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ISOLATION AND CHARACTERIZATION OF BOVINE CEREBELLAR PROTEIN PHOSPHATASES

by

PAUL F. SIMONELLI

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

ISOLATION AND CHARACTERIZATION OF
BOVINE CEREBELLAR PROTEIN PHOSPHATASES

by

Paul F. Simonelli

Advisor: Dr. Heng-Chun Li

A systematic study of cerebellar protein phosphatases (phosphoprotein phosphohydrolase, EC 3.1.3.16) was conducted using as substrates ^{32}P-labeled phosphorylase a and G-substrate, a neuron- and cerebellum-specific substrate for cyclic GMP-dependent protein kinase (Aswad, D., & Greengard, P., J. Biol. Chem. 256, 3487-3493, 1981). Four major phosphatase activities termed I, -2, II, and III were eluted by DEAE-cellulose chromatography of fresh cerebellar homogenates at 0.12, 0.15, 0.20, & 0.35 M KCl, respectively. Phosphatase I (M_r 62,800, SR 3.8 nm, $s_{20,w}$ 4.0) was preferentially active towards phosphorylase a, could be activated by Mn^{2+} or by preincubation with Mg·ATP + F_a, and was the only phosphatase sensitive to heat-stable inhibitor-1 (K_i 0.7 nM) and inhibitor-2. Phosphatase-2 (M_r 80,000) was activated by Mn^{2+} or Ca^{2+}-calmodulin, and preferentially dephosphorylated G-substrate. The ratio of maximal velocities indicated that phosphatase II (M_r 187,300, SR 5.4 nm, $s_{20,w}$ 8.4) preferentially dephosphorylated G-substrate (G-substrate : phosphorylase a 3:1), whereas phosphatase III (M_r 101,700, SR 4.4 nm, $s_{20,w}$ 5.6) was preferentially active towards phosphorylase a (V_{max} ratio 1:5). The K_m parameters displayed by the two phosphatases were similar (0.2 uM G-substrate, 5-17 uM phosphorylase a). Like

phosphatase I, G-substrate dephosphorylation catalyzed by phosphatases II and III required the simultaneous presence of Mn^{2+} (K_a 0.2 mM); other metals were less effective. Dephosphorylation of phosphorylase a by phosphatase II was also Mn^{2+}-dependent (K_a 0.2 mM), but phosphatase III displayed near-maximal activity towards this substrate in the presence of 2 mM EDTA. Under these conditions, G-substrate inhibited phosphatase III activity towards phosphorylase a in a concentration-dependent, non-competitive manner (K_i 0.15 uM), an effect that was reversible by dilution. In its dephosphorylated form (catalyzed by phosphatase II), G-substrate was ineffective as an inhibitor, but its potency could be restored by subsequent phosphorylation catalyzed by cyclic GMP-dependent protein kinase. SDS-gel electrophoresis indicated that no changes other than an altered ^{32}P-content were apparent. Together, these findings indicate that G-substrate could function as a specific inhibitor of phosphatase III, and that its potency can be regulated by a cycle of phosphorylation/dephosphorylation.

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Introduction

Protein phosphorylation is increasingly recognized as an important regulatory mechanism acting within the nervous system. Neurotransmitter biosynthesis and release, alterations in membrane electrical properties, microtubule and vesicle functioning, and behavior modification in invertebrates are among the processes that have been correlated with changes in the phosphorylation state of neuronal proteins (reviewed by Greengard, 1978; Paris et al, 1981; Kennedy, 1983; and Nestler & Greengard, 1984). In addition, considerable evidence indicates that the intracellular effects of several neurotransmitters and pharmacologic agents - acting through second messenger molecules including cyclic AMP, cyclic GMP, and calcium - are mediated by phosphorylation-dephosphorylation mechanisms (reviewed by Greengard, 1976; Walter & Greengard, 1981; Greengard, 1981; Forn, 1984; and Berridge, 1984). One approach to the study of neurophosphorylation has relied on the purification of proteins that were found to be phosphorylated endogenously in response to protein kinase activation (Kennedy, 1983; Forn, 1984). Once the protein and the effectors regulating its phosphorylation are fully characterized, it is possible to begin an extrapolation of these findings to the physiologically based setting. To this end, most studies of neurophosphorylation have focused primarily on protein kinases and their target substrates.

Protein phosphatases (phosphoprotein phosphohydrolase, EC 3.1.3.16) represent a potentially important mechanism of physiologic control. By reversing the effects of protein kinases, they modulate the activity of proteins that are regulated by phosphorylation and dephosphorylation. Protein phosphatases have been most intensively studied in relation to glycogen metabolism using enzymes purified from muscle and liver (reviewed by Krebs & Beavo, 1979; Curnow &

Larner, 1979; Lee et al, 1980; and Li, 1982). Recent studies indicate that the activity of these enzymes is highly regulated; a complete understanding of systems regulated by protein phosphorylation requires a characterization of the factors that modulate protein phosphatase as well as protein kinase activity (Hers, 1976; Ingebritsen & Cohen, 1983a). To date, however, there has been little systematic study of protein phosphatases isolated from the nervous system. The goal of this study has been to identify and characterize protein phosphatases from brain.

Classification of Protein Phosphatases

Multiple forms of protein phosphatases have been identified in a wide variety of animal tissues (reviewed by Cohen, 1978; Curnow & Larner, 1979; Lee et al, 1980; Shimazu, 1982; and Li, 1982). Recent studies have indicated that many of these varied phosphatase activities can be attributed to one of a few basic types of protein phosphatase isozymes (Li, 1982; Ingebritsen & Cohen, 1983a). A scheme of classification developed in this laboratory (Chernoff & Li, 1983) relates various reported findings to the properties of phosphatases previously purified from cardiac muscle (Hsiao et al, 1977; Li et al, 1978; Binstock & Li, 1979) and brain (Chernoff et al, 1983). Classification into one of five types considers differences in physical, catalytic, and regulatory properties. Briefly, types 1, 2, and 4 phosphatases can be defined by their preferential mechanism of activation. Type 1 phosphatases are activated by incubation with Mg·ATP plus a protein activator termed F_a (Vandenheede et al, 1980). In addition, type 1 phosphatases are uniquely sensitive to low concentrations (nanomolar) of heat-stable inhibitors 1 and 2 (Huang & Glinsmann, 1976). Type 2 phosphatases are activated by Ca^{2+}·calmodulin, and type 4 phosphatases are activated by Mg^{2+}. Type 3 phosphatases are characteristically high molecular weight (M_r>90,000),

multisubunit enzymes. Each seems to contain a common catalytically active subunit (M_r 35,000) that was separately characterized as phosphatase S (Hsiao et al, 1977). Catalytic properties unique to different forms of type 3 protein phosphatases have not yet been defined. Instead, type 3 phosphatases are subcategorized as types 3A, 3B, and 3C based on differences in chromatographic properties and subunit composition. A fifth group of phosphatases (Y-1, Y-2, and Y-3) are highly specific for proteins phosphorylated on tyrosine residues. (Types 1-4 are most active towards substrates phosphorylated on serine or threonine residues.) As part of this study, phosphatases classified into types 1, 2, and 3 have been identified in bovine cerebellum. Type 4 protein phosphatases have been extensively characterized in canine heart (Binstock & Li, 1979), rat liver (Hiraga et al, 1981), turkey gizzard (Pato & Adelstein, 1983b), and human erythrocytes (Usui et al, 1983), and have been identified in an extract prepared from rabbit brain (Ingebritsen et al, 1983c). Tyrosine protein phosphatases isolated from bovine brain have been characterized in this laboratory as part of a separate study (Chernoff & Li, 1983; Li, 1984).

Another widely used scheme of classification of protein phosphatases has been developed by Philip Cohen and his co-workers (Ingebritsen & Cohen, 1983a). In this system, type 1 phosphatases are defined by their sensitivity to heat stable inhibitors 1 and 2, and substrate specificity for the beta subunit of phosphorylase kinase. In contrast, type 2 phosphatases are relatively insensitive to the heat stable inhibitors, and display specificity for the alpha subunit of phosphorylase kinase. The type 2 phosphatases are further subclassified into phosphatases 2A ($2A_0$, $2A_1$, & $2A_2$), 2B, and 2C according to differences in subunit composition, chromatographic properties, and mechanism of activation (Ingebritsen et al, 1983a; Pato et al, 1983; and Stewart et al, 1983). Recent studies using a wide variety of phosphoprotein substrates have indicated that virtually all of the

protein phosphatase activity isolated from extracts of several tissues is accounted for by types 1 and 2 phosphatases (Ingebritsen et al, 1983c). A comparison of this system with that used by our laboratory suggests the following correspondence: the two type 1 phosphatases seem to be identical, and phosphatases $2A_0$, $2A_1$, $2A_2$, 2B, and 2C are similar to types 3A, 3B, 3C, 2, and 4, respectively.

Type 1 Protein Phosphatases

Ethanol precipitation has been widely used as a step in the purification of protein phosphatases (reviewed by Lee et al, 1980). Treatment with ethanol typically results in the activation and apparent dissociation of high molecular weight (type 3) protein phosphatases into smaller catalytically active enzymes (discussed below). Other phosphatases are seemingly resistant to ethanol precipitation as no activation or dissociation is observed. One of these ethanol-resistant enzymes was characterized in this laboratory as phosphatase U-1 (M_r 61,000), a metal ion-dependent protein phosphatase purified from canine heart (Li & Hsiao, 1977a; and Li et al, 1978). Phosphatase U-1 displayed broad substrate specificity using a battery of phosphorylated substrates; the highest levels of activity were measured using phosphorylase a and phosphohistone as substrates (Li et al, 1978). Low levels of activity were measured towards phosphorylated casein and p-nitrophenyl phosphate (a low molecular weight phosphoester) in contrast to the pattern of substrate specificity observed with other (particularly type 3) protein phosphatases. This difference in substrate specificity, together with demonstrated differences in chromatographic properties and sensitivity to various inhibitors and activators led to the proposal that phosphatase U-1 might represent a unique catalytic entity separate from other better characterized protein phosphatases (Li et al, 1978).

Phosphatase U-1 was originally defined by its requirement for the addition

of sub-millimolar amounts of a divalent metal ion to the assay mixture in order to express enzymic activity. Of the metal ions tested, Mn^{2+} was found to be the most effective (Li & Hsiao, 1977a). Later studies indicated that full activation could instead be achieved by the incubation of phosphatase U-1 with Mg, ATP, and a protein activator termed F_a (H.-C. Li, unpublished results). This latter finding suggested that phosphatase U-1 might be the same as phosphatase F_c, a Mg·ATP-dependent phosphorylase phosphatase first described by Wilfried Merelevede and his co-workers (Merlevede & Riley, 1966; Goris et al, 1980). Phosphatase F_c and its activator F_a have been isolated from several tissues (Chelala & Torres, 1970; Goris et al, 1977; Yang et al, 1980b), but the most intensively studied forms of F_c and F_a have been purified from rabbit skeletal muscle (Yang et al, 1980a; Vandenheede et al, 1980). Like phosphatase U-1, F_c displays little or no spontaneous activity but could be at least partially activated by the addition of Mn^{2+} to the assay incubation mixture (Yang et al, 1981b; Vandenheede et al, 1981b). And like phosphatase U-1, full activation requires a prior time-dependent incubation of F_c with Mg·ATP and F_a (Yang et al, 1980a).

Although originally disputed (Vandenheede et al, 1981c; Yang et al, 1981b), it now appears that the mechanism of activation of F_c by F_a involves phosphorylation (Hemmings et al, 1982; Jurgensen et al, 1984; Villa-Moruzzi, 1984). F_a displays protein kinase activity (Vandenheede et al, 1980), and appears to be identical to glycogen synthase kinase 3 (Hemmings et al, 1981), an enzyme thought to be linked to the hormonal control of muscle glycogen synthase activity (Parker et al, 1983). An interesting development in these studies is the finding that phosphorylation takes place on a component of purified F_c that can be separately isolated as phosphatase inhibitor-2 (Hemmings et al, 1982; Ballou et al, 1983; Resink et al, 1983; Jurgensen et al, 1984). Inhibitor-2 was first isolated from heat-inactivated extracts of rabbit skeletal muscle (Huang & Glinsmann,

1976a), and was later shown to be highly specific in the reversible inactivation of type 1 protein phosphatase activity (Cohen et al, 1977; Li, 1982; Ingebritsen & Cohen, 1983b). Inhibitor-2 co-purifies with F_c (Yang et al, 1981a; Brautigan et al, 1982; Yang et al, 1983; Ballou et al, 1983), but it is not yet clear if inhibitor-2 is an integral part of the F_c molecule in its native state, or if it exists as a separate protein (Jurgensen et al, 1984; Villa-Moruzzi, 1984). In either case, recently proposed models suggest that F_c activity might be reversibly regulated by a cycle of inhibitor-2 phosphorylation and dephosphorylation: activation would follow F_a-catalyzed phosphorylation, and inactivation would (slowly) follow F_c-catalyzed dephosphorylation (Resink et al, 1983; Jurgensen et al, 1984; and Villa-Moruzzi, 1984).

The molecular weight of F_c in the native state is not yet clear. Some reports suggest that the phosphatase (or its inactive precursor) exists in high molecular weight forms (M_r 140,000 - 260,000) that can be rapidly proteolyzed to a lower molecular weight during purification (Mellgren et al, 1979; Lee et al, 1980; Vandenheede et al, 1981b & 1981c; Brautigan et al, 1982; Khatra & Soderling, 1983). In general, the molecular weight to purified F_c has been reported to be in the range of 60,000 -70,000 daltons (Yang et al, 1980a; Vandenheede et al, 1981b; Brautigan et al, 1982; Jurgensen et al, 1984; Villa-Moruzzi et al, 1984). Further proteolysis results in the isolation of a relatively stable phosphatase (M_r 32,000 - 38,000) that displays spontaneous (i.e., metal ion- and Mg$^+$ATP-independent) activity (Vandenheede et al, 1981b; Brautigan et al, 1982; Ballou et al, 1983). Reconstitution of the latter with purified inhibitor-2 (M_r 30,000 - 33,000) reduces the spontaneously measured activity while simultaneously restoring sensitivity to F_a plus Mg$^+$ATP (Yang et al, 1981a; Vandenheede et al, 1983; Resink et al, 1983).

Several of the properties of F_c are similar to those of protein phosphatase-

1, a widely studied type 1 phosphatase originally described by Philip Cohen and his co-workers (reviewed by Ingebritsen & Cohen, 1983a). Both display identical substrate specificity, and both display similar sensitivity to specific heat-stable inhibitor proteins (Stewart et al, 1981). Like the proteolyzed form of F_c, phosphatase-1 is most often isolated as a M_r 33,000 - 37,000, metal ion and Mg·ATP-independent activity (Cohen, 1978; Tung et al, 1984). Furthermore, addition of inhibitor-2 to phosphatase-1 confers sensitivity to F_a plus Mg·ATP (Hemmings et al, 1982; Resink et al, 1983; Tung et al, 1984). It seems likely that phosphatase-1 and F_c represent two forms of the same enzyme. A possible explanation of the difference in the properties of the two phosphatases relates to the inclusion of Mn^{2+} (added for stability) in the buffers used to prepare and store phosphatase-1 (Stewart et al, 1981). In the presence of Mn^{2+}, a catalytically active, metal ion-independent phosphatase (M_r 35,000) can be derived from proteolyzed F_c. This form is resistant to further trypsin-catalyzed proteolysis (Brautigan et al, 1980 & 1982). Under similar conditions, inhibitor-2, and the portion of F_c that confers F_a sensitivity are highly labile to proteolysis (Vandenheede et al, 1981a; Resink et al, 1983; Ballou et al, 1983).

Sensitivity to low concentrations of inhibitor-2 has been used to demonstrate a wide tissue distribution of phosphatase-1 (Foulkes et al, 1983b; and Ingebritsen et al, 1983c). Phosphatase-1 displays broad substrate specificity (Ingebritsen & Cohen, 1983b), and represents a significant proportion of the phosphatase activity potentially involved in the regulation of several diverse cellular functions, including glycogen metabolism (Ingebritsen et al, 1983a), glycolysis/gluconeogenesis and fat metabolism (Ingebritsen et al, 1983b), and protein synthesis (Ernst et al, 1982; Ingebritsen et al, 1983c). Recently, phosphatase-1 has been identified in extracts of whole rabbit and chicken brain (Foulkes et al, 1983a; Ingebritsen et al, 1983c). In both of these studies,

phosphatase-1 was defined by its elution properties on ion-exchange chromatography, and its activity towards skeletal muscle phosphorylase a measured in the presence and absence of inhibitor-2. These reports indicated that the level of activity of brain phosphatase-1 is comparable to that measured in other tissues. To date, however, neither phosphatase-1 nor any other form of type 1 protein phosphatase has been isolated from brain. One of the goals of the present study was to identify a $Mg^{\cdot}ATP$-dependent protein phosphatase in brain, and to compare some of its properties with the well-characterized skeletal muscle F_c.

Regulation of Type 1 Protein Phosphatase Activity by Specific Protein Inhibitors

Specific inhibitors of protein phosphatase activity were first independently isolated from glycogen-deficient carcinoma cells (Baba & Tsuiki, 1974) and from extracts prepared from rat liver, heart, and skeletal muscle (Brandt et al, 1974 & 1975a). In both cases, these inhibitors were characterized as non-dialyzable (apparent $M_r \geq 50{,}000$) and trypsin-labile, with pronounced stability to heat ($90^{\circ}C$, 3 - 10 minutes) and acid precipitation (15% trichloroacetic acid). The inhibitors displayed selectivity for different protein phosphatase activities (Baba & Tsuiki, 1974) and for particular forms of the phosphatase used in the assay of inhibitor potency (Brandt et al, 1975b). Together with the observation that inhibition was shown to be non-competitive with respect to substrate, these findings suggested that the protein inhibitors might specifically interact with protein phosphatases per se.

A few earlier observations had indicated that the phosphatase activity of crude adrenal or muscle extracts could be inactivated by the addition of cyclic AMP (Merlevede & Riley, 1966; Chelala & Torres, 1970). Huang and Glinsmann (1975) demonstrated that the potency of a skeletal muscle inhibitor preparation could be increased by phosphorylation catalyzed by cyclic AMP-dependent protein

kinase. This increased potency could subsequently be decreased by incubation with a Mn^{2+}-dependent protein phosphatase suggesting that the effectiveness of this inhibitor could be modified by a cycle of reversible covalent modification (Huang & Glinsmann, 1975). These same authors isolated a second type of inhibitor that was unaffected by either cyclic AMP-dependent phosphorylation or Mn^{2+}-dependent dephosphorylation (Huang & Glinsmann, 1976a). These findings led to the isolation and characterization of two distinct protein inhibitors from rabbit skeletal muscle (Huang & Glinsmann, 1976b): inhibitor-1, which requires phosphorylation in order to inhibit protein phosphatase activity; and inhibitor-2, which does not.

