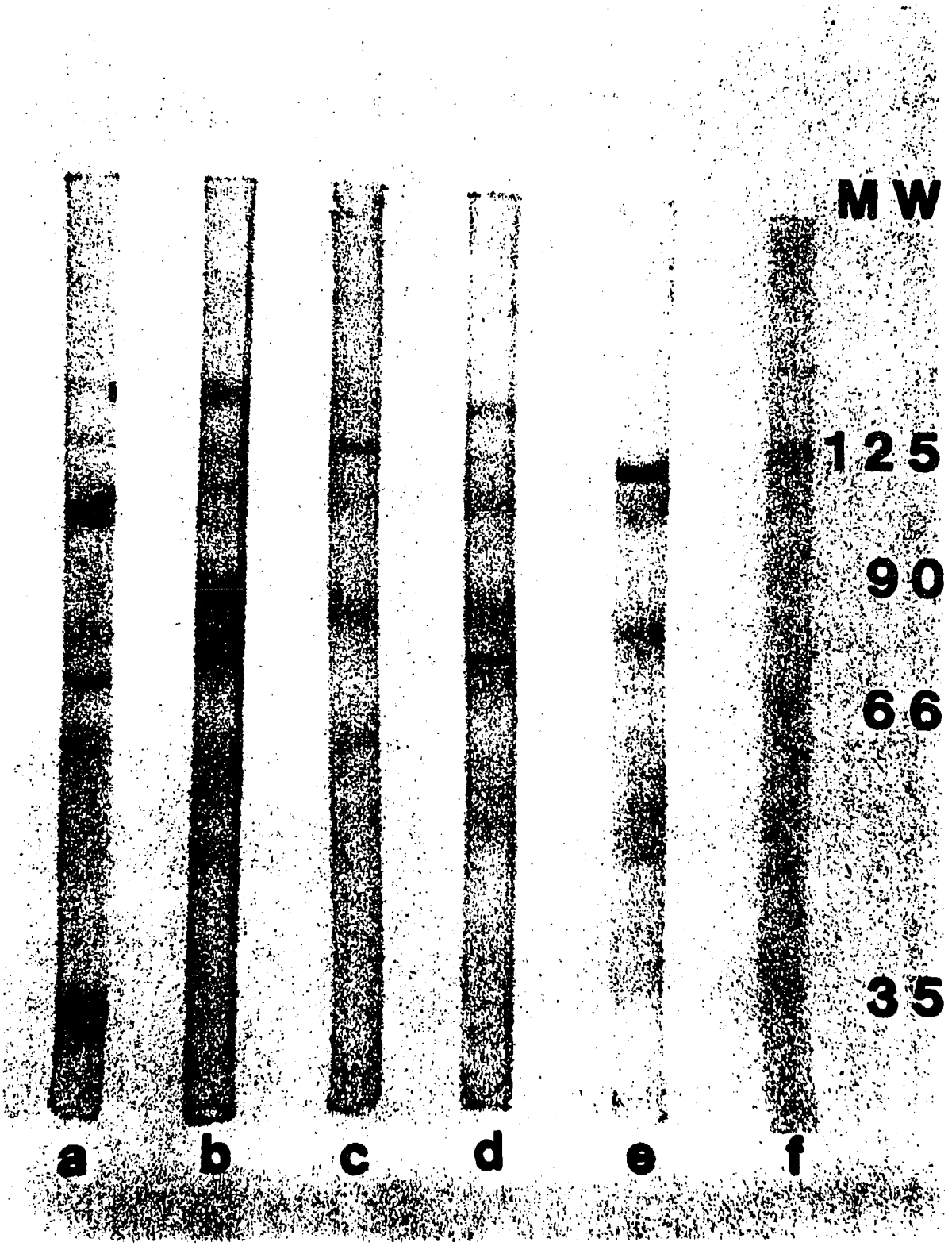
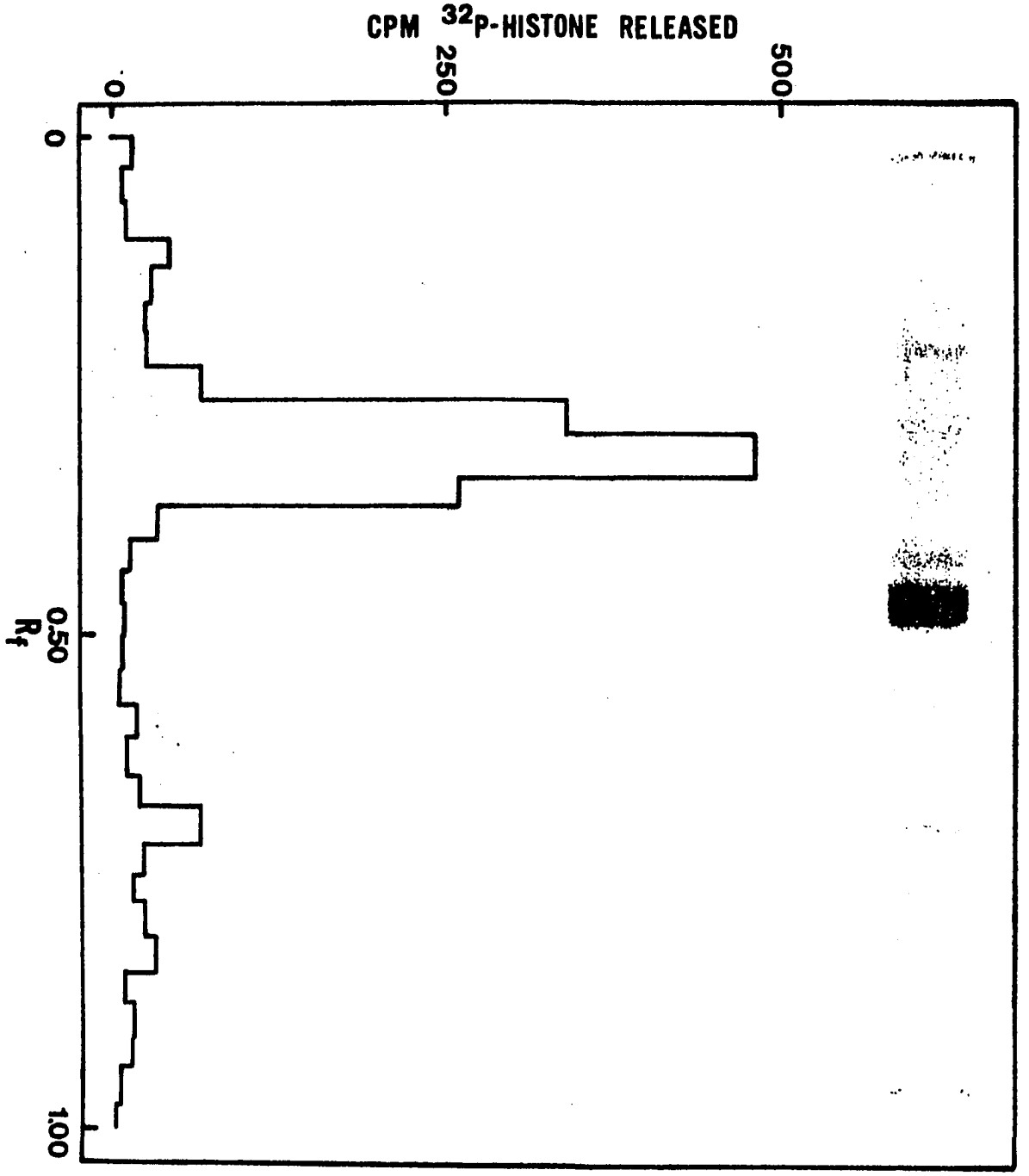