Inhibitor-1 has been purified to apparent homogeneity from rabbit skeletal muscle (Nimmo & Cohen, 1978a). (Several of the physical properties of purified inhibitor-1 are shown in Table 2). Cyclic AMP-dependent protein kinase catalyzes the phosphorylation of a single threonine residue in inhibitor-1 (Nimmo & Cohen, 1978b). In this form, inhibitor-1 specifically inhibits type 1 protein phosphatase activity: both protein phosphatase-1 (Nimmo & Cohen, 1978b; and Ingebritsen & Cohen, 1983b) and F_c (Goris et al, 1978; Stewart et al, 1981) are completely inhibited by nanomolar concentrations of inhibitor-1 ($K_i \sim 2$ nM). In contrast, concentrations of inhibitor-1 100- to 200-fold greater result in only partial inhibition of other types of protein phosphatase activity (Cohen et al, 1977; Ingebritsen et al, 1983b). Dephosphorylated inhibitor-1 does not affect type 1 protein phosphatase activity as it neither inhibits phosphatase activity nor prevents phosphorylated inhibitor-1 from exerting its inhibitory effect (Nimmo & Cohen, 1978b; Goris et al, 1978).

Inhibitor-2 has also been purified to apparent homogeneity from rabbit skeletal muscle (Foulkes & Cohen, 1980). As discussed above, inhibitor-2 seems to be an integral component of phosphatase F_c in that it confers sensitivity to

activation by F_a (Jurgensen et al, 1984; Villa-Moruzzi et al, 1984). However, inhibitor-2 affects the activity of F_c and protein phosphatase-1 by what is thought to be a second, possibly distinct mechanism (Resink et al, 1983; Foulkes et al, 1983c). Addition of stoichiometric amounts of inhibitor-2 to protein phosphatase-1 or trypsin-treated F_c diminishes spontaneously measured activity, but results in a form of the enzyme that can be completely re-activated by F_a plus $Mg^{\cdot}ATP$ (Vandenheede et al, 1981a & 1983; Hemmings et al, 1982). Addition of inhibitor-2 to levels in excess of a 1 : 1 ratio with the phosphatase results in a concentration-dependent inhibition that is not reversible by incubation with F_a (Yang et al, 1981a; Resink et al, 1983). This effect of inhibitor-2 is also thought to be highly selective for type 1 protein phosphatases (Ingebritsen et al, 1983a & 1983b); complete inhibition of phosphatase activity is measured using inhibitor-2 in concentrations ranging from 1 - 10 nM (Foulkes et al, 1983c). This property of inhibitor-2 has been used advantageously to quantitate type 1 protein phosphatase activity in a variety of tissues (Ingebritsen et al, 1983c; Foulkes et al, 1983a & 1983b), and to define a potential role for type 1 protein phosphatases measured <u>in situ</u> (Foulkes & Maller, 1982; and Ernst et al, 1982).

Multiple molecular forms of both inhibitor-1 and inhibitor-2 have been isolated from tissues other than rabbit skeletal muscle (Huang et al, 1977; Goris et al, 1978; Khandelwal & Zinmann, 1978b; Huang & Tao, 1980; Knight & Skala, 1982; Nemenoff et al, 1983; Reddy & Ernst, 1983). In some cases, differences in specificity for different protein phosphatases - all presumed to be type 1 protein phosphatases (Ingebritsen et al, 1983a & 1983b) - have been reported (Goris et al, 1978; Khandelwal & Zinmann, 1978; Knight & Teal, 1980). However, it is not yet clear if these different inhibitors are proteins unrelated to rabbit skeletal muscle inhibitor-1 and inhibitor-2, or if they are instead derived from counterparts of the skeletal muscle inhibitors by proteolysis or another type of modification (Knight &

Teal, 1980; Khandelwal et al, 1981; Foulkes & Cohen 1980). In any event, both the various protein inhibitors and their target phosphatases demonstrate a high degree of cross-selectivity regardless of the tissue used in their preparation. In one study, Khandelwal & Zinmann (1978) demonstrated that an analogue of inhibitor-2 prepared from rabbit liver was equally effective in the inhibition of phosphorylase phosphatase activity isolated from several rat tissues, including brain. Conversely, inhibitors similarly prepared from each of these tissues were equally effective in the inhibition of rabbit liver phosphatase activity. Interestingly, rat brain displayed the highest concentration of the inhibitor (measured as potency per unit weight of tissue) suggesting that heat-stable inhibitors may play an important role in the regulation of brain protein phosphatase activity.

Some concern has been raised about the potential relevance of the protein inhibitors for two principle reasons: heat inactivation (potentially a source of artifactual change in the physical properties of the inhibitors) has been used in their preparation; and phosphatase activity assayed in freshly prepared homogenates has been reported to be insensitive to the inhibitors (Laloux et al, 1978; Laloux & Hers, 1979; Jett & Hers, 1981). With respect to the latter finding, it has been suggested that only proteolyzed forms of the phosphatases are inhibitor-sensitive (Khatra et al, 1983). However, both inhibitors have been prepared using procedures not involving heat-precipitation (Huang & Glinsmann, 1975; Nimmo & Cohen, 1978b; Goris et al, 1981). The reason(s) why freshly isolated phosphatases are insensitive to inhibitors is not entirely understood. Some recent studies have questioned these findings on procedural grounds (Ingebritsen et al, 1983c). And in at least one preparation (dog liver), a change in sensitivity concurrent with proteolysis has been ascribed to the selective loss of an additional factor termed deinhibitor protein (Goris et al, 1981 & 1983).

Some evidence for a potential physiological role for inhibitor-1 and inhibi-

tor-2 (and type 1 protein phosphatases) is based on experiments utilyzing the direct microinjection of the inhibitors into Xenopus oocytes (Huchon et al, 1981; Foulkes & Maller, 1982). Injection of either inhibitor arrested further oocyte maturation, a process thought to be regulated by protein phosphorylation (Maller & Krebs, 1980). In addition, inhibitor-2 injection decreased endogenous phosphorylase phosphatase activity (Foulkes & Maller, 1982). Other studies have attempted to define a physiological role for inhibitor-1 by measuring in vivo changes in the level of inhibitor-1 phosphorylation in response to agents known to affect protein phosphorylation. Immunohistochemical studies using antibodies specific for the phosphorylated form of inhibitor-1 have demonstrated that the intravenous administration of glucagon resulted in an increased level of phospho-inhibitor-1 in rat liver (Shenolikar & Steiner, 1984). An opposite effect was observed when insulin was administered. Addition of epinephrine to perfused rat skeletal muscle resulted in a concentration-dependent increase in the proportion of phosphorylated inhibitor-1 (Khatra et al, 1980). A three to eight-fold increase in the percentage of phosphorylated inhibitor-1 resulted as the concentration of epinephrine was raised from 10^{-9} to 10^{-7} M.

In a similar experiment, while the concentration of total (phospho- + dephospho-) inhibitor-1 did not change during the course of a 60 minute perfusion the proportion of phosphorylated inhibitor-1 increased from 24% to 52%, an effect matched by a parallel increase in cellular cyclic AMP concentration (Foulkes et al, 1982). These effects were mimicked by isoproteranol administration, and were blocked by propranolol or prior adrenalectomy implying that the activation of the beta-adrenergic receptor led to the phosphorylation of inhibitor-1. Simultaneous administration of insulin could also block the effect of isoproteranol (Foulkes et al, 1982). Opposing effects of beta-adrenergic agonists and insulin on inhibitor-1 phosphorylation were also observed in studies using isolated rat epididymal fat

pads and adipocytes (Nemenoff et al, 1983), skeletal muscle (Chang & Huang, 1980), and liver (Khandelwal et al, 1977). Together, these findings suggest that the modification of the state of inhibitor-1 phosphorylation may be linked to the physiological expression of certain hormones.

It then seems that the activity of type 1 protein phosphatases can be regulated by the interaction with two heat-stable inhibitor proteins. Phosphorylation of inhibitor-1 through a cyclic AMP-dependent mechanism decreases phosphatase activity thereby coordinating the activity of cyclic AMP-dependent protein kinase with that of a phosphatase capable of reversing its effects (Cohen, 1978; Ingebritsen & Cohen, 1983a). And F_a-catalyzed phosphorylation of inhibitor-2 has the reverse effect leading to an activation of the phosphatase. It is not yet known, however, how F_a activity is regulated. Nor is it certain which protein phosphatases are responsible for the dephosphorylation of inhibitors-1 and -2 catalyzed <u>in vivo</u>. It is currently thought that activated F_c catalyzes the dephosphorylation of inhibitor-2 as part of an auto-regulatory scheme (Jurgensen et al, 1984; Villa-Moruzzi et al, 1984). And examples of type 1, 2, and 3 protein phosphatases have been identified which catalyze inhibitor-1 dephosphorylation at apparently appropriate rates (Ingebritsen et al, 1983c; Ingebritsen & Cohen, 1983b). However, with the exception of type 2 (calcium·calmodulin-dependent) protein phosphatases, inhibitor-1 dephosphorylation requires the simultaneous presence of millimolar amounts of Mn^{2+} ions (Nimmo & Cohen, 1978b; Ingebritsen et al, 1983a; Foulkes et al, 1983c) raising questions about the nature of the cellular mechanisms used to reverse the effects of this inhibitor.

Type 2 Protein Phosphatases

Type 2 protein phosphatases are defined by their potential for activation by Ca^{2+} and calmodulin (Chernoff & Li, 1983). Type 2 protein phosphatases have

been identified in several tissues (Ingebritsen et al, 1983c). Those purified from rabbit skeletal muscle (Stewart et al, 1982 & 1983) and brain (Chernoff et al, 1983) are very similar in molecular composition to calcineurin, a protein previously identified as a major calmodulin binding-protein in brain (Klee et al, 1979; Wallace et al, 1980). Calcineurin has been independently characterized as a Ca^{2+}·calmodulin-dependent protein phosphatase (Yang et al, 1982; Manalan & Klee, 1983).

Calcineurin is composed of two subunits. The smaller (M_r 19,000) B-subunit binds Ca^{2+} with high affinity, whereas the larger (M_r 61,000) A-subunit binds both the B-subunit and Ca^{2+}·calmodulin, and seems to be the site responsible for enzymic activity (Klee et al, 1979; Sharma et al, 1979; Manalan & Klee, 1983). Calcineurin displays little activity measured in the absence of any activator or in the presence of either Ca^{2+} or calmodulin alone (Stewart et al, 1982). Partial activation of calcineurin can be achieved by the addition of either Ca^{2+}·calmodulin, or a divalent metal ion (Mn^{2+}, Ni^{2+}, Co^{2+}, or Mg^{2+}) to the phosphatase assay mixture (Yang et al, 1982; King & Huang, 1983; Stewart et al, 1983; Chernoff et al, 1983). Recently, it has been discovered that complete activation of calcineurin requires the synergistic effects of an added divalent metal ion (Mg^{2+} preferably) plus Ca^{2+}·calmodulin (Chernoff et al, 1983; Li, 1984).

In comparison with types 1 and 3 protein phosphatases, calcineurin displays limited substrate specificity (Ingebritsen & Cohen, 1983a). In one study using skeletal muscle calcineurin (Ingebritsen & Cohen, 1983b), activity was compared using a battery of 13 substrate proteins that were phosphorylated on 18 distinct sites. Significant activity was measured against just three of the substrates: inhibitor-1 was dephosphorylated the most rapidly, a rate approximately twice that measured using the alpha subunit of phosphorylase kinase or myosin light chains as substrate. Rates measured against the remaining substrates (including

phosphorylase a) were 10- to 100-fold less than that measured against inhibitor-1.

Calcineurin is highly concentrated in brain (Wallace et al, 1980): the highest levels of calcineurin are found in cerebellar and cerebral (particularly neostriatum) brain tissue. The subunit composition, molecular weight, and catalytic properties of the brain and muscle forms of calcineurin are similar, but differences were detected antigenically (Wallace et al, 1980) suggesting that different molecular forms of the phosphatase may be present in different tissues (Manalan & Klee, 1983). Brain calcineurin is primarily neural in distribution, and is principally localized to post-synaptic sites within neural cell bodies and dendrites (Wood et al, 1980). Moreover, the levels of calcineurin were found to increase significantly in rat and chick brains during periods of major synapse formation (Tallant & Cheung, 1983). Together, these findings suggest that calcineurin may play an important role in synaptic function. As part of this study, a type 2 phosphatase similar to calcineurin has been identified as one of the cerebellar protein phosphatases that display activity towards G-substrate, a neuron-specific phosphoprotein.

Type 3 Protein Phosphatases

The study of high molecular weight protein phosphatases has often been complicated by the separation of multiple activities - frequently differing in physical and catalytic properties - by various chromatographic procedures (reviewed by Cohen, 1978; Curnow & Larner, 1979; Lee, 1980; and Li, 1982). To facilitate characterization of these enzymes, dissociation prior to more extensive purification has been widely used. Instead of separating a multiplicity of activity peaks, treatment of crude tissue extracts or partially purified enzymes with urea (Gratecos et al, 1977), trypsin (Lee et al, 1976), 2-mercaptoethanol (Killilea et al, 1979), or ethanol (Brandt et al, 1974) has resulted in the isolation of single peaks

of spontaneously measured activity (reviewed by Shimazu, 1982). Typically, these peaks have displayed a common molecular weight of approximately 35,000. (Molecular weights based on hydrodynamic data - Stokes radii and $s_{20,w}$ - have been reported in the range of 30,000 - 35,000: Brandt et al, 1975a; Hsiao et al, 1977; Kinohara et al, 1982; Imaoka et al, 1983; Usui et al, 1983.) For example, in a study conducted in this laboratory, protein phosphatases were purified from multiple rabbit, bovine, and lobster tissues using a procedure that included ethanol-precipitation prior to ion-exchange chromatography and gel filtration (Li & Chan, 1981). In each case, the major phosphorylase (and p-nitrophenyl) phosphatase activity was isolated as a low molecular weight (M_r 35,000) enzyme. With few exceptions, each of these activities displayed similar chromatographic and catalytic properties. And each was similar to phosphatase S, a M_r 35,000 protein phosphatase previously purified from canine heart and bovine adrenal cortex (Li & Hsiao, 1977b; Li et al, 1978; Li, 1979; Li et al, 1979).

Initially, the finding that ethanol-precipitation resulted in the isolation of a single major phosphatase activity led to a proposal that the low molecular weight phosphatase might represent a catalytic subunit common to all forms of higher molecular weight phosphatases (Brandt et al, 1975a; Lee at al, 1976). Differences in catalytic properties displayed by the higher molecular weight phosphatases would then be conferred by other modulatory proteins. However, more extensive study indicated that the M_r 35,000 phosphatases were heterogeneous.

Khandelwal et al (1976) purified to apparent homogeneity two rabbit liver phosphatases that differed slightly in molecular weight as determined by SDS-gel electrophoresis (M_{SDS} 30,500 and 34,000). Although each phosphatase dephosphorylated each of several substrates tested, differences could be detected when the kinetic properties of the two enzymes were compared. Lee et al (1980) separated two low molecular weight (M_{SDS} 32,000 and 33,500) phosphatases from

ethanol-treated rabbit muscle extracts by ion-exchange chromatography. These were later termed phosphatases C-I and C-II, respectively (Silberman, et al, 1984). Like the two phosphatases purified from liver, phosphatases C-I and C-II also displayed crossed substrate-selectivity. However, they could be distinguished by comparing the activity measured towards phosphorylase a and p-nitrophenyl phosphate: phosphatase C-I was 14-fold more active using phosphorylase a as substrate, whereas phosphatase C-II was 31-fold more active using p-nitrophenyl phosphate.

Despite the slight difference in molecular weight, and the demonstrated differences in catalytic properties, the similarity of other characteristics suggests the possibility that phosphatases C-I and C-II are inter-related, possibly through proteolytic processing of a common precursor. Both phosphatases co-purify through multiple chromatographic steps (Ingebritsen et al, 1980), both display identical hydrodynamic properties (Lee et al, 1980), and both display similar sensitivity to heat inactivation (Silberman et al, 1984). A high degree of similarity is also observed when comparing the amino acid composition of the two phosphatases (Silberman et al, 1984). In addition, monoclonal antibodies prepared against either phosphatase demonstrated cross-reactivity: eight of ten antibodies selective for phosphatase C-I cross-precipitated phosphatase C-II, and each of eight antibodies prepared against phosphatase C-II cross-reacted with phosphatase C-I (Speth et al, 1984). However, recent evidence based on selective proteolysis indicates that phosphatases C-I and C-II are distinct proteins.

Tung et al (1984) compared the effects of protease inhibitors on the purification of phosphatases C-I and C-II. When protease inhibitors were included in the purification buffers, three forms of phosphatase C-I (M_{SDS} 37,000, 34,000, and 33,000) and a single form of phosphatase C-II (M_{SDS} 36,000) were obtained. (The multiple forms of phosphatase C-I each displayed similar catalytic proper-

ties.) In the absence of any added protease inhibitor, only the M_{SDS} 33,000 form of phosphatase C-I was obtained. Similarly, incubation of the larger molecular weight forms of phosphatase C-I with chymotrypsin resulted in their conversion to the lower molecular weight; the molecular weight of phosphatase C-II was unaffected by chymotrypsin. Moreover, cyanogen bromide, S. aureus protease, and trypsin digests of the two phosphatases displayed distinct peptide mapping patterns (Tung et al, 1984; Silberman et al, 1984). Lastly, in contrast with the results obtained using monoclonal antibodies, Tung et al (1984) prepared serum antibodies that precipitated phosphatase C-II but did not cross-react with phosphatase C-I.

Ingebritsen et al (1980) observed that phosphatases C-I and C-II could be differentiated using two additional criteria: sensitivity to heat-stable inhibitor-2, and selectivity for either the alpha or beta subunit of phosphorylase kinase as substrate. Phosphatase C-I, which is sensitive to inhibitor-2 and selective for the beta subunit of phosphorylase kinase, was shown to have properties identical to protein phosphatase-1. As discussed above, reconstitution of phosphatase-1 with inhibitor-2 converts the enzyme into a Mg·ATP-dependent form (Resink et al, 1983) suggesting that phosphatase C-I represents a catalytic component derived from a type 1 protein phosphatase. Phosphatase C-II, on the other hand, is unaffected by inhibitor-2; inhibitor-2 neither inhibits the activity of phosphatase C-II, nor converts it to a form responsive to F_a plus Mg·ATP activation (Ingebritsen et al, 1983a; Tung et al, 1984). Current evidence indicates that phosphatase C-II is a catalytic component derived from type 3 protein phosphatases (Ingebritsen et al, 1983a; Silberman et al, 1984).

In earlier studies conducted in this laboratory, three forms of higher molecular weight protein phosphatases were separated from extracts of cardiac tissue by ion-exchange chromatography (Li, 1975; Hsiao et al, 1977). Phosphatase

A (M_r 156,000) was not absorbed by the resin (DEAE-cellulose), whereas phosphatases B (M_r 161,000) and C (M_r 95,000) were eluted at progressively higher ionic strengths. Similar elution profiles have subsequently been reported by several other laboratories (Mumby & Traugh, 1979; Knight & Skala, 1979; Tamura et al, 1980; Foulkes et al, 1983a; Usui et al,1983; Ingebritsen et al, 1983). Consequently, type 3 phosphatases displaying elution properties similar to phosphatases A, B, and C are subclassified as types 3A, 3B, and 3C.

Further analysis of a bovine heart type 3 protein phosphatase (phosphatase E, Table 1) by SDS-gel electrophoresis indicated that the purified enzyme contained two protein bands with molecular weights of 35,000 and 63,000 (Li, 1981). This finding was similar to that reported earlier in studies of rat liver protein phosphatases. Tamura et al (1980) found that a type 3C phosphatase contained protein bands with molecular weights of 35,000 and 69,000. They termed these bands alpha and beta, respectively (phosphatase II, Table 1). A type 3B phosphatase (Ib, Table 1) also contained alpha and beta protein bands that displayed exactly the same molecular weights as the bands derived from the type 3C enzyme. In addition, phosphatase Ib contained a third protein (M_{SDS} 58,000) that was termed gamma (Tamura & Tsuiki, 1980). Similar protein bands have now been demonstrated in type 3 protein phosphatases that have been isolated from a variety of tissues (Table 1). Generally, type 3B phosphatases contain protein bands similar to the alpha, beta, and gamma proteins described in the rat liver enzymes. Type 3C phosphatases contain only alpha and beta proteins. Type 3A protein phosphatases have remained poorly characterized; one report has indicated that they are homopolymers composed of alpha proteins (Cohen, 1978).

One proposal concerning the composition of type 3 protein phosphatases maintains that different forms of the enzyme isolated from a given tissue contain one or more of the same alpha, beta, and gamma proteins (Ingebritsen et al,

1983a; Usui et al, 1983). Furthermore, the alpha protein is thought to confer enzymic activity, whereas the beta and gamma proteins would be modulatory in function. Considerable evidence has now been obtained in support of this model. In this laboratory, ethanol-precipitation was used to dissociate canine heart phosphatases 3A, 3B, and 3C into a common lower molecular weight (M_r 34,800) activity (Hsiao et al, 1977). While the non-dissociated enzymes could be differentiated by measuring the effects of added ATP and Mn^{2+} on activity, the low molecular weight phosphatases displayed identical sensitivity to these effectors. A more detailed study of the type 3B phosphatase indicated that in comparison with the ethanol-treated form, the non-dissociated enzyme was more sensitive to the conformational state of the substrate (Li & Hsiao, 1977b). Dissociation was also concurrent with an increased level of activity, and an increased sensitivity to ATP-mediated inactivation. (The latter is thought to reflect an increased access of the nucleotide to a site involved in catalytic activity: Hsiao et al, 1978). Together, these findings suggested that some of the regulatory properties of the phosphatase were lost following dissociation (Li & Hsiao, 1977b).

This conclusion was supported by a more recent study conducted by Kinohara et al (1982). They compared the molecular weight and catalytic properties of protein phosphatases that were isolated from freshly prepared cytosolic fractions of ten different rat tissues. Following gel filtration, the bulk of the enzymic activity was recovered in two major molecular weight ranges: peak 1 (M_r 190,000 -210,000) was identified in all but one of the tissues, and peak 2 (M_r 93,000-140,000) was recovered from half of the extracts. Based on the molecular weight and substrate specificity, peak 1 and 2 phosphatases were thought to correspond to type 3B and 3C protein phosphatases, respectively (i.e., rat liver phosphatases Ib and II, Table 1). Using substrate selectivity (phosphoryl-

ase a, glycogen synthase b, and histones H2B and H1) and metal ion-stimulation (Mg^{2+} and Mn^{2+}) as criteria, the catalytic properties of each of the peak 1 phosphatases were found to be similar. The catalytic properties of the peak 2 phosphatases were also found to be similar, but as a group were different from those of the peak 1 enzymes. Ethanol precipitation of each of these phosphatases resulted in the isolation of an identical low molecular weight (M_r 30,000) activity. The catalytic properties of the dissociated phosphatases were indistinguishable, but were different from those displayed by the non-dissociated enzymes. Similar findings to these were subsequently reported by Ingebritsen et al (1983a) using type 3A, 3B, and 3C protein phosphatases that were partially purified from rabbit skeletal muscle.

These experiments indicate that each of the type 3 protein phosphatases contains a similar catalytic component. Furthermore, several studies have indicated that this component is the same as phosphatase C-II and phosphatase S (Tamura et al, 1980; Li, 1981; Usui et al, 1983; Ingebritsen et al, 1983a). What isn't clear from these studies is whether the change in catalytic properties following dissociation of the enzyme are due to the selective loss of non-catalytic modulatory proteins, or instead a second type of catalytic component that displays a greater lability to the dissociative procedures. However, recent studies conducted by Masao Takeda and his co-workers (Imaoka et al, 1980; Imazu et al, 1981; Imaoka et al, 1983) indicate that the former possibility is more likely.

Starting with a type 3B protein phosphatase that was partially purified from pig heart (Ib, Table 1), three components were resolved following gel filtration and ion-exchange chromatography conducted in the presence of 6 M urea. The molecular weight of these enzymes (34,000, 69,000, and 56,000) indicated that these were the same as the alpha, beta, and gamma proteins already identified in other type 3 phosphatases. Using phosphorylase a, glycogen synthase b, and

Table 1. Molecular Weight and Subunit Composition of Selected Type 3 Phosphatases

Phosphatase & Source	Type[a]	$M_{r,\ app}$[b]	$M_{r,\ calc}$[c]	Subunits (M_{SDS})[d]	Stoichiometry[e]	Reference
E, Bovine Heart	3C	145,000	95,000	A (35,000) B (63,000)	A B	Li (1981)
SMP-I, Turkey Gizzard	3B	230,000	165,000	A (38,000) B (65,000) G (55,000)	A B G	Pato et al (1983) Pato & Adelstein (1983a)
eIF2, Rabbit Reticulocyte	3C	-	98,000	A (38,000) B (60,000)	A B	Crouch & Safer (1980)
Ib, Rat Liver	3B	260,000	185,000	A (35,000) B (69,000) G (58,000)	A_2B G	Tamura & Tsuiki (1980)
II, Rat Liver	3C	160,000	154,000	A (35,000) B (69,000)	A B_2	Tamura et al (1980)
H, Rabbit Skeletal Muscle	3B	250,000	160,000	A (35,000) B (65,000) G (55,000)	A B G	Lee et al (1980)
H, Rat Liver	3B	225,000	130,000	A (35,000) B (65,000) G (55,000)	B A_2 (G)	Lee et al (1980)

(continued)

Table 1. (Continued)

Phosphatase & Source	Type[a]	$M_{r,\ app}$[b]	$M_{r,\ calc}$[c]	Subunits (M_{SDS})[d]	Stoichiometry[e]	Reference
IV, Human Erythrocyte	3C	145,000	104,000	A (32,000) B (69,000)	A B	Usui et al (1983)
Ib, Pig Heart	3B	250,000	224,000	A (34,000) B (69,000) G (56,000)	A B G	Imazu et al (1981) Imaoka et al (1983)
(Rechromatographed) (Reconstituted)	3B	200,000	171,000		A B G	
Form 1	3B	250,000	199,000		A B G	
Form 2	3C	185,000	123,000		A B	
Form 2'	3C	145,000	106,000		A B	
Myosin, Bovine Aorta	3C	-	-	A (38,000) B (67,000)	A B	Werth et al (1982)

Notes: a) Type assignment based on subunit composition and chromatographic properties (see text);

b) $M_{r,\ app}$: apparent molecular weight determined by gel filtration;

c) $M_{r,\ calc}$: molecular weight calculated using Stokes radius + $s_{20,w}$ or sedimentation equilibrium data;

d) Subunits: A, alpha; B, beta; G, gamma;

e) Molecular stoichiometry estimated by densitometry of Coomassie-stained SDS-polyacrylamide gels.

histones H1 and H2B as substrates, only the alpha component displayed enzymic activity. Addition of the beta to the alpha component (Form 2, Table 1) resulted in an increased rate of activity measured towards histone H1, and an increased Mg^{2+}-requirement for optimal activity towards histone H2B. Addition instead of the gamma to the alpha component did not alter enzymic activity, nor was a complex formed. However, gamma combined with the Form 2 complex resulting in Form 1 (Table 1). The catalytic properties of this enzyme - which displayed markedly reduced levels of activity towards both phosphorylase a and glycogen synthase b - were very similar to the original, non-dissociated enzyme. Furthermore, this complex was not dissociated by dilution, gel filtration, or sucrose gradient sedimentation indicating that the complex was relatively stable. It is interesting to note that differences in the apparent stoichiometry of the alpha, beta, and gamma components have been reported when comparing highly purified phosphatases with those analyzed at a lower stage of purification. (Compare, for example the molecular weights of the pig heart phosphatase Ib with its rechromatographed form, or Form 2 with Form 2', a complex that was reconstituted using homogeneous components.) Together, these findings indicate that the type 3 protein phosphatases can be isolated in multiple forms that differ in both the composition and relative stoichiometry of three different component proteins.

Previous Studies of Brain Protein Phosphatases

Brain protein phosphatase activity has been characterized using extracts prepared from several sources, including bovine (Miyamoto & Kakiuchi, 1975; Li & Chan, 1981), ox (Rose & Heald, 1962; Weller & Rodnight, 1971), chicken (Foulkes et al, 1983), rat (Maeno & Greengard, 1972; Ueda et al, 1975; Yang et al, 1977; Parsadian et al, 1982; Sheu et al, 1983), rabbit (Ingebritsen et al, 1983c), and invertebrate brain tissue (Albin & Newburgh, 1975). In a few of these studies,

activity was measured using endogenous or brain-specific phosphoprotein substrates. However, most of the characterization of these phosphatases was conducted using phosphorylase a (rabbit muscle), p-nitrophenyl phosphate, or phosphorylated histone, casein, or protamine as substrates. The brain phosphatases displayed broad and overlapping substrate specificity for these substrates. The kinetic constants determined using these substrates, as well as other catalytic properties including pH optima and sensitivity to selected inhibitors (NaF, PP_i, ATP) were similar to those measured using non-brain phosphatases (Maeno & Greengard, 1972; Miyamoto & Kakiuchi, 1975; Arbin & Newburgh, 1975; Li & Chan, 1981). Similarities with non-brain phosphatases were also observed when comparing the effects of divalent metal ions on activity. Mn^{2+} (1 - 2.5 mM) generally increased activity (2 to 6-fold), Mg^{2+} and Ca^{2+} had little effect, and Zn^{2+}, Fe^{2+}, and Cu^{2+} acted as inhibitors (Rose & Heald, 1961; Weller & Rodnight, 1971; Maeno & Greengard, 1972; Yang et al, 1977; Foulkes et al, 1983).

Multiple forms of phosphatase activity have been isolated from brain, although there has been little detailed study of the physical and catalytic properties of these enzymes. Maeno and Greengard (1972) separated three forms of protein phosphatase activity from rat cortex by ion-exchange chromatography. Besides differences in elution behavior, each of these phosphatases could be distinguished by comparing the activity measured towards phosphorylated histone and protamine. This latter observation indicated that these multiple phosphatases might display differences in substrate preference. Each phosphatase also dephosphorylated ^{32}P-labeled endogenous substrates at a rate 10-fold greater than that measured using histone or protamine suggesting that these phosphatases might display activity towards neuron-specific phosphoproteins. In this same study, the subcellular localization of brain protein phosphatase activity was compared with that isolated from non-brain tissues. Approximately half of the phosphatase

activity isolated from multiple brain regions was particulate in distribution. In contrast, phosphatase activity prepared from either liver, lung, heart, or kidney was predominantly (70% -80%) cytosolic.

In more recent studies, Foulkes et al (1983a) separated multiple phosphatase activities from an extract of chicken brain using ion-exchange chromatography and gel filtration. Based on a comparison with the elution behavior and inhibitor-2 sensitivity of phosphatases purified from rabbit skeletal muscle, these multiple activities were identified as types 1, 3B, and 3C. Ingebritsen et al (1983c) similarly measured type 1, 2, 3, and 4 protein phosphatase activity in an extract of rabbit brain. Using both phosphorylase kinase and phosphorylase a as substrates, levels of activity comparable to that found in other tissues (skeletal muscle, heart, adipose tissue, and liver) were measured.

In an earlier study conducted in this laboratory (Li & Chan, 1981), phosphatases active on phosphorylase a and p-nitrophenyl phosphate were isolated from an ethanol-treated extract of bovine brain. At least two phosphatases were isolated: one displayed properties similar to canine heart phosphatase U-1 (a type 1 protein phosphatase); another was similar to phosphatase S, a M_r 35,000 enzyme characterized in bovine adrenal cortex and canine heart (Li & Hsiao, 1977b; Li, 1979; Li et al, 1979). Together, these studies indicate that procedures used in the isolation and characterization of protein phosphatases from non-brain tissues would be applicable to the study of phosphatases purified from the brain. In order to better relate the current study to neuronal function, G-substrate, a neuron-specific phosphoprotein localized to the cerebellum (described below), was used as substrate. Activity towards other well-characterized substrates was also measured in order to compare the findings of this study with the past work of this and other laboratories.

G-Substrate

Several lines of evidence suggest that cyclic GMP may play an important role within the central nervous system (reviewed by Goldberg & Haddox, 1977; Bartfai, 1980; Greengard, 1981; Volle & Quenzer, 1983; Nairn & Greengard, 1983; and Walter, 1984). Cyclic GMP is particularly concentrated in the cerebellum (Ferrendelli, 1978); together with the enzyme responsible for its synthesis (guanylate cyclase), cyclic GMP is primarily distributed within the cytoplasm of Purkinje cells (Ariano, et al, 1982), a neuron representing the only output of the cerebellar cortex (Llinas, 1975). Pharmacologic studies have demonstrated that the activation or inhibition of Purkinje cells is paralleled by an increase or decrease, respectively, of the level of cerebellar cyclic GMP (Biggio, et al, 1977). Cyclic GMP-dependent protein kinase is also localized in Purkinje cells (Schlicter, et al, 1980; & Lohmann, et al, 1981) leading to speculation that the effects of cyclic GMP within the cerebellum may be mediated by the specific activation of cyclic GMP-dependent protein kinase (Greengard, 1978).

G-substrate was initially identified as the only protein specifically phosphorylated in response to the addition of cyclic GMP to an homogenate prepared from rabbit cerebellum (Schlichter, et al, 1978). The low concentration of cyclic GMP required to produce half-maximal phosphorylation (60 nM) suggested that the phosphorylation of G-substrate may be a physiologically relevant event. G-substrate is also a substrate for cyclic AMP-dependent protein kinase. However, the rate of G-substrate phosphorylation catalyzed by cyclic AMP-dependent protein kinase is slow in comparison with that catalyzed by cyclic GMP-dependent protein kinase (a 9-fold kinetic difference measured in vitro) (Aswad & Greengard, 1981b). This observation suggests that G-substrate phosphorylation may allow the unique expression of cyclic GMP even in the presence of increased levels of cyclic AMP. Activation of cyclic AMP-dependent protein kinase by cyclic AMP has been

implicated as a mechanism of expression of several hormonal and pharmacologic agents acting within the nervous system (Nestler & Greengard, 1983).

G-substrate appears to be localized almost exclusively within the cerebellum. Using antibodies selective for its phosphorylated form, G-substrate could be immunoprecipitated from cytosolic extracts of cerebellum; no detectable G-substrate could be found in extracts of cortex, hippocampus, or striatum (Detre et al, 1984). Using instead a sensitive radioimmunoassay, levels of G-substrate found in cerebral cortex were just 0.4% that found in the cerebellum (Nairn et al, 1982; Detre et al, 1984). Based on studies using mutant mice deficient in Purkinje cells or in other types of cerebellar neurons, G-substrate, like cyclic GMP-dependent protein kinase, was localized to Purkinje cells (Schlichter et al, 1980; Dolphin et al, 1983; Detre et al, 1984). And like cyclic GMP-dependent protein kinase, a cytosolic distribution of G-substrate within these neurons was indicated by studies comparing the levels of the acid-soluble protein in particulate and soluble subcellular fractions prepared from cerebellum (Aswad & Greengard, 1981a). (Preliminary immunohistochemical studies have resulted in similar findings: A.C. Nairn, personal communication). A widespread phylogenetic distribution of G-substrate is suggested by the finding that antibodies prepared against rabbit G-substrate immunoprecipitated proteins of similar molecular weight from homogenates prepared from rat, guinea pig, bovine, chicken, and human cerebellum (Detre et al, 1984). Together, these findings indicate that G-substrate may have a generalized role in the functioning of the cerebellum.

G-substrate has been purified to apparent homogeneity from rabbit cerebellum (Aswad & Greengard, 1981a). A summary of some of the physical properties of G-substrate is presented in Table 2. G-substrate shares several properties in common with phosphatase inhibitor-1. In addition to similar molecular weights and hydrodynamic properties, both are stable to heat and acidity, both are

phosphorylated on threonine residues instead of the more usual serine, and the amino acid sequence surrounding the phosphorylation sites of the two proteins shares a high degree of homology (Table 2). (Note that two residues are phosphorylated in G-substrate, in contrast to the single site on inhibitor-1). However, inhibitor-1 is preferentially phosphorylated by cyclic AMP-dependent protein kinase (Nimmo & Cohen, 1978b). Furthermore, while inhibitor-1 is widely distributed in brain tissue, it is present in cerebellum in barely detectable amounts (Detre, et al, 1984). Part of this thesis is concerned with the demonstration that G-substrate, like inhibitor-1, can function as a specific inhibitor of protein phosphatase activity.