MW
125
90
66
35
a
b
c
d
e
f

Figure 12: Activity gels of soluble phosphoprotein phosphatase. Ten μg of phosphoprotein phosphatase purified through Sephacryl S200 chromatography (22.5 U/mg protein) were electrophoresed in a Tris-glycine-mercaptoethanol gel system according to Methods. Duplicate gels were stained for protein with Coomassie Brilliant Blue G250 or sliced into 1.5 mm segments and assayed for ^{32}P-histone phosphatase in the presence of 5 mM Mn^{+2} as described in Methods.


CPM 32P-HISTONE RELEASED
0
250
500
0
0.50
1.00
Rf

was present at a high enough concentration to be observed. The phosphatase activity peaked at an R_f = 0.34 - 0.36 for 4 determinations on 2 preparations, indicating only one active species was present, and that the phosphatase coincided with a minor protein band.

The phosphatase activity was also stable during electrophoresis in a sucrose gradient and this technique was used to better resolve some of the contaminants from the phosphatase. The activity profile of the phosphatase electrophoresed in the sucrose gradient is shown in Figure 13. Ninty-seven percent of the phosphatase activity was recovered in a single symmetrical peak. The purity of some of the fractions from the sucrose gradient electrophoresis experiment was monitored by SDS gel electrophoresis. Each fraction from the electrophoretic separation was lyophilized and dissolved in an SDS-urea buffer and electrophoresed on SDS polyacrylamide gels and stained for protein as described in Methods. The destained gels were scanned with a Gilford spectrophotometer. Band densities were determined by triangulation and calculation of the area corresponding to each peak. A picture of the gels from SDS polyacrylamide gel electrophoresis is shown in Figure 14, while scans of gels from selected fractons are represented in Figure 15. The fractions corresponding to active phosphatase (fractions 15-17) contained a number of protein bands. Since several of the bands (76, 68 and 49 x 10^3 daltons) reached maximum concentration in the fraction with the highest phosphatase activity a single band could not be correlated with enzymatic activity (Fig. 16). Most of the protein corresponding to a 33 or 35 x 10^3 dalton subunit was separated from the phosphatase activity, indicating that the majority of protein migrating at this region was not derived from the phosphatase. The excellent recovery

Figure 13: Sucrose gradient electrophoresis of soluble phosphoprotein phosphatase. Sephacryl S200 purified soluble phosphoprotein phosphatase (5.7 U/mg protein) was dialyzed for 2 hours against TEEM and 0.2 ml of the enzyme were electrophoresed in a 5-30% sucrose gradient in TEEM as described in Methods. The OD_{280nm} (■–■) was monitored as the 0.3 ml fractions were collected. The fractions were assayed for ^{32}P-histone phosphatase in the presence of 5 mM Mn^{+2} (● - ●).