Table 2. Comparison of the Physical Properties of G-Substrate and Inhibitor-1

Property	G-Substrate[a]	Inhibitor-1[b]
Source	Rabbit Cerebellum	Rabbit Skeletal Muscle
Molecular Weight		
M_r	21,700	19,200
M_{SDS}	23,000	26,000
Apparent (Gel Filtration)	54,000	60,000
Stokes Radius	3.1 nm	3.1 nm
f/f_o	1.7	1.7
Heat Stability	95°C, 5 min	90°C, 10 min
Acid Solubility	0.2 N H_2SO_4 (pH 1)	1.5% trichloroacetate[c]
Phosphorylated Residue	Threonine (2)	Threonine
Preferential Kinase	cyclic GMP-Dependent	cyclic AMP-Dependent
Relative Concentration[d]		
Cerebellum	100	3.5
Cortex	0.4	19

Sequence Surrounding Phosphorylated Residue

G-Substrate[e]

Site 1: Gln-Lys-Lys-Pro-Arg-Arg-Lys-Asp-Thr(P)-Pro-Ala-Leu-His

Site 2: Gln-Gln-Lys-Pro-Arg-Arg-Lys-Asp-Thr(P)-Pro-Ala-Leu-His

Inhibitor-1[f]: Glu-Gln-Ile-Arg-Arg-Arg-Arg-Pro-Thr(P)-Pro-Ala-Thr-Leu

References: a) Aswad & Greengard, (1981a)
b) Nimmo & Cohen, (1978a)
c) Foulkes & Cohen (1979)
d) Detre et al, (1984)
e) Aitken et al (1981)
f) Aitken et al, (1982)

Abbreviations - BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; cyclic AMP, adenosine 3':5'-monophosphate; cyclic GMP, guanosine 3':5'-monophosphate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; M_{SDS}, molecular weight determined by SDS-gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; p-NPP, p-nitrophenyl phosphate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TLCK, tosyllysylchloromethyl ketone; TPCK, tosyl-2-phenylethylchloromethyl ketone; and Tris, 2-amino-2(hydroxymethyl)-1,3-propanediol.

Experimental Procedures

Materials - Phosphorylase kinase, histone type IIb, bovine serum albumin (BSA), p-nitrophenyl phosphate (p-NPP), ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), tosyllysylchloromethyl ketone (TLCK), tosyl-2-phenylethylchloromethyl ketone (TPCK), TPCK-treated trypsin, soy trypsin inhibitor, 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris) and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffers, and proteins used as M_r markers were obtained from Sigma. (γ-^{32}P)ATP and (^{14}C)ovalbumin were obtained from New England Nuclear or ICN. Calmodulin was prepared from bovine brain as described by Charbonneau and Cormier (1979). Heat stable protein phosphatase inhibitor-1 (Nimmo & Cohen, 1978a), inhibitor-2 purified through the first DEAE-cellulose chromatography step (Foulkes & Cohen, 1980), and glycogen phosphorylase b (Fischer & Krebs, 1962) were prepared from rabbit skeletal muscle (Pel-Freez Biologicals, Inc.). Vitamin-free casein was purchased from Nutritional Biochemicals Corp. DEAE-cellulose (DE-52) was obtained from Watman, and Sephacryl S-200, Sephadex G-25, G-50, G-100, and G-200, DEAE-Sepharose, and CM-Sephadex were purchased from Pharmacia. Poly-lysine (60) Sepharose CL-6B was prepared by covalently linking Poly-L-Lysine (M_r 60,000, Type VIIB, Sigma) to CNBr-activated Sepharose CL-6B (Pharmacia) as described by Nevaldine and Kassel (1971). Acrylamide, N,N'-methylene-bis-acrylamide (bis-acrylamide), sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethyl-ethylenediamine (TEMED), Coomassie brilliant blue R-250, and SDS-protein standards were obtained from Bio-Rad. Reagents and film used for autoradiography were purchased from Eastman Kodak. All reagents were the highest grade commercially available.

Buffers - The following buffers were used repeatedly in these studies. Buffer A: 20 mM Tris·HCl, pH 7.4, and 10 mM 2-mercaptoethanol. Buffer B: 20 mM Tris·HCl, pH 7.4, 10 mM 2-mercaptoethanol, 0.1 M NaCl, and 10% (v/v) glycerol. Buffer C: 100 mM Tris·HCl, pH 6.8, 1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.001% (w/v) bromphenol blue. Buffers were adjusted to the pH, as indicated, at 25°C.

Preparation of Protein Kinases - The catalytic subunit of cyclic AMP-dependent protein kinase was prepared from bovine heart as described by Kinzel & Kubler (1976). The kinase was stored at (-)20°C in a buffer containing 50 mM potassium phosphate, pH 7.0, and 50% glycerol. Bovine lung cyclic GMP-dependent protein kinase (Walter et al, 1980) was a gift from Dr. P. Greengard (Rockefeller Univ.), and was stored at (-)20°C in a buffer containing 10 mM Tris·HCl, pH 7.0, 25 mM 2-mercaptoethanol, 1 mM EDTA, and 50% (v/v) glycerol. Specific activities of 5.5 and 1.2 umol-min^{-1}-mg^{-1} were measured for the cyclic AMP and cyclic GMP-dependent protein kinases, respectively, using histone type IIb as a phosphate acceptor (Aswad & Greengard, 1981b). ^{32}P-Incorporation into protein was measured by a modification of the descending paper chromatography method described previously (Li & Hsiao, 1977a). After eluting with TCA, the top of the strip (i.e., the portion containing the precipitated protein) was cut, washed extensively with 5% (w/v) TCA, dried, and the ^{32}P-protein was quantitated by liquid scintillation counting.

Preparation of G-substrate - (^{32}P)G-substrate was prepared from rabbit cerebellum as described by Aswad & Greengard (1981a), and was provided by Dr. P. Greengard (Rockefeller Univ.). G-substrate was stored at (-)20°C in a buffer containing 20 mM Tris·HCl, pH 7.4, 250 mM KCl, and 0.2 mM EDTA. The G-substrate was estimated to be 70% pure by gel electrophoresis, and the phosphate

content ranged between 1.9-2.05 mol/mol protein. The dephosphorylated form of G-substrate was prepared in the following manner. The (^{32}P)G-substrate was dialyzed at 4°C against a buffer containing 20 mM Tris·HCl, pH 7.4, 0.2 mM EGTA, and 1 mM DTT. The dephosphorylation was then carried out at 30°C in a volume of 200-300 uL containing 20 mM Tris·HCl, pH 7.4, 1 mM DTT, 1 mg/mL BSA, 2.5 mM $MnCl_2$, 30-67 uM G-substrate, and 60-90 milliunits of phosphatase II (prepared as described below). For time-course studies, one tenth this amount of phosphatase was used. The reaction was stopped after 60 minutes by the dropwise addition of 0.5 M H_2SO_4 to the cooled reaction mixture until a pH of 1 was achieved. After an additional 10 minutes, 1 M Tris base was added to neutrality. The dephosphorylated G-substrate was then dialyzed against a buffer containing 20 mM HEPES, pH 7.4, 0.2 mM EGTA, and 5 mM $MgCl_2$. Under these conditions, the extent of dephosphorylation was greater than 95%, as determined by descending paper chromatography, with an overall protein yield estimated to be 75%. The dephosphorylated form of G-substrate (6.3 uM) was rephosphorylated at 30°C in a reaction volume of 200 uL that contained 20 mM HEPES, pH 7.4, 4 mM $MgCl_2$, 160 ug BSA, 1 uM cyclic GMP, 65 uM (γ-^{32}P)ATP, and 40 ng of cyclic GMP-dependent protein kinase. Controls in which BSA was substituted for G-substrate were also prepared.

Preparation of ^{32}P-labeled Proteins - G-substrate was phosphorylated by cyclic GMP-dependent protein kinase as described above. Inhibitor-1 (Nimmo & Cohen, 1978a), and casein and histone (Li & Hsiao, 1977a) were phosphorylated using the catalytic subunit of cyclic AMP-dependent protein kinase. Glycogen phosphorylase b was converted to phosphorylase a by phosphorylation with phosphorylase kinase using a modification of the method of Torres & Chelala (1961). The $^{32}P_i$ content of the phosphorylated proteins was 8-10 nmol/mg phosphorylase a, 30-40 nmol/mg histone, 2.5-5 nmol/mg casein, 90-100 nmol/mg G-substrate, and 25-50

nmol/mg inhibitor-1. The specific radioactivity of the ^{32}P-labeled phosphoproteins used in the assay of phosphatase activity varied between 25-400 cpm/nmol P_i. The concentration of the substrate proteins was expressed in terms of the $^{32}P_i$ content.

Enzyme Assay

p-Nitrophenyl Phosphatase - Activity towards p-NPP (alkaline phosphatase) was measured by the release of p-nitrophenol from p-NPP at pH 8.6 as previously described (Li & Chan, 1981). The results were quantitated spectrophotometrically at 410 nm using a molar absortivity of the p-nitrophenolate ion = 1.75×10^4 M^{-1}-cm^{-1}. p-Nitrophenyl phosphatase activity was localized directly on polyacrylamide gels in the following way. After the electrophoretic run, the gel was immediately immersed into a buffer that contained 50 mM Tris·HCl, pH 8.6, 10 mM $MgCl_2$, 1 mM DTT, 20 mM p-NPP, and 0.2 M $CaCl_2$. Alternatively, 0.5 mM $MnCl_2$ was substituted for the $MgCl_2$, and the pH was adjusted to 7.4. The gel was incubated at room temperature for 1 - 4 days until the white precipitated bands that marked the presence of enzymic activity had reached the desired intensity.

Protein Phosphatase - Protein phosphatase activity was assayed by measuring the release of $^{32}P_i$ from ^{32}P-labeled proteins at 30°C (Li et al, 1979). The following protocol was used in the study of phosphatase I, and in the comparison of the activity of phosphatase I with that of phosphatases II and III. The effect of preactivation with F_a + Mg·ATP, $MnCl_2$, or Ca^{2+}·calmodulin was assayed as follows. The phosphatase was first converted to an active form by preincubation at 30°C for 10 minutes in a volume of 20 uL that contained 63 mM Tris·HCl, pH 7.4, 0.6 mM DTT, 2.5 mg/mL BSA, 50-100 ng cerebellar F_a, 0.6 mM Mg-$(CH_3COO)_2$ and 0.12 mM ATP. The phosphatase reaction was initiated by the

addition of 5 uL of ^{32}P-substrate (50 uM phosphorylase a, 5 uM G-substrate, or another substrate in a concentration as indicated), and the incubation at 30°C was continued for 10 minutes. Spontaneous phosphatase activity was measured by omitting $Mg(CH_3COO)_2$ and ATP from the reaction mixture. Mn^{2+}-activated phosphatase activity was measured by substituting $MnCl_2$ (0.6 or 2.4 mM, as indicated), and Ca^{2+}·calmodulin activation was measured by substituting 5 uM $CaCl_2$ and 0.5 ug of calmodulin for the $Mg(CH_3COO)_2$ and ATP. The effect of protein inhibitors on phosphatase I activity was measured by the addition of the inhibitor in a concentration, as indicated, to the preactivation reagent mixture.

The following protocol was used primarily in the study of phosphatases II and III. Activity towards G-substrate and phosphorylase a was measured in a final volume of 25 uL that contained 20 mM Tris·HCl, pH 7.4, 1 mM DTT, 0.2% (w/v) BSA, and either 1 uM (^{32}P)G-substrate + 2 mM $MnCl_2$, or 10 uM (^{32}P)phosphorylase a + 0.5 mM $MnCl_2$. Alternatively, (^{32}P)inhibitor-1, or ^{32}P-labeled histone or casein were used as substrate in the presence of $MnCl_2$ or $MgCl_2$, as indicated. Theophylline (5 mM) was also included in the assay of phosphorylase phosphatase activity, unless otherwise indicated. The reaction was initiated by the addition of enzyme, and was terminated after 10 minutes incubation. The effect of protein inhibitors on the activity of phosphatases II and III were measured by the inclusion of the inhibitor in the reaction mixture at the indicated concentration.

In both protocols, the reaction was terminated by the addition of 20 uL of 8% (w/v) TCA. $^{32}P_i$ released during the assay was separated from unreacted substrate by descending paper chromatography (Li & Hsiao, 1977a), and was quantitated by liquid scintillation counting. All dilutions of enzyme, substrate, and inhibitor were made prior to assay into a buffer that contained 20 mM Tris·HCl, pH 7.4, and 0.2% (w/v) BSA. Conditions were maintained such that the reaction rates were linear with respect to time and the amount of added enzyme; generally

less than 10% of the substrate was dephosphorylated. Blank values (i.e., $^{32}P_i$ obtained in the absence of added enzyme) were subtracted from all assays. One unit of activity was defined as the amount of enzyme that catalyzed the release of 1 nmol $^{32}p_i$ or p-nitrophenol per minute at 30°C.

Subcellular Distribution of Cerebellar Protein Phosphatases - Procedures used for the homogenization and fractionation of bovine cerebellum were adapted from the studies of Maeno and Greengard (Maeno et al, 1971; Maeno & Greengard, 1972). Fresh bovine brains were shipped on ice, and were processed within one to two hours of slaughter. Following a rinse in cold 0.9% (w/v) NaCl, the outer meningeal and vascular tissue was removed and 0.5 gram sections of the outer cortex were combined from four different cerebella, yielding a total of 2.0 grams. The tissue was homogenized in 10 volumes of 0.32 M sucrose + 4 mM Tris·HCl, pH 7.4, with 12 up-and-down strokes at 1000 rpm in a glass homogenizer using a Teflon pestle of 0.15 mm clearance. All steps in the fractionation were carried out at 4°C. After dilution in the same buffer to 30 mL, the homogenate was centrifuged twice at 1000 x g (SS 34 rotor, Sorvall RC2-B centrifuge) for 10 minutes, and the pellets were combined to form the P1 fraction (nuclei and cell debris). The supernatant was then centrifuged for 15 minutes at 14,000 x g yielding the P2 fraction (mitochondria). The remaining supernatant was then centrifuged for 60 minutes at 210,000 x g (Beckman Ti-60 rotor) yielding the P3 (microsomal pellet) and S (cell sap) fractions. The pellets were resuspended using a glass homogenizer with a ground glass pestle. All of the fractions were diluted into the same Tris/sucrose buffer as described above.

Phosphoprotein phosphatase activity towards G-substrate and phosphorylase a was measured in the presence of $MnCl_2$ as described under Enzyme Assay. In addition, latent phosphatase activity (Maeno & Greengard, 1972) was assayed as follows: 10 uL of 1% (w/v) Triton X-100 was added to 90 uL of the fraction at

4°C. After 10 min, an aliquot of this mixture was assayed for phosphatase activity measured as described above, except that 0.1% Triton was added to the reaction mixture. (Preliminary studies determined that this concentration of Triton had no appreciable effect on the assay system, and was optimal for the expression of latent phosphatase activity.) Succinate dehydrogenase and lactate dehydrogenase, used as marker enzymes for mitochondria and cytosol, respectively, were measured as described by Maeno et al (1971).

Preparation of Protein Phosphatases

Preparation of Crude Homogenate - Fresh bovine cerebella (100 - 500 g) obtained from a local slaughter house were rinsed in 0.9% (w/v) NaCl, stripped of outer meningeal and vascular tissue, and were homogenized in 2 volumes of extraction buffer (20 mM Tris·HCl, pH 8.0, 10 mM 2-mercaptoethanol, 2 mM EDTA, 2 mM EGTA, 5 mM benzamidine, and 0.1 mM each of PMSF, TLCK, and TPCK. The homogenate was centrifuged at 12,000 x g for 20 minutes, and the supernatant was filtered through glass wool (Step 1, Tables 4 & 5).

Separation of Cerebellar Protein Phosphatases by DEAE-Cellulose - In order to demonstrate the separation of protein phosphatases on DEAE-cellulose, the following procedure was carried out. Solid ammonium sulfate (55% saturation) was added to the filtered supernatant, and the precipitated protein was collected by centrifugation, suspended in a small volume of Buffer A, and was then dialyzed against the same buffer. Following centrifugation for 60 minutes at 105,000 x g, the clarified supernatant was applied to a DEAE-cellulose column (2.5 x 45 cm) equilibrated with Buffer A + 50 mM KCl, 1 mM EGTA, and 2 mM $MgCl_2$ (Fig. 1). The column was rinsed with one bed volume of the same buffer, after which it was developed with 1.5 L of a linear salt gradient ranging from 0.05 -0.4 M KCl in Buffer A.

Purification of Phosphatase I - The 12,000 x g supernatant was directly mixed with one half volume of DEAE-cellulose equilibrated with Buffer A. (The omission of the $(NH_4)_2SO_4$ precipitation prior to the ion-exchange chromatography step resulted in an increased column resolution.) The slurry was filtered using a Buchner funnel (Watman paper type 4), and the resin was rinsed with two bed volumes of Buffer A. The combined filtrates were used for the preparation of the protein activator (F_a) of the Mg·ATP-dependent phosphatase (see below). The resin was further rinsed with Buffer A + 50 mM KCl, and then the Mg·ATP-dependent phosphatase was eluted with Buffer A + 120 mM KCl, precipitated with 60% $(NH_4)_2SO_4$, and dialyzed against Buffer B (Step 2, Table 4). The enzyme was then chromatographed on a Sephacryl S-200 column (2.6 x 90 cm) equilibrated with Buffer B (Step 3). The column was eluted at a constant flow rate of 4.8 mL-hr^{-1}-cm^{-2}, and 2.2 mL fractions were collected (Fig. 4). The most active fractions were pooled and directly applied to a polylysine (60) Sepharose CL-6B column (1 x 12 cm) equilibrated with Buffer A (Fig. 5). The column was washed with Buffer A followed by Buffer A + 200 mM NaCl, and then the Mg·ATP-dependent phosphatase activity was eluted with a linear gradient ranging from 200 - 600 mM NaCl in the same buffer. The fractions corresponding to peak activity were pooled, concentrated by ultrafiltration (PM 10, Amicon), and were then dialyzed extensively against Buffer A (Step 4). The phosphatase was then applied to a second polylysine Sepharose column (1 x 4 cm) equilibrated with Buffer A (Fig. 6). After washing with Buffer A + 300 mM NaCl, Mg·ATP-dependent phosphatase activity was eluted using a linear gradient ranging from 300 - 600 mM NaCl in the same buffer. The most active fractions were pooled, concentrated by ultrafiltration, and then were dialyzed against Buffer A + 60% glycerol (Step 5).

Preparation of Fa - F_a was further purified in the following manner. The initial breakthrough fraction from the DEAE-cellulose absorption (see above) was

mixed with one half volume of CM-Sephadex equilibrated with Buffer A. The resin was washed batchwise with Buffer A + 100 mM KCl, and then the F_a was eluted with Buffer A + 300 mM KCl. The enzyme was precipitated with $(NH_4)_2SO_4$ (60% saturation), collected by centrifugation, and then dialyzed extensively against Buffer A + 60% glycerol.

Purification of Phosphatases II and III - The crude homogenate was prepared as described above, and was centrifuged at 12,000 x g for 20 min. The supernatant was filtered through glass wool (Step 1, Table 5), solid $(NH_4)_2SO_4$ was added to 55% saturation, and the precipitated protein was collected by centrifugation, suspended in a small volume of Buffer A, and was then dialyzed against the same buffer (Step 2). The dialyzed material was applied to a DEAE-cellulose column (4 x 42 cm) equilibrated with Buffer A + 0.05 M KCl (Figs. 7 & 8). The column was rinsed with one bed volume of the same buffer, and then was progressively developed with Buffer A + 0.12 M KCl, a non-linear gradient ranging from 0.12 -0.4 M KCl in Buffer A, and finally, with 0.55 M KCl in the same buffer. The non-linear gradient (Li, 1975) was prepared as follows: three equal volumes of Buffer A containing 0.12 M, 0.20 M, and 0.40 M KCl were connected in series ranging from low to high salt concentration. The peak activities corresponding to phosphatase II and phosphatase III were separately pooled, concentrated by ultrafiltration (PM 10, Amicon), and dialyzed against Buffer B (Step 3).

Phosphatases II and III were then separately applied to a Sephacryl S-200 column (2.6 x 90 cm) equilibrated with the same buffer (Figs. 9 & 10). The column was eluted at a constant flow rate of 4.8 $mL\text{-}hr^{-1}\text{-}cm^{-2}$, and 2.2 mL fractions were collected. The most active fractions of each phosphatase were pooled, concentrated by ultrafiltration, and were dialyzed against Buffer A (Step 4). The phosphatases were then applied to a DEAE-Sepharose column (1.6 x 22 cm) equilibrated with Buffer A + 0.05 M KCl (Figs. 11 & 12). After rinsing with one

bed volume of the same buffer, the column was developed with a linear gradient ranging from 0.05 - 0.4 M KCl in Buffer A. The active fractions were pooled and concentrated as before, and were then dialyzed against Buffer A + 60% (v/v) glycerol (Step 5).

All purification steps were carried out at 4°C. Fractions from each stage of purification were stored at (-)20°C in Buffer A + 60% glycerol.

Preparation of Phosphatase S - Phosphatase S (the M_r 35,000 phosphatase) was prepared from the crude cerebellar homogenate using a modification of the procedure described by Brandt and Lee (Brandt et al, 1974; & Li et al, 1978). The 12,000 x g supernatant was precipitated by the addition of solid $(NH_4)_2SO_4$ (60% saturation), and was then dialyzed against Buffer A. $MnCl_2$ (0.1 M) was added to the dialyzed protein to give a final concentration of 5 mM, and then one volume of this mixture was mixed at room temperature with five volumes of 100% ethanol. The mixture was immediately centrifuged at 10,000 x g for 10 min at 4°C, following which the ethanol was decanted, and the precipitated protein was resuspended in one volume of Buffer A + 1 mM EDTA, 7 mM $MnCl_2$, and 50 mM KCl. The resuspended protein mixture was centrifuged at 10,000 x g, and the supernatant containing the extracted phosphatase S decanted. This extraction procedure was repeated three additional times; the supernatants were pooled, and were then precipitated by the addition of solid $(NH_4)_2SO_4$ (70% saturation). The precipitated phosphatase preparation was collected by centrifugation, was extensively dialyzed against Buffer A, followed by dialysis against Buffer A + 60% glycerol, and was then stored at (-)20°C.

Purification of Cardiac Phosphatases I, II, and III - Protein phosphatases with chromatographic and catalytic properties similar to those displayed by the cerebellar phosphatases were purified from bovine heart muscle as part of a

separate study (D. Tabarini, Ph. D. Dissertation, Mount Sinai Sch. Med., 1984; and H.-C. Li, unpublished results). The same purification protocol as that described above was used in the purification of cardiac phosphatase I, but the following modifications were employed in the purification of cardiac phosphatases II and III: instead of the final DEAE-Sepharose chromatography step, the phosphatases were twice chromatographed on polylysine (60) Sepharose CL-6B columns, and then were re-chromatographed on a Sephacryl S-200 column. The specific activity of the cardiac phosphatases II and III (measured towards (^{32}P)phosphorylase a in the presence of 0.5 mM $MnCl_2$) was 132 units/mg and 150 units/mg, respectively.

Molecular Weight Determination - Stokes radii were determined by gel filtration on Sephacryl S-200 or Sephadex G-100 using the following protein standards (Stokes radius): rabbit muscle aldolase (4.8 nm), bacterial alpha amylase (4.0 nm), bovine serum albumin (3.5 nm), ovalbumin (2.8 nm), chymotrypsinogen (2.1 nm), myoglobin (2.1 nm), and cytochrome c (1.7 nm). The data were plotted according to the method of Siegel and Monty (1966). $s_{20,w}$ values were determined by sucrose density ultracentrifugation in a procedure modified from Martin and Ames (1961). Centrifugation was carried out at 4°C using an SW 41 swinging bucket rotor (Beckman) run at 35,000 rpm for 18 hours. Approximately 25 ug of the purified phosphatase, together with 10 nCi of ^{14}C-ovalbumin ($s_{20,w}$ = 3.55) that was added as an internal sedimentation standard, were applied in a volume of 200 uL to 11.8 mL of a linear gradient ranging from 5 -20% (w/v) sucrose in Buffer A. $MnCl_2$ (0.5 mM) was included in the sucrose solutions when centrifuging phosphatases II and III. In some runs, ^{14}C-gamma globulin ($s_{20,w}$ = 7.1) was also included as an internal standard. After the run, 0.25 mL fractions were collected using a Density Gradient Fractionator (Instrument Specialties), and peak phosphatase activity was located by measuring the activity towards phosphorylase a, as described above. The molecular weight and frictional ratio was calculated from

$s_{20,w}$ and Stokes radius values as described by Siegel and Monty (1966). The axial ratio was estimated according to the method of Schachman (1959). The apparent molecular weight was estimated by gel filtration, or, where indicated, from sucrose gradient ultracentifugation using the relationship $s_1/s_2 = (M_1/M_2)^{2/3}$ (Martin & Ames, 1961).

Polyacrylamide Gel Electrophoresis - Non-denaturing disc gel electrophoresis was carried out using a procedure modified from Davis (1964). Separating gels measuring 1.5 mm in thickness and 6.5 mm in length were prepared using 7 - 7.5% (w/v) acrylamide (37:1 acrylamide:bis-acrylamide) at pH 8.3. A separating gel measuring 1 cm in length was prepared using 2.5% (w/v) acrylamide (4:1 acrylamide:bis-acrylamide) at pH 6.8. The gels were electrophoresed using a Bio-Rad model 220 slab gel apparatus that was maintained at 4°C during the run. At 40 mAmps (constant power), electrophoresis was complete within 1.5 - 3 hours. Following electrophoresis, the gels were stained for 10 min using the following: 1.5% (w/v) Coomassie brilliant blue R-250, 10% (v/v) glacial acetic acid, and 45% (v/v) methanol. Destaining was by diffusion using a solution containing 7.5% (v/v) acetic acid and 10% (v/v) methanol. Alternatively, p-nitrophenyl phosphatase activity could be directly localized as described under Enzyme Assay. As a second alternative,enzymic activity could be recovered from the gels in the following way. Immediately following the electrophoretic run, the gel was frozen on dry ice, and 1 mm slices were then cut from the gel. The slices were eluted for 12 - 48 hours at 4°C into 100 uL of a buffer containing 20 mM Tris·HCl, pH 7.4, 1 mM DTT, and 10% glycerol. Aliquots from these samples could then be tested for protein phosphatase activity as described above. These same eluted samples could then be re-electrophoresed in the presence of SDS in a method that was adopted from Beeman and Hunter (1978). 100 uL of a buffer containing 50 mM $NaHCO_3$, 0.1% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) brom-

phenol blue, and 10% glycerol was added, and the samples were heated in a boiling water bath for 5 min, and at 37°C for an additional 24 hours. The samples were then applied to SDS-polyacrylamide gels as described below.

SDS-gel electrophoresis was carried out in 10 - 15% acrylamide in a method modified from Laemmli and Favre (1973) and Weber and Osborn (1969). The dimensions and composition of the gels were the same as those described above. Samples were prepared in Buffer C, and were heated in a boiling water bath for 2 min prior to electrophoresis. Standard proteins were processed in an identical manner, and were included with each gel in order to estimate molecular weight (Weber & Osborn, 1975). Protein was visualized using Coomassie brilliant blue as described above. When greater sensitivity was required, a silver-stain procedure modified from Switzer et al (1979) was used. The gel was fixed in 40% (v/v) methanol:10% (v/v) acetic acid (60 min). Following two 30 min washings in 10% methanol:5% acetic acid, the gel was incubated with 34 mM potassium dichromate + 32 N nitric acid for 10 min. The gel was rinsed with deionized water (10 min x 3), and was then incubated with 12 mM $AgNO_3$ for 30 min. The gel was placed directly under a fluorescent lamp for the first 10 min of this incubation. Following a rinse with deionized water, the gel was developed using 0.28 M Na_2CO_3 + 0.05% (v/v) formaldehyde. The development was stopped by immersing the gel into 5% (v/v) acetic acid.

Subunit stoichiometry was estimated using Coomassie-stained SDS-polyacrylamide (10 & 12%) gels. The gels were scanned using an IEC densitometer, and the mass ratio was determined by integration. Molecular stoichiometry was then calculated from the mass ratio and the molecular weights determined by SDS-gel electrophoresis. Each ratio was determined using a minimum of three amounts of applied protein in order to check the linearity of the staining and instrument response.

Thermolytic Digestion of G-Substrate - The following procedure was performed in the laboratory of Dr. P. Greengard by Dr. A.C. Nairn. Following SDS-polyacrylamide gel electrophoresis, the band corresponding to G-substrate was excised, and was washed with three 10 mL portions of isopropanol/acetic acid/H_2O (1/1/8). The gel piece was washed with 50% methanol (2 x 10 mL), lyophilized, and then reswollen in 50 mM NH_4HCO_3 (1 mL) containing 0.3 mg/mL thermolysin. Digestion proceeded for 20 hours at 37°C (1.5 mM microfuge tubes). The gel piece was removed, and was then washed (at 37°C) using 50 mM NH_4HCO_3 (500 uL). The wash and digest were combined, centrifuged to remove particulate matter, and the supernatant was lyophilized. Pyridine/acetic acid/H_2O (1/10/89), pH 3.5 was added to the final dried sample such that counts were normalized to about 500-1000 cpm/uL. Aliquots (1-10 uL) were then spotted on 20 x 20 cm thin layer cellulose plates (Eastman), and electrophoresis was performed at 400 V until the sample migrated 5-6 cm (Basic Fuschin dye was added as an indicator). Following drying, ascending chromatography using pyridine/butanol/acetic acid/H_2O (15/10/3/72) was performed twice. Peptides corresponding to sites 1 and 2 of G-substrate (Aitken et al, 1981) were localized by autoradiography. Those parts of the plate were excised and were quantitated by liquid scintillation spectrophotometry.

Miscelleneous Procedures - Ethanol precipitation of the purified phosphatases was carried out as follows. 2 volumes of iced-cold 100% ethanol (EtOH) was added to the phosphatase that was contained in 1 vol of Buffer A + 60% glycerol + 0.1 mM EDTA. The mixture was immediately centrifuged for 1.5 min at 10,000 x g, the ethanol was decanted, and the precipitated protein was resuspended in 1 vol of a buffer that contained 20 mM Tris·HCl, pH 7.4, 10% glycerol, and 0.1 mM EDTA. In the control preparations, the phosphatase was treated identically except that the addition of EtOH was omitted. Autoradiography was carried out on stained

polyacrylamide gels using Eastman Kodak XR-1 X-OMAT R-film. The film was processed using Kodak Developer (2 min) and Kodak Rapid Fixer (8 min). Protein concentration was measured using the dye-binding method of Bradford (1976), or the method of Lowry et al (1951) following precipitation with 8% (w/v) TCA. BSA was used as a reference protein. All experiments were separately performed a minimum of two times; data used for illustrations were generally taken from representative experiments. Numerical data (other than that used for elution profiles) were averaged from duplicated or triplicated experimental results.

Experimental Results

Identification and Purification of G-Substrate Phosphatases

Subcellular Distribution of Cerebellar G-Substrate Phosphatase Activity

Subcellular fractionation of fresh cerebellar cortex indicated that G-substrate phosphatase activity was concentrated in the soluble or cell sap (S) fraction (Table 3). Both the percent distribution (56%), and the relative specific activity (2.4) were consistent with a cytoplasmic localization. Determination of the activity in the presence of 0.1% Triton X-100 resulted in a three-fold increase in the activity in the P3 (microsomal) fraction, less than a two-fold increase in the activity of the P2 (mitochondrial and synaptosomal) fraction, and no apparent increase in the activity of the P1 (nuclear and cellular debris) and soluble fractions (data not shown). Under these conditions, 45 - 50% of the G-substrate phosphatase activity was distributed in the soluble fraction. Since G-substrate is also primarily a cytoplasmic protein (Schlichter et al, 1980), these results, taken together, indicated that the major cerebellar G-substrate phosphatases could be isolated from a soluble extract.

The results also demonstrated that the distribution of phosphorylase and G-substrate phosphatase activity was approximately the same, suggesting that many of the same protein phosphatases might catalyze the dephosphorylation of both substrates. However, some differences between these two phosphatase activities were apparent. The recovery of phosphorylase phosphatase activity (124%) from the subcellular fractions was greater than that recovered in the crude homogenate, suggesting that fractionation, dilution, or possibly proteolysis could activate this phosphatase activity. In contrast, an increased recovery was not observed for

Table 3. Distribution of G-Substrate Phosphatase in Bovine Cerebellar Cortex

Bovine cerebellum was homogenized and fractionated as described under Experimental Procedures. Enzymic activity, expressed as Units/gm tissue, was measured using (^{32}P)G-substrate and (^{32}P)phosphorylase a in the presence of 2 mM and 0.5 mM $MnCl_2$, respectively. Details of the assay procedure, the measurement of protein concentration and lactate dehydrogenase (LDH) and succinate dehydrogenase activity (SDH) is described under Experimental Procedures. The activity towards G-substrate is the average of three separate fractionations. All other measurements are the average of two fractionations. RSA (relative specific activity) is defined as the ratio of % recovered activity/% recovered protein.

Fraction		Protein	G-Substrate Phosphatase			Phosphorylase Phosphatase			LDH	SDH
		mg/g tissue	Units ± SEM.	% Distribution	RSA	Units	% Distribution	RSA	RSA	RSA
P1	Nuclei	38	1.0 ± 0.1	24	0.6	3.5	26	0.7	0.4	1.1
P2	Mitochondria	27	0.7 ± 0.1	17	0.6	2.1	15	0.5	0.4	1.9
P3	Microsomes	7.5	0.1 ± 0.01	3	0.4	0.6	5	0.6	0.3	0
S	Cell Sap	22.5	2.3 ± 0.1	56	2.4	7.2	54	2.4	3.2	0
	Total	95	4.1	100		13.4	100			
	Homogenate	92	4.2 ± 0.4			10.9				
	Recovery (%)	103	98			124			100	82

the G-substrate phosphatase activity. And phosphorylase phosphatase in each of the fractions was spontaneously active, although the activity could be stimulated approximately two-fold by the inclusion of 0.5 mM $MnCl_2$ in the assay mixture (not shown). In contrast, phosphatase activity measured towards G-substrate was completely dependent upon the addition of $MnCl_2$ (10 to 20-fold activation using 2 mM $MnCl_2$). This effect was observed in the crude homogenate, suggesting that the dependence on Mn^{2+} for activity was not artifactually related to the fractionation process.