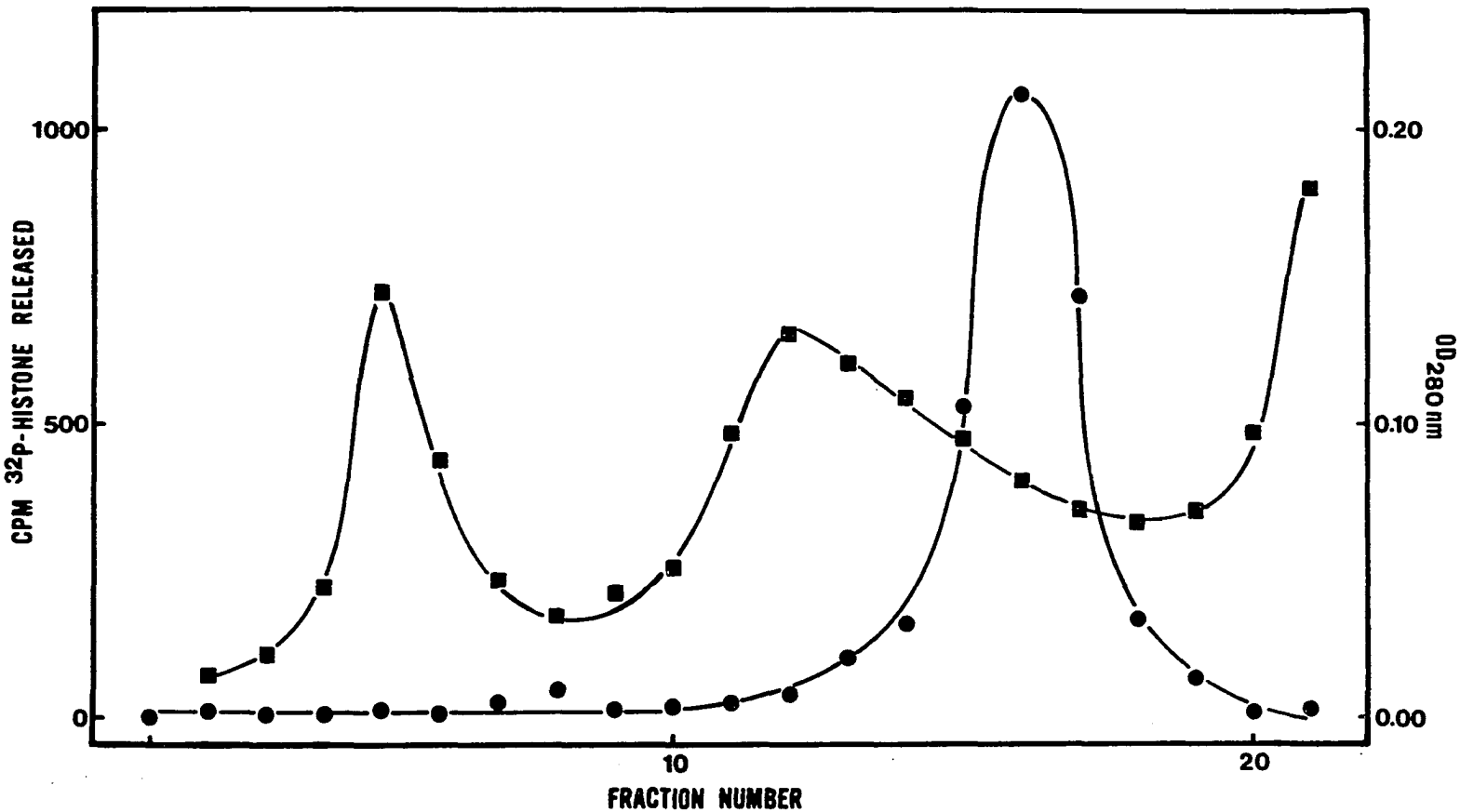

1000
500
0
CPM 32P-HISTONE RELEASED
0.20
0.10
0.00
OD280nm
10
20
FRACTION NUMBER

Figure 14: SDS polyacrylamide gel electrophoresis of fractions from sucrose gradient electrophoresis of soluble Sephacryl S200 purified phosphoprotein phosphatase. Fractions from the sucrose gradient electrophoresis of the phosphatase (refer to Figure 13) were lyophilized and then dissolved in 50 µl of buffer containing 4 M urea, 1% SDS, 1% mercaptoethanol, 0.01 M sodium phosphate, pH 7.0. The fractions were boiled for 5 minutes and electrophoresed on 7.5% SDS polyacrylamide gels, stained for protein, and the subunit molecular weights of the bands calculated as described in Methods. The fractions that were electrophoresed include the following: (a) 20 µg of Sephacryl S200 purified phosphoprotein phosphatase before electrophoresis and fractions (b) 11, (c) 12, (d) 14, (e) 15, (f) 16, (g) 17, (h) 18 and (i) 20.

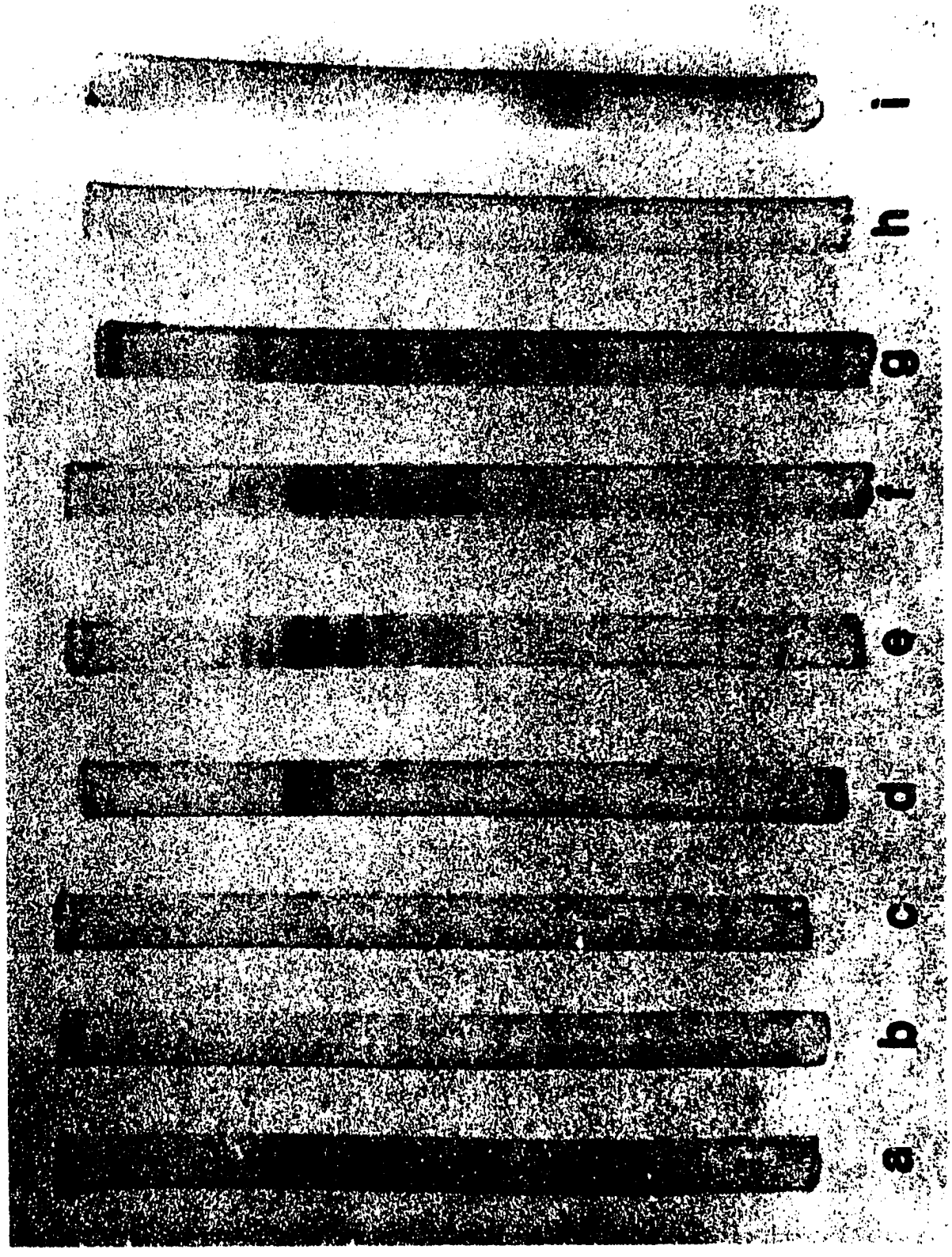

a
b
c
d
e
f
g
h
i

Figure 15: Densitometric scans of SDS polyacrylamide gels of sucrose gradient electrophoresed phosphoprotein phosphatase. The destained gels of the phosphoprotein phosophatase electrophoresed in the sucrose gradient (refer to Figure 14) were scanned at 595 nm with a Gilford spectrophotometer. Scans are shown for the following fractions: (a) 20 μg of Sephacryl S200 purified phosphoprotein phosphatase before electrophoresis; (b) fraction 14; (c) fraction 16; and (d) fraction 18.