Separation of Cerebellar Protein Phosphatases by DEAE-Cellulose

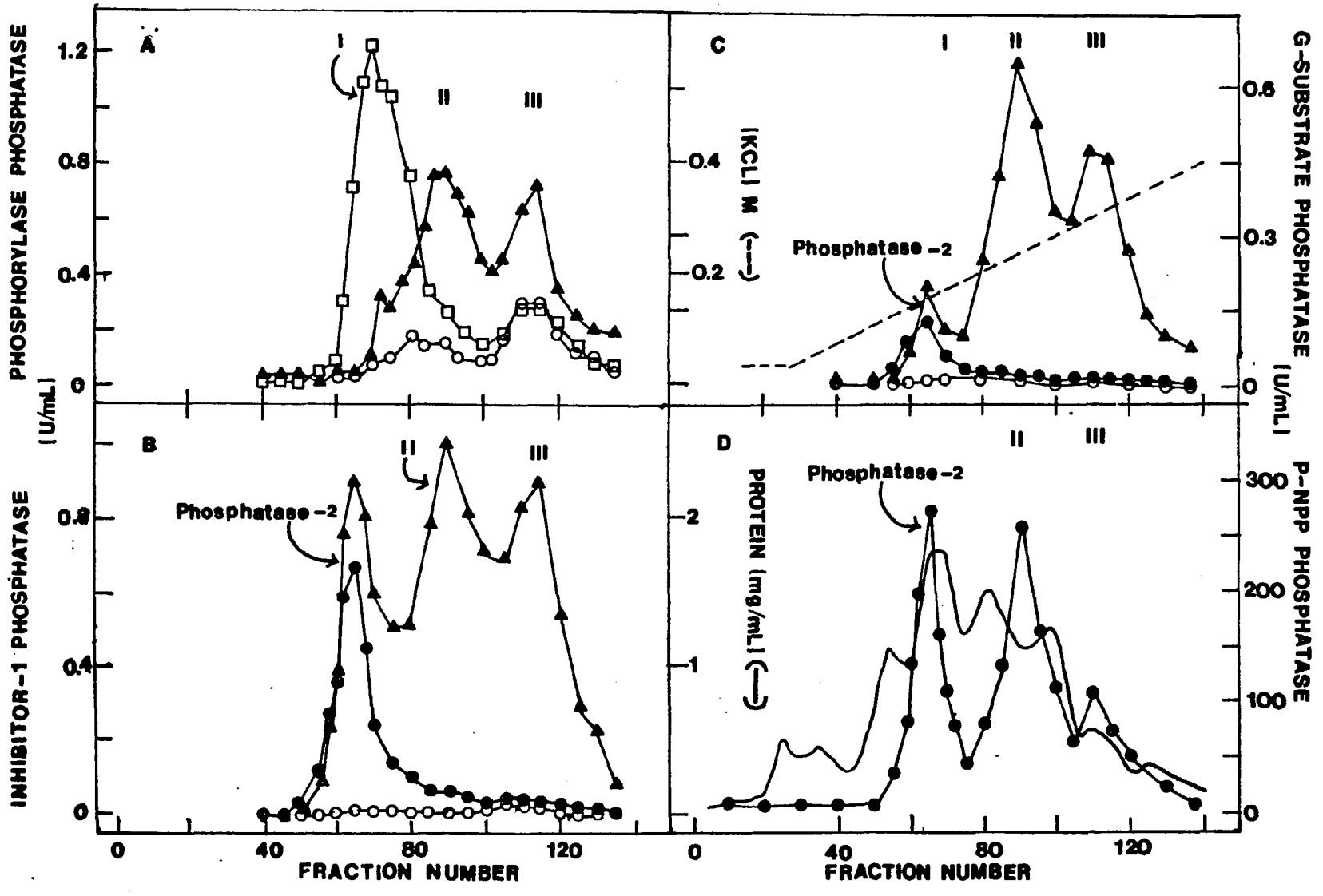
The 55% $(NH_4)_2SO_4$ fraction of bovine cerebellum was separated into four major peaks of protein phosphatase activity by DEAE-cellulose chromatography (Fig. 1). Three of the peaks were active using phosphorylase a as substrate (Fig. 1a), and were termed phosphatases I, II, and III after the order of elution from the ion exchange resin. A fourth peak, termed phosphatase-2, was not active towards phosphorylase a, but was evident when activity was measured towards inhibitor-1 (Fig. 1b) and G-substrate (Fig. 1c). Each of the peaks was active towards p-NPP (Fig. 1d) demonstrating that these phosphatases could also catalyze the dephosphorylation of certain low molecular weight phosphoesters.

Whereas none of the four phosphatases was specifically active towards a single substrate, a relative preference for different substrates was observed. For example, phosphatase I dephosphorylated each of the substrates tested, but was most active when dephosphorylation of phosphorylase a was measured. Phosphatase-2 displayed a higher level of activity measured towards inhibitor-1 in comparison with G-substrate. In contrast, phosphatases II and III displayed comparable levels of activity measured towards each of these substrates. In addition, the conditions required to optimally express the activity varied depend-

ing on the phosphatase, and the particular substrate. As examples, only the activity of phosphatase I measured using phosphorylase a as substrate was dependent upon a pre-activation with F_a + Mg·ATP. The activity of this phosphatase towards the other substrates was unaffected by this procedure (not illustrated). Calcium-calmodulin could activate phosphatase-2 activity measured towards G-substrate and inhibitor-1, but had no effect on the other phosphatases. And the activity of phosphatases I, II, and III measured towards G-substrate and inhibitor-1 was completely dependent upon the addition of Mn^{2+} to the assay mixture. Mn^{2+} also stimulated the activity of phosphatases I and II when dephosphorylation of phosphorylase a was measured, but near-maximal activity of phosphatase III could be measured in the presence of EDTA using the same substrate (see Figs. 3 & 7). The activity measured towards p-NPP was also dependent upon the addition of a metal ion (Mg^{2+} or Mn^{2+}), but the optimal pH (8.6 measured in the presence of $MgCl_2$) was higher than that measured for phosphorylase a, G-substrate, or inhibitor-1 dephosphorylation (not illustrated).

Phosphatases II and III together represented the major cerebellar activities measured towards G-substrate, accounting for more than 90% of the G-substrate phosphatase activity recovered from the DEAE-cellulose column. Phosphatase I, when assayed following activation with F_a + Mg·ATP, represented the major cerebellar phosphorylase a phosphatase activity (Fig. 1a). Phosphatase-2, quantitatively a minor G-substrate phosphatase activity, was purified to homogeneity in this laboratory as part of a separate study (Li, 1984; Chernoff et al, 1984), and consequently will not be considered further. The remainder of this study details the purification and characterization of phosphatases I, II, and III.

Figure 1. Separation of Cerebellar Protein Phosphatases on DEAE-Cellulose. The 12,000 x g supernatant prepared from bovine cerebellum (Step 1, Table 4) was precipitated with 55% $(NH_3)_2SO_4$, dialyzed against Buffer A (20 mM Tris·HCl, pH 7.4, 10 mM 2-mercaptoethanol), and was then centrifuged at 105,000 x g for 60 minutes. The supernatant was applied to a DEAE-cellulose column (2.5 x 45 cm) equilibrated with Buffer A + 2 mM $MgCl_2$, 1 mM EGTA, and 50 mM KCl. After washing with two volumes of the same buffer, the enzymic activity was eluted with linear gradient ranging from 50 - 400 mM KCl in Buffer A, as indicated. Aliquots from the indicated 12 mL fractions were tested for enzymic activity measured towards A. (^{32}P)phosphorylase a measured under control conditions (O—O), in the presence of 0.5 mM $MnCl_2$ (▲—▲), or in the presence of F_a + Mg·ATP (□—□); B. (^{32}P)inhibitor-1 measured under control conditions (O—O), in the presence of Ca^{2+}·Calmodulin (●—●) or in the presence of 2 mM $MnCl_2$ (▲—▲); C. (^{32}P)G-substrate under control conditions (O—O), in the presence of Ca^{2+}·Calmodulin (●—●), or in the presence of 2 mM Mn^{2+} (▲—▲); and D. towards p-nitrophenyl phosphate at pH 8.6 (●—●). Details of the assay protocol are given under Experimental Procedures.


A
B
C
D
I
II
III
Phosphatase -2
PHOSPHORYLASE PHOSPHATASE (U/mL)
INHIBITOR-1 PHOSPHATASE
[KCL] M (---)
G-SUBSTRATE PHOSPHATASE (U/mL)
PROTEIN (mg/mL) (—)
P-NPP PHOSPHATASE
FRACTION NUMBER
0
40
80
120
1.2
0.8
0.4
0.2
0.6
0.3
2
1
300
200
100

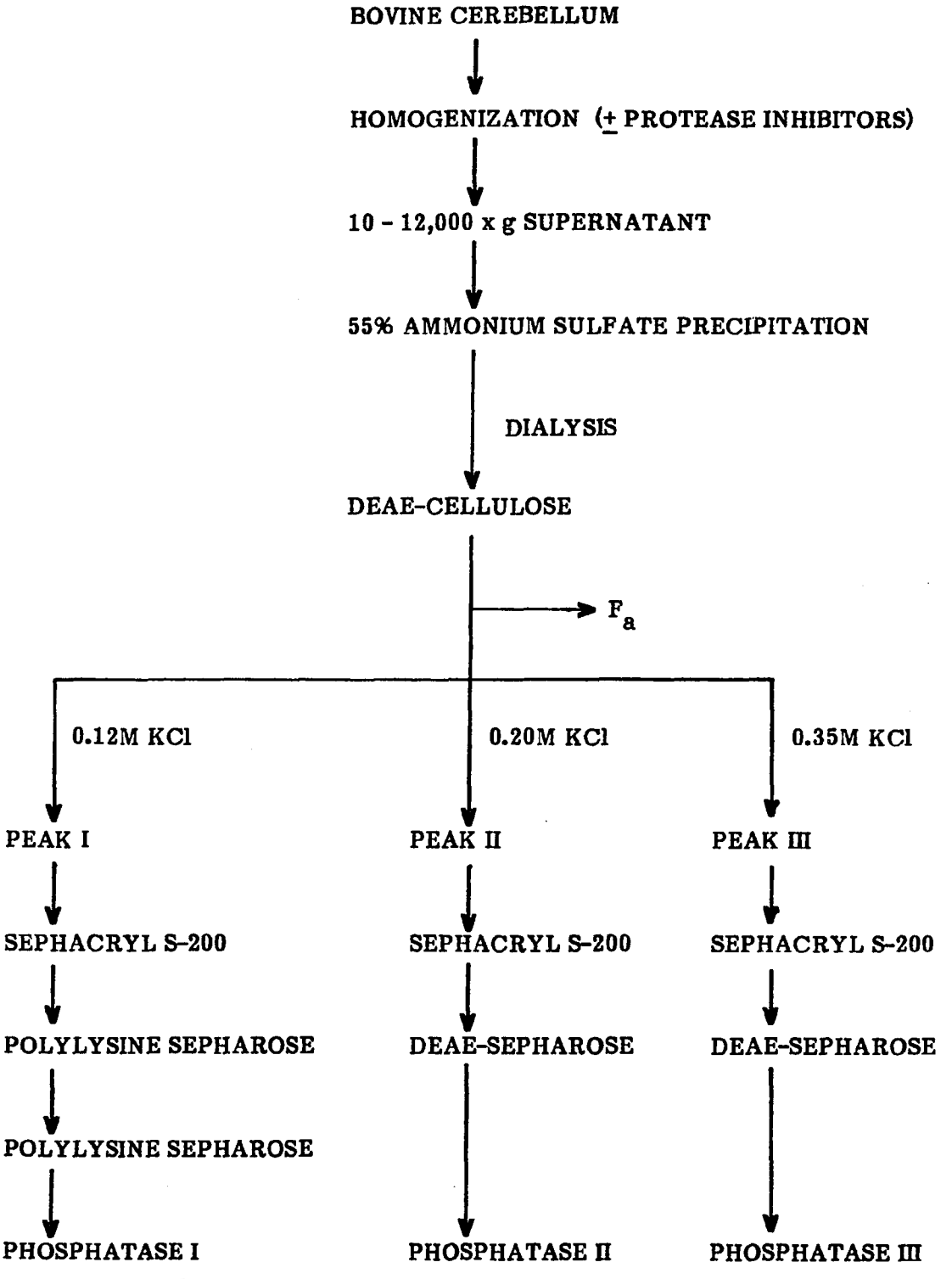
Purification of Protein Phosphatases I, II, and III

Phosphatases I, II, and III were purified from bovine brain by a procedure that involved initial homogenization, centrifugation at 12,000 x g, and fractionation with ammonium sulfate (55% saturation). The phosphatases were separated by chromatography on DEAE-cellulose using a stepwise gradient (0.12 M KCl) to elute phosphatase I, followed by a non-linear gradient (0.12 - 0.40 M KCl) to elute phosphatases II and III. Development of the column in this manner, as opposed to the use of a single linear gradient (as depicted in Fig. 1), resulted in a more efficient separation of the three protein phosphatases. Further purification was achieved using gel filtration, and repeated chromatography on polylysine Sepharose (phosphatase I) or DEAE-Sepharose (phosphatases II and III). An outline of this purification scheme is presented in Figure 2.

Purification of Phosphatase I

DEAE-Cellulose Chromatography - The purification of phosphatase I was facilitated by the direct absorption of the crude cerebellar extract onto DEAE-cellulose. Phosphatase I and F_a could then be easily separated using a batchwise elution, and the eluate containing F_a could be concentrated by $(NH_4)_2SO_4$ precipitation. Chromatography of the crude cerebellar extract on DEAE-cellulose resulted in the elution of a major peak of phosphorylase phosphatase activity that was dependent upon F_a + Mg·ATP pre-activation (Fig. 3). This peak displayed little spontaneous activity, but could be partially activated by the addition of $MnCl_2$ to the assay mixture. In contrast, peak II was primarily activated by Mn^{2+}, and peak III displayed substantial spontaneous activity, although it was also stimulated by Mn^{2+}. Neither peak II nor III was affected by F_a + Mg·ATP pre-incubation. A second peak of F_a + Mg·ATP-dependent phosphatase activity eluted

Figure 2. Purification Scheme for Protein Phosphatases I, II, and III



between peaks I and II; the appearance of this peak was variable, and consequently was thought to represent a carry-over of phosphatase I activity.

Sephacryl S-200 Chromatography - The peak fractions of phosphatase I activity were pooled following DEAE-cellulose chromatography, were precipitated with 60% $(NH_4)_2SO_4$, and were dialyzed prior to gel filtration on Sephacryl S-200 (Fig. 4). A single major peak of phosphorylase phosphatase activity was eluted (Stokes radius 4.6 nm, apparent M_r 138,000). The peak displayed little spontaneous activity; activity was partially expressed in the presence of Mn^{2+}, but was preferentially activated by F_a + Mg·ATP. A minor peak of activity with a higher apparent molecular weight was also observed (Fig. 4), but was not considered further.

Polylysine Sepharose Chromatography - Further purification of phosphatase I was achieved by repeated chromatography on polylysine (60) Sepharose CL-6B. The initial chromatography (Fig. 5) resulted in the elution of a single broad peak of F_a + Mg·ATP-dependent phosphorylase phosphatase activity. Mn^{2+} could partially stimulate this activity. Repeated chromatography using the same resin (following pooling and dialysis) resulted in the elution of two peaks of activity (Fig. 6). Both peaks were dependent upon F_a + Mg·ATP, and both could be partially activated by Mn^{2+}.

A summary of the purification of phosphatase I is presented in Table 4. These data indicate that the phosphorylase phosphatase activity in the crude extract was not activated by F_a + Mg·ATP, but was stimulated approximately 2-fold by Mn^{2+}. With increasing purification, the ratio of the activity measured following F_a + Mg·ATP activation to that measured under control conditions increased and reached a constant level after the first chromatography on polylysine Sepharose. In addition, the ratio of the activity measured in the

presence of $MnCl_2$ to that measured following F_a + Mg·ATP activation decreased to a constant level. The results indicate that phosphatase I was purified about 240-fold from the crude extract. Based upon the comparison of activity measured under control and activated conditions, phosphatase I was apparently free of other contaminating protein phosphatases.

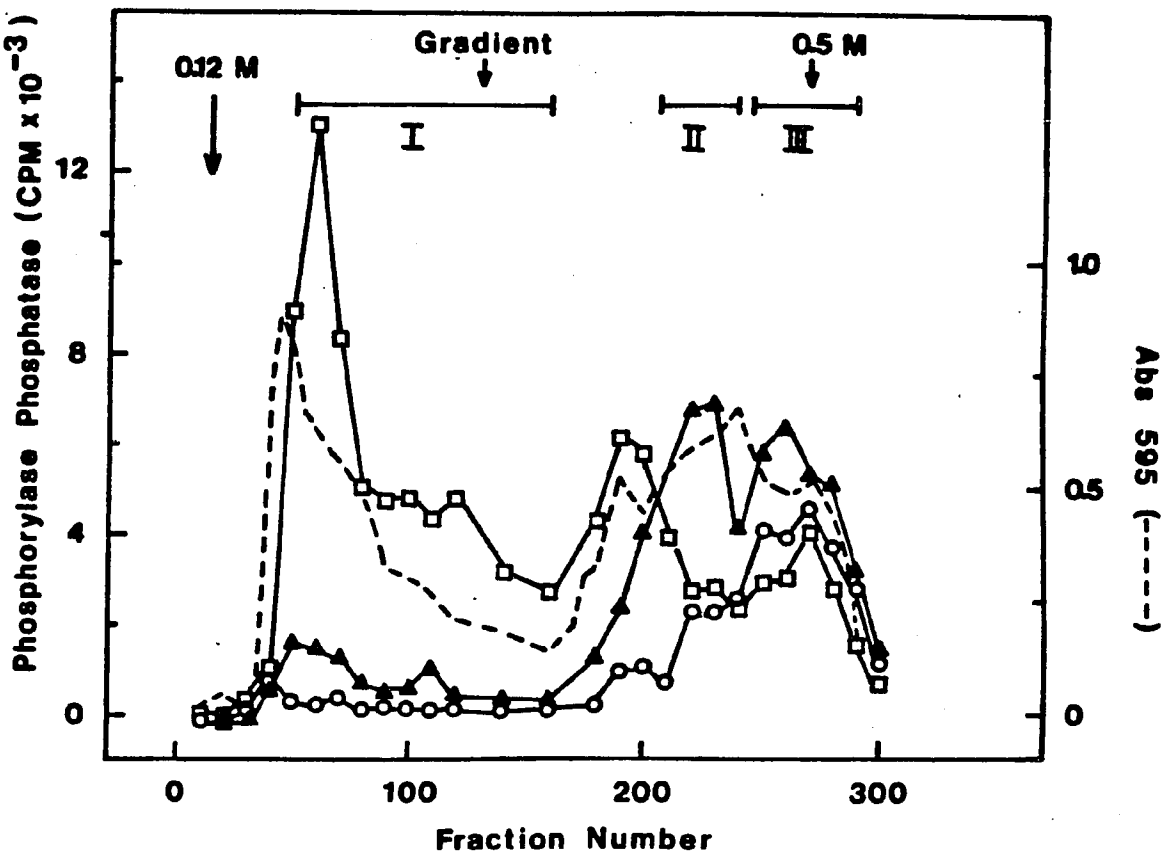


Figure 3. Separation of Phosphatase I from Phosphatases II and III on DEAE-Cellulose. The 12,000 x g supernatant prepared from bovine cerebellum (Table 4, Step 1) was applied to a DEAE-cellulose column (5 x 15 cm) equilibrated with Buffer A. The column was rinsed with 1.5 L of the same buffer, and was then developed using a non-linear gradient of KCl (0.12-0.40 M) in Buffer A. 2 uL aliquots from the indicated 10 mL fractions were tested for activity towards (^{32}P)phosphorylase a under control conditions (O—O), following activation with F_a + Mg·ATP (□—□), or in the presence of 0.5 mM $MnCl_2$ (▲—▲). Details are described under Experimental Procedures. Protein (Abs_{595}) was measured by the method of Bradford using a 25 uL aliquot. The fractions corresponding to phosphatase I were pooled as indicated by the horizontal bar.

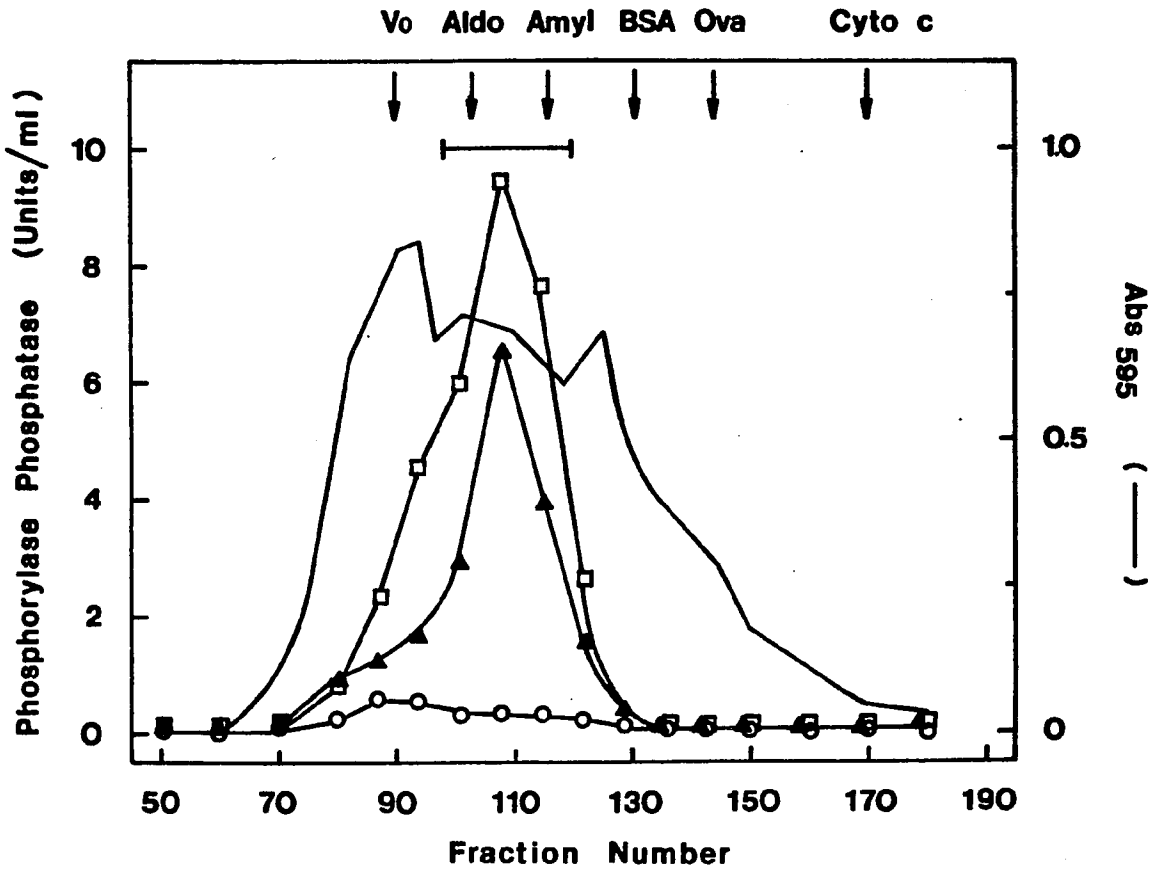
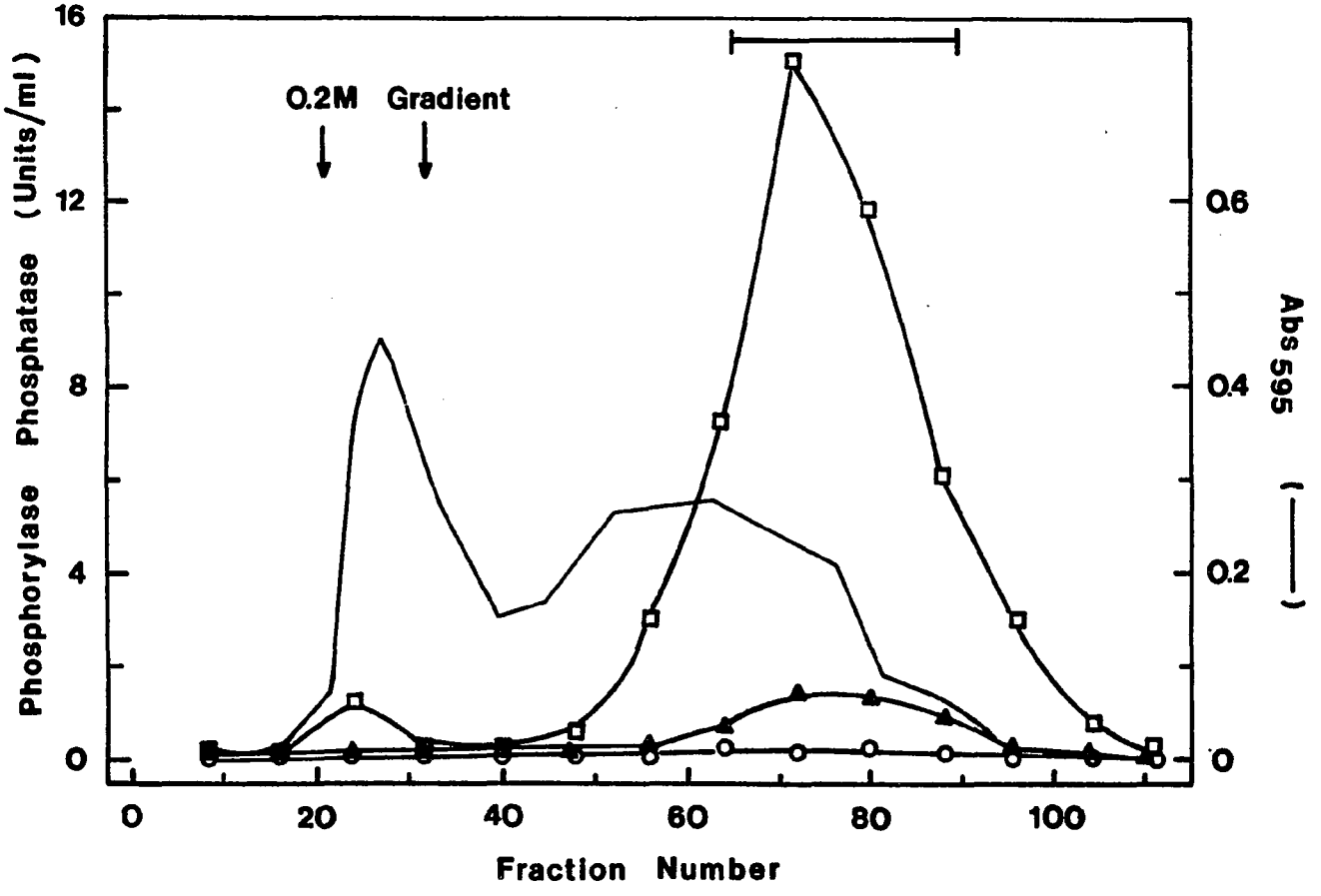


Figure 4. Chromatography of Phosphatase I on Sephacryl S-200. Cerebellar phosphatase I pooled following DEAE-cellulose chromatography (Fig. 3) was precipitated with 60% $(NH_4)_2SO_4$, and then was dialyzed against Buffer B prior to application to a Sephacryl S-200 column (2.6 x 90 cm) equilibrated with the same buffer. Aliquots from the indicated 2.2 mL fractions were tested for activity towards (^{32}P)phosphorylase a under control conditions (O—O), following activation with F_a + Mg·ATP (□—□), or in the presence of 0.5 mM $MnCl_2$ (▲—▲), as described under Experimental Procedures. The arrows mark the elution volumes (from left to right) of blue dextrin (V_o), and the following protein standards: aldolase, amylase, BSA, ovalbumin, and cytochrome c. Protein (Abs_{595}) was measured by the method of Bradford. Phosphatase I was pooled as indicated by the horizontal bar.

Figure 5. Chromatography of Phosphatase I on Polylysine Sepharose. Phosphatase I pooled following gel filtration on Sephacryl S-200 (Fig. 4) was applied to a polylysine (60) sepharose CL-6B column (1 x 12 cm) equilibrated with Buffer A. The column was developed with Buffer A, Buffer A + 200 mM NaCl, and finally with a linear gradient ranging from 200 - 600 mM NaCl in the same buffer. Aliquots from various 2.5 mL fractions were tested for enzymic activity towards (^{32}P)phosphorylase a under control conditions (○—○), following activation with F_a + Mg·ATP (□—□), or in the presence of 0.5 mM $MnCl_2$ (▲—▲) as described under Experimental Procedures. Phosphatase I was pooled as indicated by the horizontal bar.

Figure 6. Rechromatography of Phosphatase I on Polylysine Sepharose. Phosphatase I pooled following chromatography on Polylysine Sepharose (Fig. 5) was concentrated by ultrafiltration, dialyzed against Buffer A, and was then applied to a second column (1 x 4 cm) of the same resin. The column was developed with Buffer A, Buffer A + 300 mM NaCl, and a linear gradient ranging from 300 - 600 mM NaCl in the same buffer. Enzymic activity towards (^{32}P)phosphorylase a was measured as described in the legend to Figure 5.


0.2M Gradient
Phosphorylase Phosphatase (Units/ml)
Abs 595 (——)
Fraction Number
0
4
8
12
16
0.2
0.4
0.6
0
20
40
60
80
100

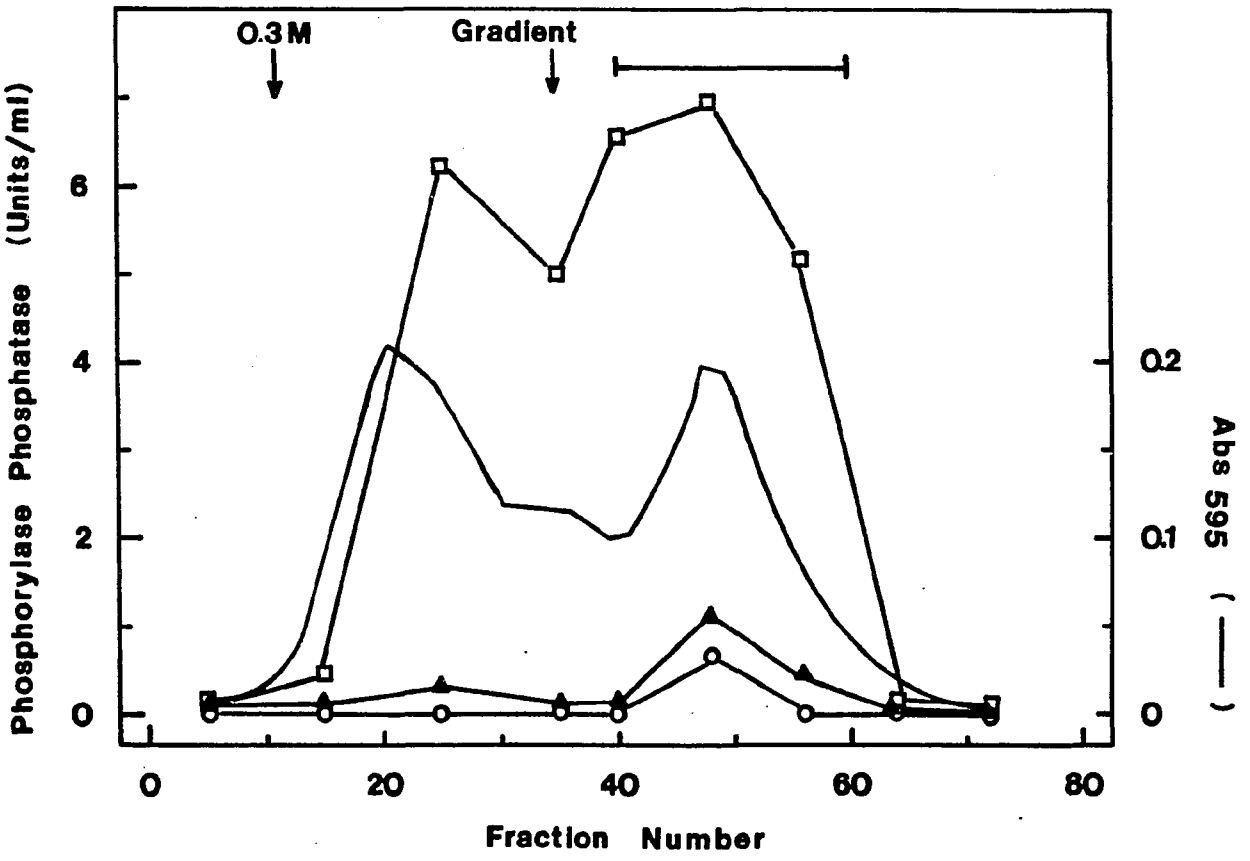

0.3 M
Gradient
Phosphorylase Phosphatase (Units/ml)
Abs 595 (——)
Fraction Number
0
2
4
6
0
0.1
0.2
0
20
40
60
80

Table 4. Purification of Phosphatase I from Bovine Cerebellum.

Phosphatase I was purified from 402 g of fresh bovine cerebellum as described in the text. Enzymic activity was measured as described under Experimental Procedures. Aliquots taken from the various stages of purification were stored at (-)20°C in Buffer A + 60% glycerol, and were assayed together at the completion of the purification procedure. Enzyme fractions were diluted into 20 mM Tris·HCl, pH 7.4 + 0.2% (w/v) BSA prior to assay such that the reaction was limited to between 2 - 6% dephosphorylation. Values represent the average of duplicate assays.

FRACTION	PROTEIN (mg)	SPECIFIC ACTIVITY (Units/mg)	ACTIVITY RATIO Fa : Control	ACTIVITY RATIO Mn : Fa
1. Crude Extract	12012	0.29	1	2.00
2. DEAE-Cellulose	228	3.35	9	0.53
3. Sephacryl S-200	74	8.54	12	0.28
4. First Polylysine Sepharose	17	41.8	31	0.07
5. Second Polylysine Sepharose	3	70.7	32	0.06

Purification of Phosphatases II and III

DEAE-Cellulose Chromatography - DEAE-cellulose chromatography of the 55% ammonium sulfate fraction prepared from a bovine brain extract resulted in the separation of G-substrate phosphatase activity into three major peaks of activity. A representative elution profile of an extract derived from whole brain is depicted in Figures 7 and 8, but qualitatively similar elution profiles were obtained using material derived from cerebellum, as well as from whole brain from which the cerebellum was removed (not shown). The first peak co-eluted with phosphatase I activity (see Fig. 3), and represented only 5% of the pooled G-substrate phosphatase activity. Peaks II and III eluted at a KCl concentration of 0.20 and 0.35 M, respectively. Peak II accounted for 55% and peak III 40% of the G-substrate phosphatase activity, and consequently represented the major cerebellar G-substrate phosphatase activities that were recovered following this chromatography step.

None of these peaks was specific for G-substrate as each also displayed phosphatase activity measured towards phosphorylase a (Fig. 7), phosphohistone, phosphocasein, and p-NPP (Fig. 8). The activity of peak II measured towards phosphorylase a and phosphocasein was metal ion-dependent (Mn^{2+} or Mg^{2+}, as indicated), while that of peak III using these same substrates was stimulated less than 2-fold by added metal ions. However, activity of either of these phosphatases towards G-substrate could only be detected when Mn^{2+} was present in the assay mixture.

Sephacryl S-200 Chromatography - Gel filtration of cerebellar phosphatase II on Sephacryl S-200 (Fig. 9) resulted in the separation of G-substrate phosphatase activity into three active peaks, termed IIA, IIB, and IIC (Stokes radius 5.4, 4.0, and 3.0 nm, and apparent M_r 200,000, 130,000, and 35,000, respectively). Each of

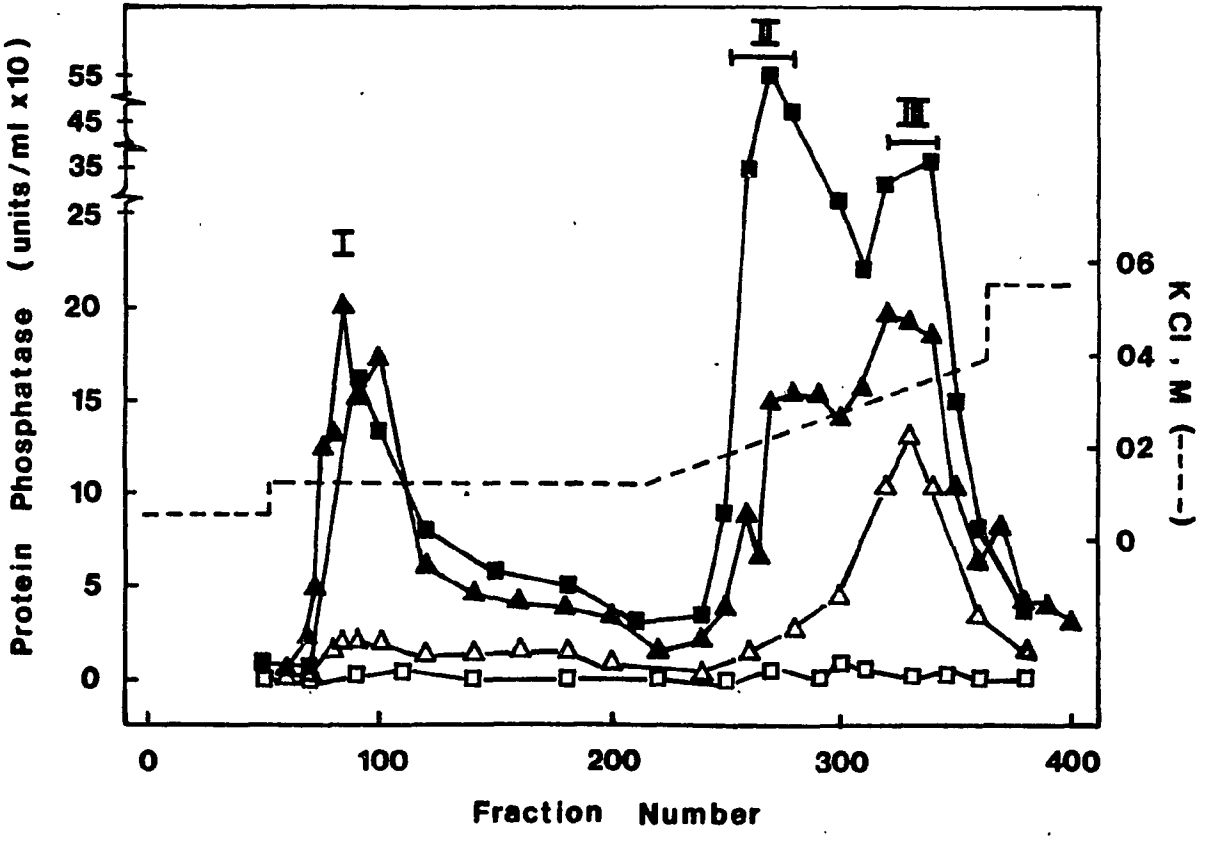
these three peaks also exhibited activity towards phosphorylase a and p-NPP. A Mn^{2+}-independent phosphorylase a phosphatase activity eluted between peaks IIA and IIB, and also co-eluted with peak IIC. This cation-independent activity possibly represented cross-contamination of phosphatase II by phosphatase III from the previous purification step. Gel filtration of phosphatase III (Fig. 10) resulted in the elution of a single active peak (Stokes radius 4.4 nm, apparent M_r 125,000) that was active towards phosphorylase a and p-NPP as well as towards G-substrate. The data presented in Figures 9 and 10 indicated that the Mn^{2+}-dependency of the activity of both phosphatase II and phosphatase III measured towards G-substrate (and phosphorylase a) was conserved following gel filtration.