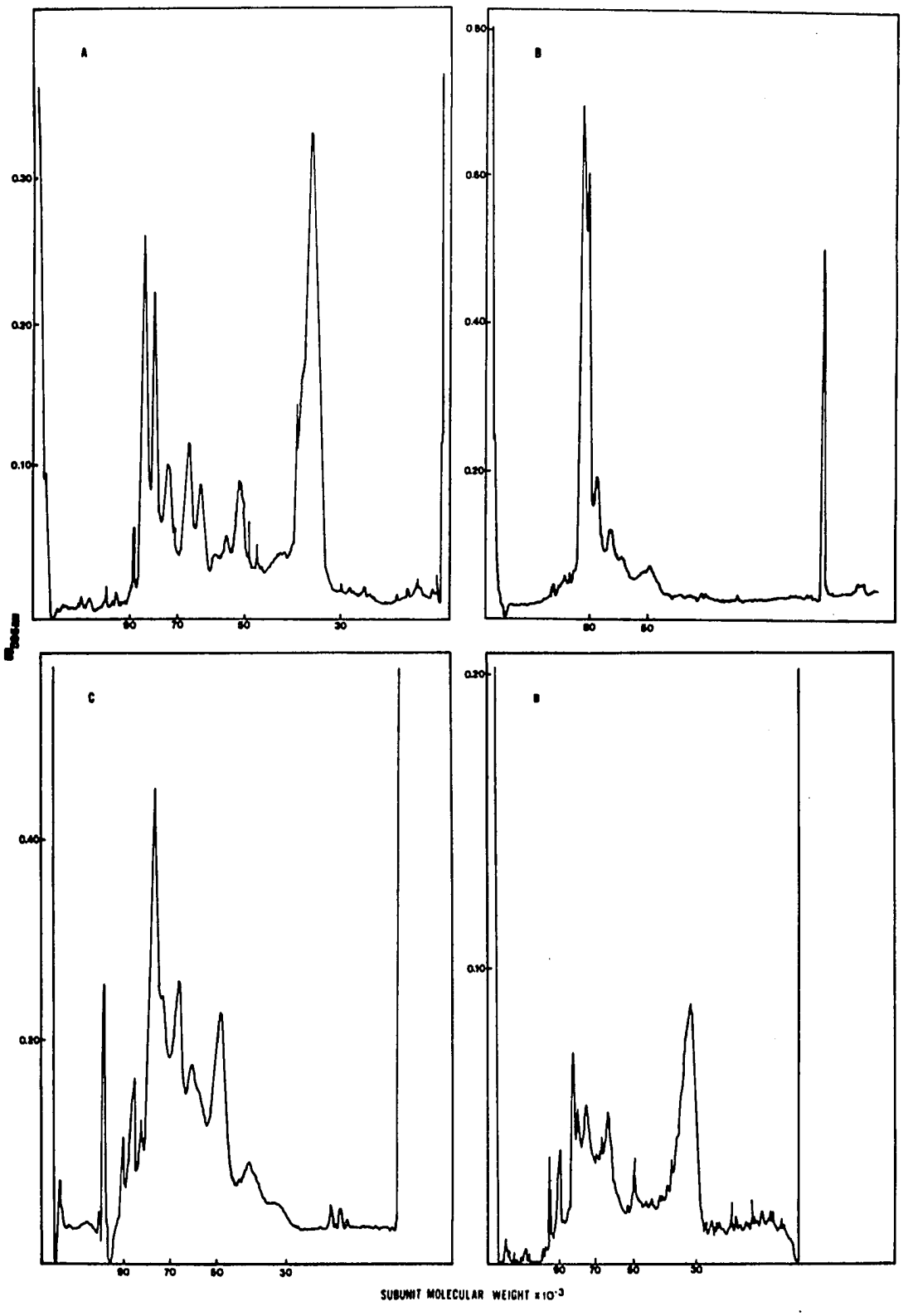
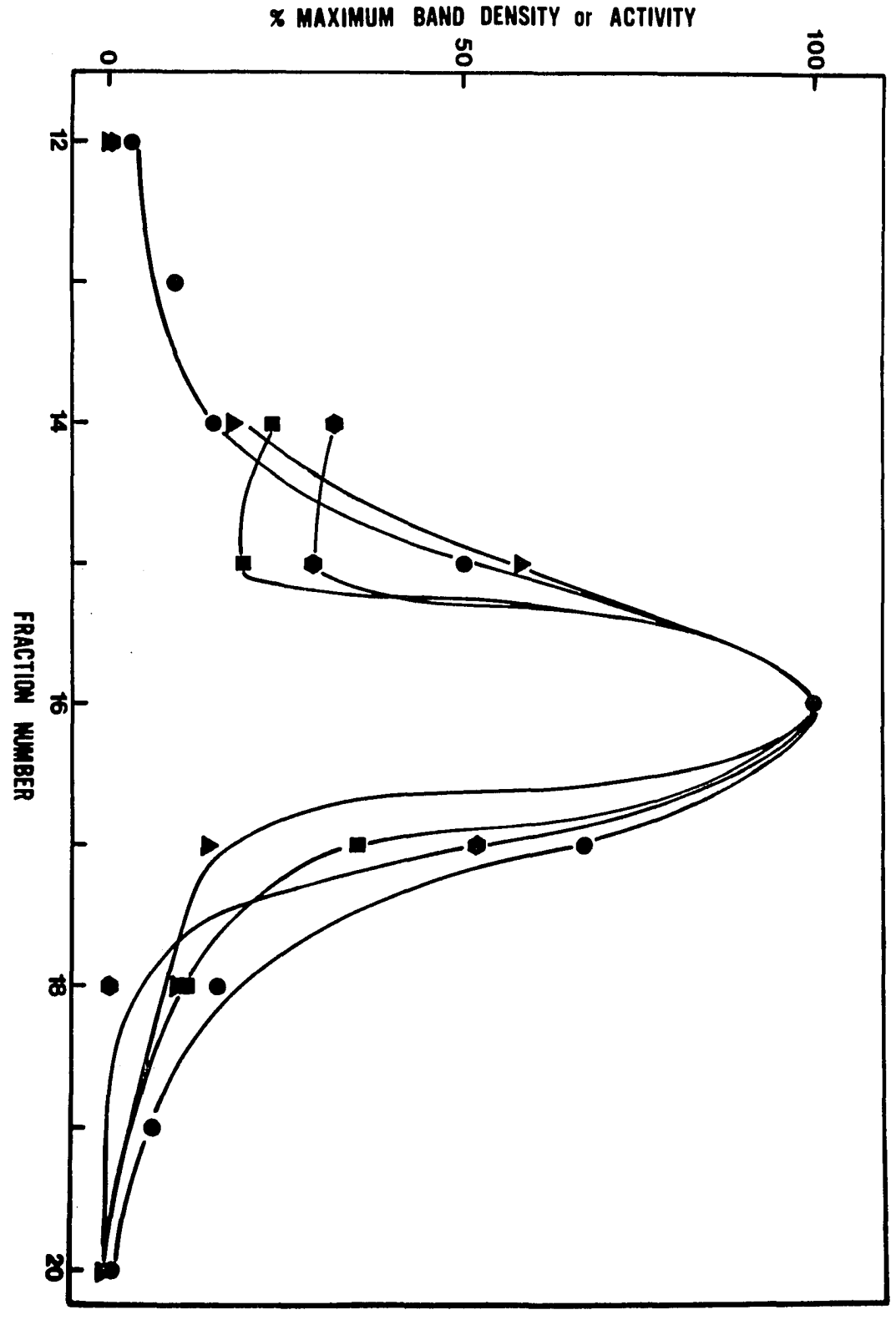

A
B
C
D
0.30
0.20
0.10
0.80
0.60
0.40
0.20
0.40
0.30
0.20
0.10
90
70
50
30
SUBUNIT MOLECULAR WEIGHT x10⁻³

Figure 16: Correlation of phosphoprotein phosphatase activity and protein concentration. The relative amounts of protein in specific bands of fractions from the sucrose gradient electrophoresis of phosphoprotein phosphatase (Fig. 13) were were calculated. The area of each peak from the gel scans of the fractions (Fig. 15) was determined by triangulation. The areas were corrected to the same O.D. scale. The area of each peak was calculated relative to the maximum area observed for that particular subunit molecular weight. Data obtained from the bands corresponding to subunit molecular weights of 76 x 10^3 (▲-▲), 68 x 10^3 (⬢-⬢) and 49 x 10^3 (■-■) daltons and the % maximum ^{32}P-histone phosphatase activity (●-●) were graphed for selected fractions.


% MAXIMUM BAND DENSITY or ACTIVITY
0
50
100
FRACTION NUMBER
12
14
16
18
20

of activity during this procedure and the resolution of high and low molecular weight components from the phosphatase suggests that electrophoresis in a sucrose gradient might be an effective purification step.

Molecular parameters of the soluble 4.1S phosphoprotein phosphatase: Two days after preparation, the molecular size of the purified phosphatase was determined by both gel filtration and sedimentation in a sucrose gradient. The two parameters were determined on the same day on the same enzyme preparation. A single symmetrical peak of phosphohistone phosphatase activity was eluted from a calibrated Sephacryl S200 column (Fig. 17). The Stokes' radius of the phosphatase was 44 Å with a range of 42-45 Å for four different preparations, indicating that the phosphatase purified by this procedure was consistently of the same size. Figure 18 shows the migration of phosphatase activity in a 5-25% sucrose gradient. A single symmetrical peak of activity was observed that migrated with a $s_{20,w}$ = 4.1S. Using the Stokes' radius and the sedimentation coefficient determined for the phosphatase on the same day, the frictional ratio of the enzyme was calculated to be 1.5 and the molecular weight 75 x 10^3 daltons. The asymmetry of the phosphatase is consistent with the asymmetric nature of other high molecular weight phosphatases (79). The sizes of bovine heart phosphoprotein phosphatases present in crude extracts and partially purified preparations are compared in Table 6. A Mn^{+2}-dependent 4S phosphatase activity was present in crude extracts and was partially purified from the post-microsomal supernatant and glycogen-pellet. The Stokes' radius and sedimentation coefficient of this enzyme were indistinguishable following purification from either fraction. There was no evi-

Figure 17: Determination of the Stokes radius of soluble phosphoprotein phosphatase. Phosphoprotein phosphatase purified through Sephacryl chromatograpy was dialyzed for 1.5 hours against TEEM, pH 7.8. 0.125 ml of the dialyzed enzyme were chromatographed on a calibrated Sephacryl S-200 column (0.9 x 19 cm) equilibrated in the same buffer. The fractions were assayed for ^{32}P-histone phosphatase activity.

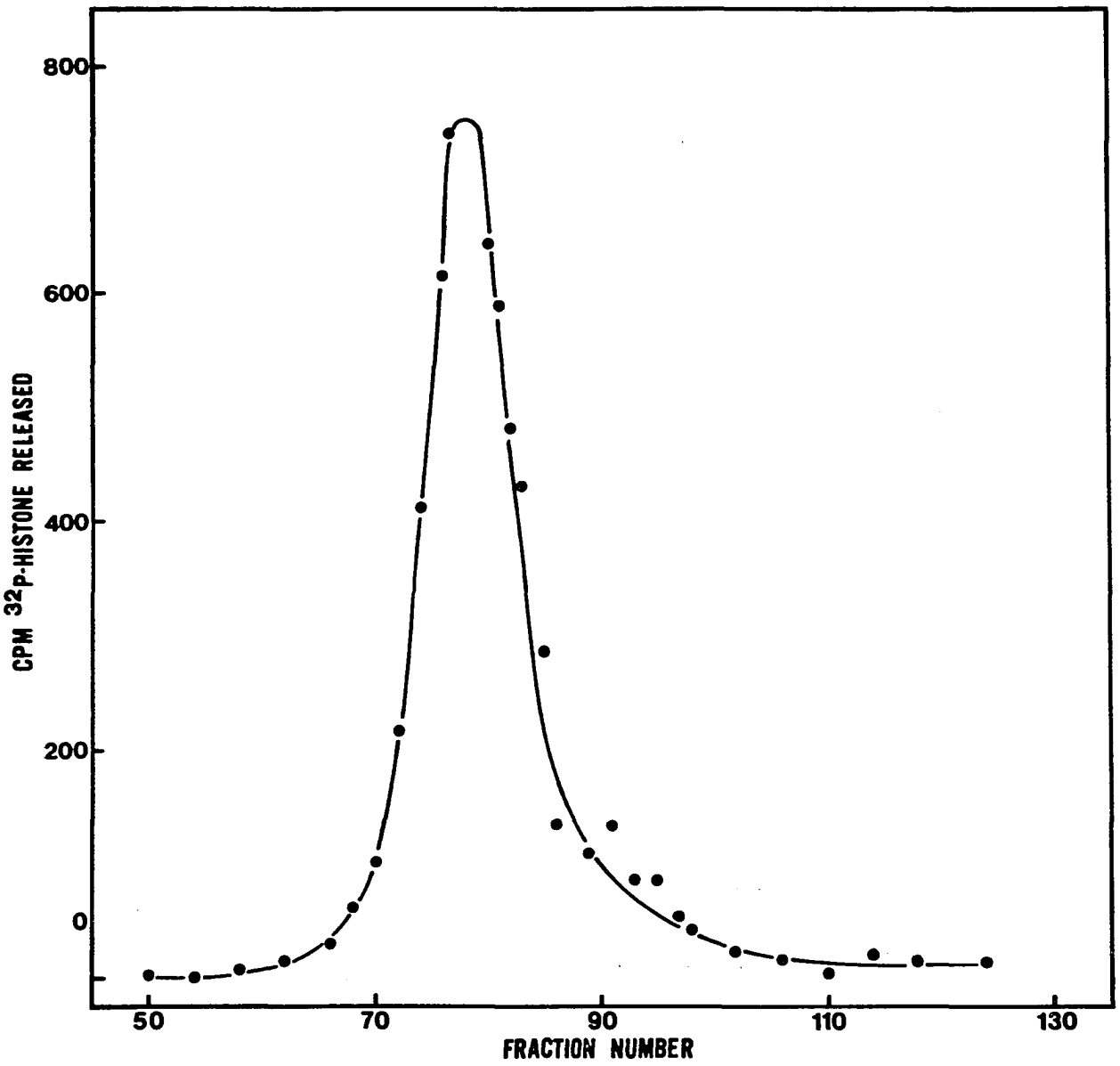

800
600
400
200
0
CPM 32P-HISTONE RELEASED
50
70
90
110
130
FRACTION NUMBER

Figure 18: Determination of $s_{20,w}$ of phosphoprotein phosphatase. The dialyzed phosphatase preparation described in Figure 17 was centrifuged the same day on a 5-25% sucrose gradient as described in Methods. Fractions were assayed for ^{32}P-histone phosphatase activity. Hemoglobin was run in a parallel gradient as a standard.

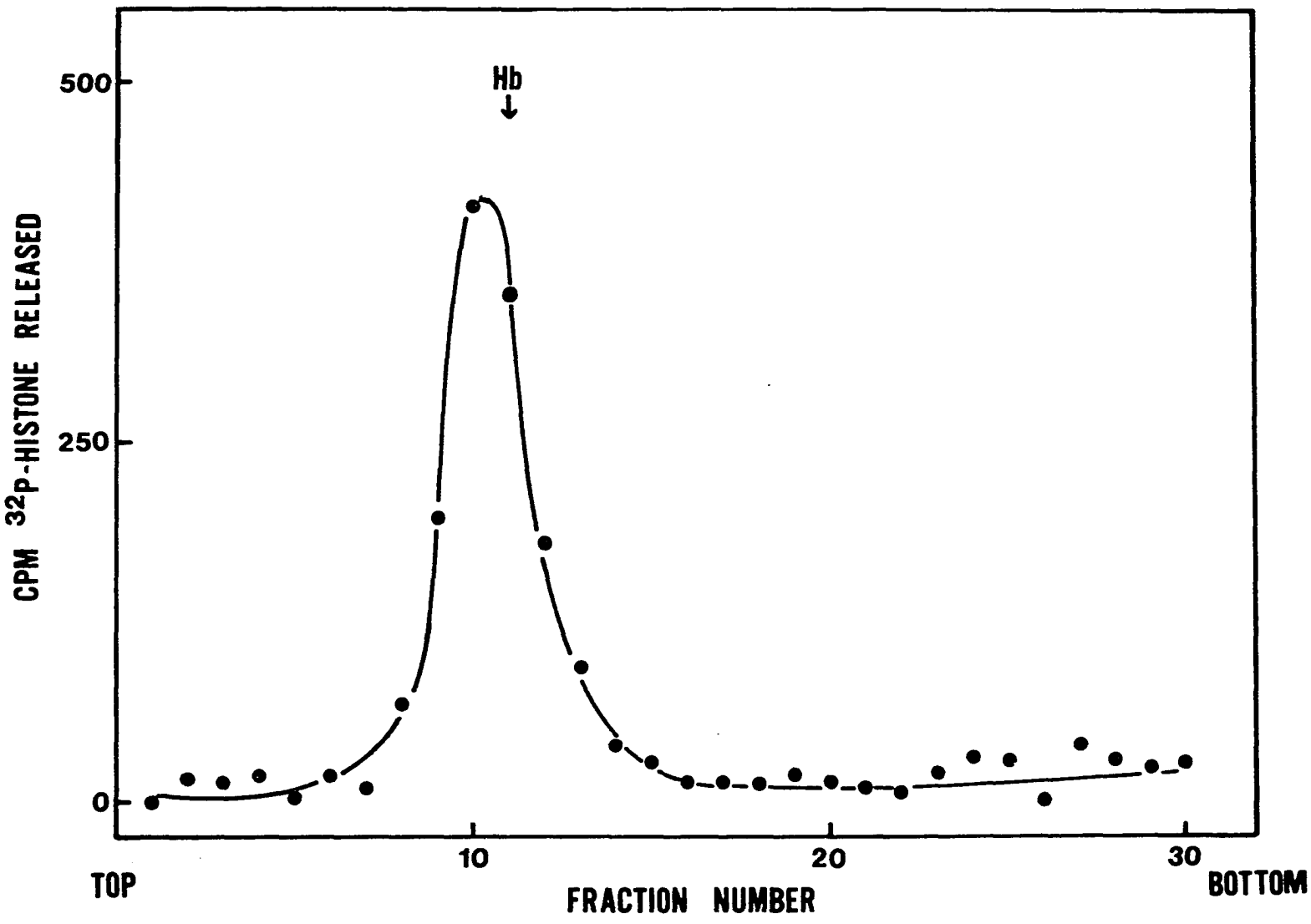

500
250
0
Hb
CPM ^{32}P-HISTONE RELEASED
TOP
10
20
30
BOTTOM
FRACTION NUMBER

Table 6: Molecular parameters of phosphoprotein phosphatases

Source	$s_{20,w}$ x 10^{13} (S)	Stokes radius (Å)	Molecular weight (daltons)	f/f_o
Post-microsomal cytosol	4.1	44	74 x 10^3	1.6
Glycogen-pellet	4.1	44	74 x 10^3	1.6
Glycogen-pellet	6.9	52	15 x 10^4	1.5

dence of extensive proteolysis during purification of the enzymes. The histone phosphatase activity was stimulated 10-fold (8.4- to 14-fold for 7 preparations) by 5 mM Mn^{+2}. The Mn^{+2} stimulation was characteristic of the enzyme purified from either the glycogen-pellet or the post-microsomal supernatant. Neither 5 mM Mg^{+2} or NaCl stimulated the activity. The dephosphorylation of glycogen synthase in the absence of Mn^{+2} was so low that it could not be determined accurately. There was a larger component present in the phosphatase preparation purified from the glycogen-pellet. This phosphatase sedimented at 6.9S, as did the largest activity observed in the crude extract. The larger phosphatase was found in only one of four preparations purified from the post-microsomal supernatant. The 6.9S phosphatase was found in three of four preparations purified from the glycogen-pellet. Studies of the association of the phosphatase forms with glycogen did not conclusively indicate that the higher molecular weight phosphatase was preferentially sedimented with glycogen. However, pelleting the high molecular weight phosphatase with glycogen may have stabilized the activity to further purification. The possibility cannot be excluded that the higher molecular weight phosphate was simply not present in crude extracts from the particular hearts used in purifying the 4.1S phosphatase from the post-microsomal supernatant.

DISCUSSION

In studying the regulation of dephosphorylation of a protein, it would be desirable to use a purified substrate with specific phosphorylation sites and a purified phosphatase isolated from the same species and tissue. The goal of this project was to purify a bovine heart phosphoprotein phosphatase that dephosphorylated glycogen synthase. One of the preliminary studies involved determining which phosphoprotein phosphatases were present in crude extracts from bovine heart.

Molecular Sizes of Bovine Heart Phosphoprotein Phosphatases

Two discrete species of phosphatase activity were observed in crude extracts from bovine heart. A small amount of activity sedimented to the bottom of the gradient. Only the $s_{20,w}$ = 7.0S phosphatase was detected when fractions were assayed in the absence of Mn^{+2}. This species is similar in size to enzymes observed in extracts from bovine heart (68), porcine heart (75), canine heart (79), rat liver (70), rat adrenal cortex (70), and rabbit skeletal muscle (122). Enzymes with similar sedimentation coefficients to that of the 4.1s Mn^{+2}-stimulated species have been observed in canine heart (79), bovine heart (68) and rabbit skeletal muscle (122), in rat adrenal cortex and rat liver (70). Whether there is a relationship between the two species of bovine cardiac phosphatase is not known. There is some evidence to support the hypothesis that similar species may be interconvertable. In 1974 it was shown that freezing a 6.7S enzyme in mercaptoethanol resulted in a loss of that activity and the appearance of activity with a $s_{20,w}$ = 3.8S (123). While a number of reports confirmed

the size of phosphatase activity could be decreased, a reversal of this possible conversion was not reported until 1978 by Khandelwal (88). When rat liver extracts were incubated with PP_i, he observed a loss in activity with time. The 4.1S activity decreased while a 7.8S activity increased. Incubation of the PP_i-inactivated extract with Co^{+2} or Mn^{+2} resulted in the activity migrating with a sedimentation coefficient of 4.1S. Partially purified preparations of the 4.1S phosphatase could not be converted to 7.8S, although partially purified 7.8S phosphatase could be converted to the 4.1S form. These data suggest that either the enzymes have been altered by purification or that there may be a component essential for association that is missing in the partially purified preparations. A dimer of 4.1S subunits would migrate at approximately 7.8S. However, the 7.8S phosphatase may be composed of 4.1S phosphatase and another component with no catalytic activity. There is a report that a physiological stimulus may affect the relative activities of the forms of phosphatase. Extracts of diaphragms from epinephrine-treated rats had a lower 4S phosphatase activity than extracts from control animals (123).

Although the enzymes observed in crude extracts of bovine heart may not be active in vivo, both species dephosphorylated glycogen synthase and could be useful in studying the regulation of the dephosphorylation of bovine heart glycogen synthase. The goal of the project was to purify one or both of these high molecular weight phosphatases. A major problem encountered in purifying phosphatases has been the generation of low molecular weight enzymes during the isolation. A survey of the purification procedures published when this project began, indicated that the low molecular weight phosphatases were isolated when divalent cations were included

in buffers and when DEAE-cellulose chromatography was used as an early purification step. Both of these conditions were avoided during development of the purification scheme for bovine heart phosphoprotein phosphatase. Based on recent data it appears that both of these conditions may stimulate proteolysis.

Ca^{+2}-stimulated proteases that degraded phosphorylase phosphatase were purified from rabbit skeletal muscle (76) in 1978. The protease activity was also stimulated by Mg^{+2} and Mn^{+2}. The phosphatase activities observed after proteolytic digestion corresponded to apparent M_r = 35 and 70 x 10^3. Enzymes of similar sizes have been obtained using procedures in which Mn^{+2} or Mg^{+2} were added to buffers to increase phosphatase recovery (48,123). Waxman and Krebs (124) have recently shown that Ca^{++}-stimulated casein protease activity is undetectable in crude bovine heart extracts. However, protease activity is easily measured following chromatography on DEAE-cellulose. The lack of proteolytic activity in crude extracts was attributed to the presence of protease inhibitors which were separated from proteases by the DEAE-cellulose chromatography step. The presence of protease inhibitors in heart may explain the small number of phosphatases observed in heart extracts even when vigorously homogenized (75,123). In contrast, 5-7 different molecular weight phosphatases are observed in vigorously homogenized liver (74) or muscle extracts (76). Protease activity is higher in crude extracts of muscle than in heart extracts (125). The inclusion of EDTA and EGTA to chelate divalent cations that could stimulate protease activity and the omission of DEAE-cellulose chromatography, coupled with rapid purification of the enzymes, may explain why only high molecular weight phosphatases were routinely observed in this study.

The 70-fold purified phosphatase preparation obtained from the glycogen-pellet contained two components. The smaller Mn^{+2}-dependent enzyme had a $s_{20,w}$ = 4.1S, a Stokes' radius of 44 Å, a M_r = 74 x 10^3 daltons, and a frictional ratio of 1.6. The 6.9S phosphatase was also asymmetric with a Stokes' radius of 52 Å, an M_r = 15 x 10^4 daltons, and a frictional ratio of 1.5. The phosphoprotein phosphatase purified 250-fold from the post-microsomal supernatant contained a single molecular weight species with a Stokes' radius of 44 Å, a $s_{20,w}$ = 4.1S, a M_r = 74 x 10^3 daltons and a frictional coefficient of 1.6.

In one preparation purified from the post-microsomal supernatant, a small amount of activity was eluted from the preparative Sephacryl S200 column at a Stokes' radius of approximately 52 Å. It was not determined if the larger phosphatase was present in each of the crude extracts that were used in purifying phosphatase from the post-microsomal fraction. The larger phosphatase may be less stable to purification in a soluble fraction than in a glycogen-pellet. The molecular parameters of the Mn^{+2}-dependent phosphatase were identical whether the enzyme was purified from the post-microsomal supernatant or the glycogen-pellet, indicating that the same enzyme was present in both fractions. The sedimentation coefficient of either of the partially purified enzymes differed by only 5% from the sedimentation coefficient of the corresponding phosphatase in crude extracts. There was less than 5% variability between preparations in the size of each phosphatase. The procedures developed for purifying phosphatase did not generate low molecular weight active species. The sizes of the partially purified phosphatases were stable for several months.

Purification of Bovine Heart Phosphoprotein Phosphatases

The phosphatase activity that sedimented with glycogen was purified approximately 70-fold. Pelleting the activity with glycogen did not result in significant purification. Only 16% of the total phosphatase activity in the post-microsomal supernatant was sedimented with glycogen. The preparations obtained by this procedure contained 4.1S and 6.9S phosphatases. Separation of the two enzymes was not successful on a preparative scale due to large activity losses. Sephacryl chromatography separated the major 44 Å phosphatase from a 55 Å phosphatase present in one of the preparations of soluble phosphatase. This rapid, high resolution chromatography step might also be useful for separating the 4.1S (44 Å) and the 6.9S (52 Å) phosphatases isolated from the glycogen-pellet.

The 4.1S phosphatase was purified 250-fold from the post-microsomal supernatant in adequate quantities for use in studying the dephosphorylation of glycogen synthase or other phosphoprotein substrates. A single active species was detected by several criteria including sucrose gradient centrifugation, molecular sieve chromatography, activity gels, and sucrose gradient electrophoresis. The phosphohistone phosphatase activity corresponds to a minor protein band on activity gels.

Many laboratories have attempted to purify high molecular weight phosphatases, but purification of these phosphatases has been very difficult due to extensive activity losses or conversion to lower molecular weight forms. Affinity chromatography techniques have been applied with limited success. Stringent conditions are needed to elute the phosphatase and result in the isolation of a low molecular weight enzyme (85). Chromatography using Sepharose linked hydrocarbon derivatives has been moderately

successful. In this study a 10-fold purification could be obtained by chromatography on diaminobutane-Sepharose. Purification by gel filtration has been limited by activity losses. Using Sephacryl beads for gel filtration permitted rapid flow rates with good resolution so that most of the activity was recovered and the enzyme was purified approximately 10-fold. The stabilization of the enzyme activity by high concentrations of glycerol permitted dialysis and storage of the preparations without extensive activity loss. Since the phosphatase activity was stable during sucrose gradient electrophoresis and several contaminants were removed, this technique might be useful on a preparative scale. Ion-exchange chromatography might permit separation of contaminants of similar size, but different charge.

Phosphatases of approximately 4S have been highly purified from canine heart and rabbit skeletal muscle. Li et al. (81) purified a 39 Å, 3.8S, Mn^{+2}-dependent phosphatase 1400-fold from canine heart. One of the major purification steps involved an 80% ethanol precipitation that also produced low molecular weight phosphatases and may have altered the properties of the 3.8S phosphatase. One of the purification procedures for a high molecular weight phosphatase involves isolation of the phosphatase activity bound to the rabbit skeletal muscle glycogen particle (78). Only eight major proteins co-sediment with endogenous glycogen. The phosphatase activity isolated by this approach has been purified 3000-fold in a 6% yield (assayed on phosphorylase), but the size of the glycogen-associated rabbit skeletal muscle phosphatase is variable (78). The activity eluted either with an apparent M_r = 48 and/or 80 x 10^3 or 125 x 10^3 daltons. An apparent M_r = 160 x 10^3 dalton phosphatase has been purified 600-fold from rat liver, but the yield was only 0.2% of the initial activity (80).

The isolation procedures developed during this study did not yield highly purified phosphatases. The enzymes could perhaps be further purified by Sephacryl chromatography of the 70-fold glycogen-pelleted prep and by preparative sucrose gradient electrophoresis. The activity and sizes of the partially purified enzymes were stable for several months. The sizes of the phosphatases were not only reproducible, but were very similar to those observed in crude extracts. A procedure was developed for obtaining a 4.1S phosphatase that contained only one active form according to several criteria.

Association of Phosphoprotein Phosphatase Activity with Glycogen

Initially in this study it was hoped that a specific glycogen synthase phosphatase could be isolated by sedimenting the phosphatase with glycogen. Bovine heart glycogen synthase can be pelleted from a post-microsomal supernatant to which glycogen has been added (106). The synthase is always contaminated with some glycogen synthase phosphatase activity. Under the conditions used in this study, however, only 16% of the phosphoprotein phosphatase activity pelleted with the exogenous glycogen (Table 2). Approximately three times more phosphatase activity pelleted in the presence of glycogen than in its absence; therefore, there did appear to be some association of phosphatase with glycogen. The same percent of total activity sedimented whether phosphohistone or glycogen synthase was used as the substrate. Neither the total synthase phosphatase nor the phosphohistone phosphatase activity was altered by pelleting with glycogen. The total activity in the post-glycogen supernatant and the glycogen-pellet was equal to that in the post-microsomal supernatant. The activity in the

glycogen-pellet was unaltered by digestion with α-amylase. Therefore, the presence of oyster glycogen did not affect total phosphatase activity.

It is intriguing that the phosphatase activity in the glycogen-pellet fraction sedimented faster in a sucrose gradient than the activity from any other fractions. In contrast, Goris et al. (126) have proposed that there is a specific low molecular weight glycogen-associated phosphatases. In their study, dog liver was homogenized in the absence of metal ion chelators and a glycogen-pellet was prepared. Glycogen was solubilized from pelleted microsomes by treatment with 8 mM CaCl. The phosphatase eluted from a Sephadex G-200 column at an apparent M_r = 51 x 10^3. This low molecular weight form of phosphatase was not observed in the post-glycogen supernatant, but it was not treated with $CaCl_2$. Incubation of the glycogen containing fraction with $CaCl_2$ may have stimulated Ca^{++}-dependent proteolysis resulting in degradation of the phosphatase(s) that did pellet with glycogen.

Both the 4.1S and the 6.9S phosphatases could be isolated from the glycogen-pellet following α-amylase digestion. This indicates that the 4.1S and 6.9S phosphatases were derived from the phosphatase(s) that pelleted in the presence of glycogen. The high molecular weight phosphatase(s) in the bovine heart glycogen-pellet may be attributable to 4.1S and 6.9S phosphatases to which glycogen or other carbohydrate is associated. The 4.1S and 6.9S phosphatases may have aggregated or associated with other protein(s) in the presence of glycogen. It would be interesting to determine if the high molecular weight phosphatase(s) can be detected in crude extracts or post-microsomal supernatants to which glycogen has been added. Bovine hearts obtained from commercial slaughter houses usually

have a low glycogen content. The forms of phosphatase present in extracts from these hearts may be comparable to those in a fasted animal. It has been shown that liver synthase phosphatase activity from fasted animals is recovered in the post-microsomal fraction (42). The phosphatase activity from glycogen-rich livers sediments with the glycogen-microsomal fraction. The sedimentation coefficient(s) of the phosphatase activity in this fraction was not analyzed. If glycogen does promote association of phosphatase components, then it might be possible to purify "associated" phosphatase in the presence of glycogen. The conditions could be optimized for sedimentation of phosphatase activity from the post-microsomal supernatant by glycogen. Glycogen synthase phosphorylated in specific sites should be used as a substrate since Stalsman's laboratory (42) has recently shown that the fraction of synthase phosphatase activity that sediments with glycogen varies depending on the phosphorylation state of the synthase substrate. The sedimented phosphatase could then be purified without degrading the glycogen.

Evaluating the kinetic parameters of purified phosphatases may lead to the development of assay conditions that can be used to easily and accurately detect specific changes in the activity of these different phosphatases. Currently determining changes in the activity of phosphatases of different sizes in response to hormones and other stimuli is tedious and time consuming. If the phosphatases have different substrate specificities, then it may be possible to assay different phosphatases without actually separating them. Data from Stalsman's laboratory (42) has recently suggested that the different phosphorylated forms of glycogen synthase may be dephosphorylated by different phosphatases. It is now possible to

prepare bovine heart glycogen synthase phosphorylated by either cAMP-dependent protein kinase or a cyclic-nucleotide-independent glycogen synthase kinase. The 4.1S phosphatase described in this thesis has not been purified to homogeneity, but it is not contaminated by glycogen synthase or phosphorylase and therefore can be used to study the dephosphorylation of glycogen synthase and other phosphoproteins. It is hoped that information acquired during this project will lead to procedures that will isolate the 7S and the glycogen-pelleted phosphatases. Studying the dephosphorylation of glycogen synthase by these phosphatases may lead to a better understanding of the function of different phosphorylation sites in glycogen synthase, and the regulation of their dephosphorylation. Using specific glycogen synthase substrates under certain assay conditions may also permit determination of changes in the activity of different phosphatases, increasing our understanding of the regulation of phosphoprotein phosphatase activity.

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