DEAE-Sepharose Chromatography - The most active fractions of phosphatase II (peak IIA, Fig. 9) and phosphatase III (Fig. 10) were pooled following gel filtration, and were further purified using DEAE-Sepharose. Phosphatase II (Fig. 11) and phosphatase III (Fig. 12) eluted at a concentration of 0.17 and 0.24 M KCl, respectively. Chromatography of phosphatase II resulted in the elution of a second peak of activity, but the elution position (0.24 M KCl) suggested that it represented cross-contamination with phosphatase III. Both phosphatases were active towards phosphorylase a and p-NPP (Figs. 11 & 12), and both were active towards G-substrate (not illustrated).

A summary of the purification of phosphatases II and III from bovine cerebellum is presented in Table 5. The G-substrate phosphatase activity of phosphatases II and III were purified about 20- and 60-fold, respectively, from the crude extract. The ratio of activity of phosphatase II measured towards G-substrate and phosphorylase a (both measured in the presence of added $MnCl_2$) was about four-fold higher than that measured for phosphatase III, indicating that phosphatase III was relatively more specific for the latter substrate.

Figure 7. Separation of Brain Phosphatases I, II, and III on DEAE-Cellulose: Activity Towards G-Substrate and Phosphorylase a. The 55% $(NH_4)_2SO_4$ fraction of an extract prepared from whole brain was applied to a DEAE-cellulose column (4 x 42 cm) equilibrated with Buffer A + 0.05 M KCl. The column was developed with an increasing concentration of KCl in Buffer A ranging from 0.05 -0.55 M, as indicated. 24 mL fractions were collected. Enzymic activity was measured towards 1 uM (^{32}P)G-substrate (■—■, + 4 mM $MnCl_2$; □—□, + 2 mM EDTA); or 10 uM (^{32}P)phosphorylase a (▲—▲, + 0.5 mM $MnCl_2$; △—△, + 2 mM EDTA) as described under Experimental Procedures. The fractions that corresponded to phosphatases II and III were pooled as indicated by the horizontal bars.

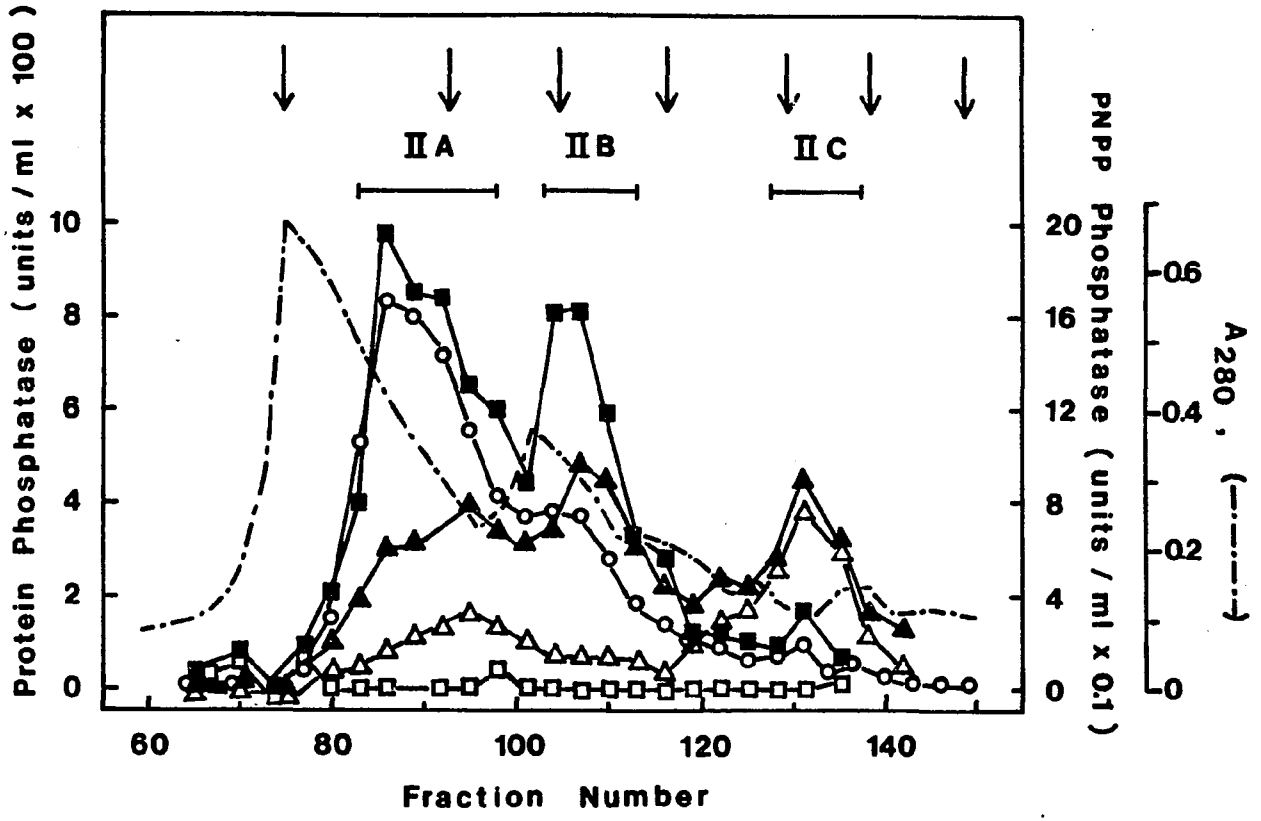
Figure 8. Separation of Brain Phosphatases I, II, and III on DEAE-Cellulose: Activity Towards Phosphohistone, Phosphocasein, and p-Nitrophenyl Phosphate. The 55% $(NH_4)_2SO_4$ fraction of an extract prepared from whole brain was chromatographed on DEAE-cellulose as described in the legend to Fig. 7. Activity was measured towards 10 uM (^{32}P)phosphocasein (●—●, + 10 mM $MgCl_2$; O—O, + 5 mM EDTA), 10 uM (^{32}P)phosphohistone (◆—◆, + 5 mM $MnCl_2$), and p-NPP at pH 8.6 (⊠—⊠).


Protein Phosphatase (units/ml x10)
55
45
35
25
20
15
10
5
0
I
II
III
0
100
200
300
400
Fraction Number
06
04
02
0
K Cl , M (----)

PNPP Phosphatase (units / ml x 0.01)
0 1 2 3 4 5 6
Protein Phosphatase (units / ml x 10)
0 5 10 15 20 25
Fraction Number
0 100 200 300 400
I
II
III
0 10 20
A280 , (-·-·-·-)

Figure 9. Chromatography of Phosphatase II on Sephacryl S-200. Cerebellar phosphatase II was pooled and concentrated following DEAE-cellulose chromatography (Table 5, Step 3), and was applied to a Sephacryl S-200 column (2.6 x 90 cm) equilibrated with Buffer B. The column was eluted at a flow rate of 4.8 mL-Hr^{-1}-cm^{-2} while collecting 2.2 mL fractions. Enzymic activity towards (^{32}P)G-substrate (■—■, +2 mM $MnCl_2$; □—□, + 2 mM EDTA), (^{32}P)phosphorylase a (▲—▲, + 0.5 mM $MnCl_2$; △—△, + 2 mM EDTA), and p-NPP at pH 8.6 (O—O) was measured as described under Experimental Procedures. The arrows indicate the elution volumes (left to right) of the following standards: blue dextrin (V_o), aldolase, amylase, BSA, ovalbumin, chymotrypsinogen, and cytochrome c. The fractions corresponding to phosphatase II were pooled as indicated by the horizontal bars.

Figure 10. Chromatography of Phosphatase III on Sephacryl S-200. Cerebellar phosphatase III was pooled and concentrated following DEAE-cellulose chromatography (Table 5, Step 3), and was chromatographed on a Sephacryl S-200 column as described in the legend to Fig. 9. The fractions corresponding to phosphatase III were pooled as indicated by the horizontal bar.


Protein Phosphatase (units / ml x 100)
PNPP Phosphatase (units / ml x 0.1)
A280 , (——)
IIA
IIB
IIC
Fraction Number
0
2
4
6
8
10
12
16
20
0.2
0.4
0.6
60
80
100
120
140

PNPP Phosphatase (Units / ml x 0.1)
Protein Phosphatase (Units / ml x 10)
0
4
8
12
16
20
60
80
100
120
140
Fraction Number
III
0
0.1
0.2
0.3
0.4
Protein , A280 (—·—·—)

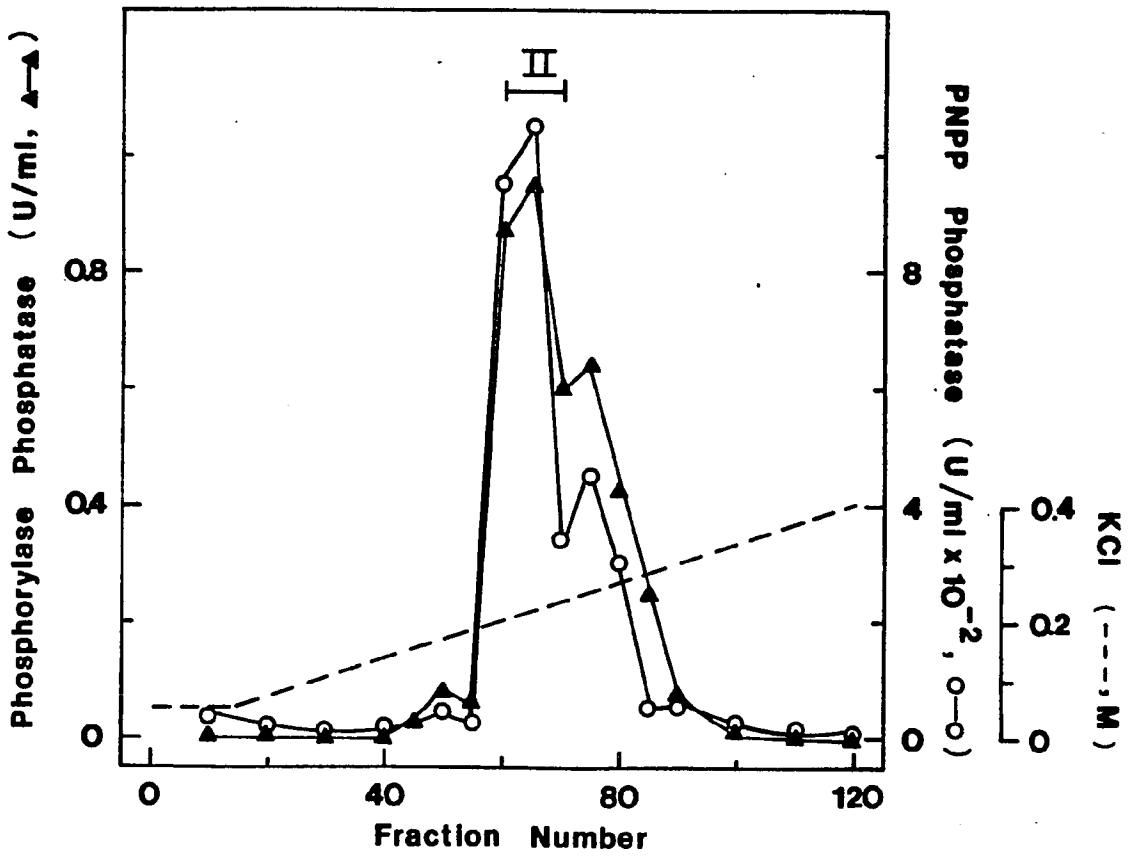


Figure 11. Chromatography of Phosphatase II on DEAE-Sepharose. Cerebellar phosphatase II was pooled and concentrated following gel filtration on Sephacryl S-200 (Table 5, Step 4), and was applied to a DEAE-Sepharose column (1.6 x 22 cm) equilibrated with Buffer A + 0.05 M KCl. The column was rinsed with 1 bed volume of the same buffer, and then was developed with a linear gradient ranging from 0.05 - 0.40 M KCl in Buffer A. Aliquots from the indicated 2 mL fractions were tested for activity towards p-NPP at pH 8.6 (O—O) and (^{32}P)phosphorylase a in the presence of 0.5 mM $MnCl_2$ (▲—▲). Details of the assay are described under Experimental Procedures. Phosphatase II was pooled as indicated by the horizontal bar.

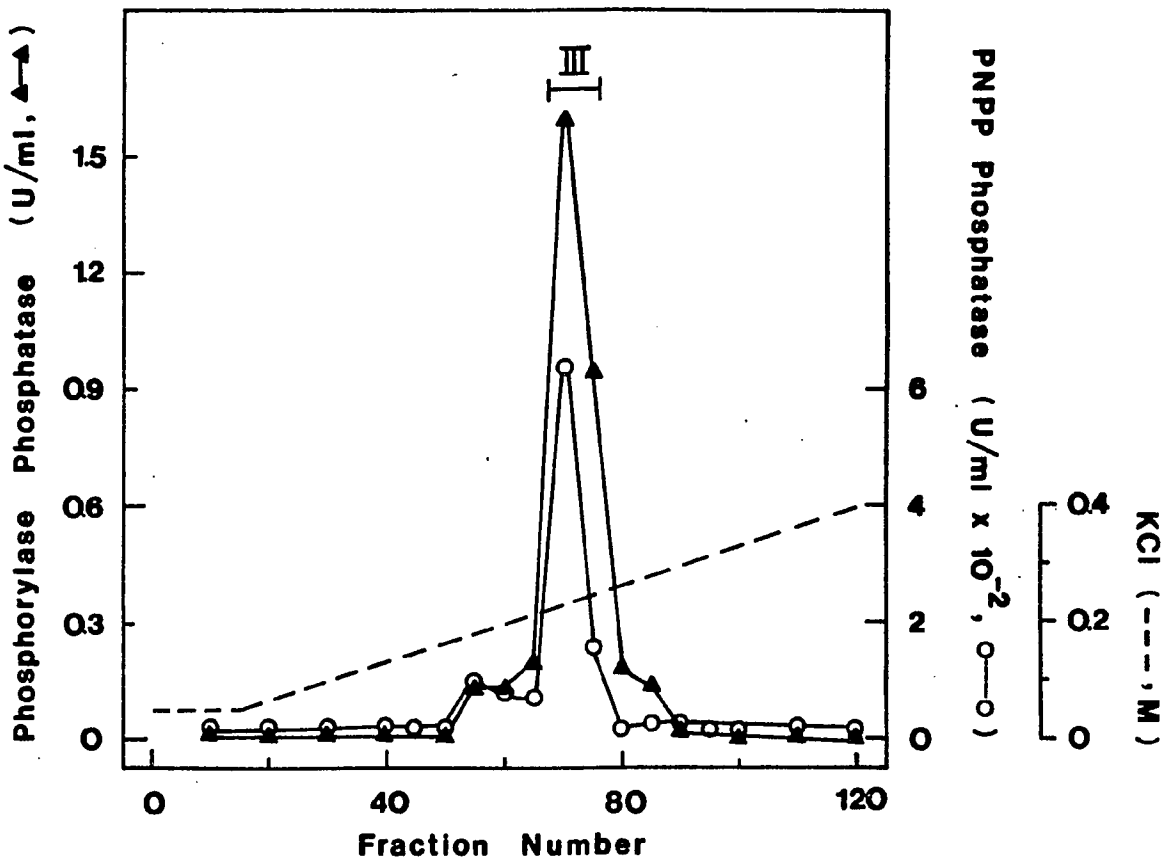


Figure 12. Chromatography of Phosphatase III on DEAE-Sepharose. Cerebellar phosphatase III was pooled and concentrated following gel filtration on Sephacryl S-200 (Table 5, Step 4), and was chromatographed on a DEAE-Sepharose column as described in the legend to Fig. 11. Activity was measured towards p-NPP at pH 8.6 (o—o) and (^{32}P)phosphorylase a in the presence of 0.5 mM $MnCl_2$ (▲—▲) as described under Experimental Procedures. Phosphatase III was pooled as indicated by the horizontal bar.

Table 5. Purification of Phosphatases II and III from Bovine Cerebellum

Phosphatases II and III were purified from 402 grams of bovine cerebellum as described in the text. Samples taken from the different fractions were stored at (-)20°C in Buffer A + 60% glycerol, and were assayed together at the completion of the purification procedure. Activity was measured towards (^{32}P)G-substrate in the presence of 2 mM $MnCl_2$, and towards (^{32}P)phosphorylase a in the presence of 0.5 mM $MnCl_2$ as described under Experimental Procedures. Enzyme fractions were diluted into 20 mM Tris·HCl, pH 7.4 + 0.2% (w/v) BSA prior to assay such that the reaction was limited to 5 - 10% dephosphorylation. The activity ratio compares the activity measured towards G-substrate to that measured towards phosphorylase a. Data are the average of duplicate assays.

Fraction	Protein (mg)	Specific Activity Towards (Units/mg) G-Substrate	Phosphorylase a	Activity Ratio
1. 12,000 x g Supernatant	12012	0.21	0.38	0.55
2. 55% $(NH_4)_2SO_4$	8288	0.17	0.32	0.52
3. DEAE-Cellulose				
Phosphatase II	332	0.67	1.55	0.44
Phosphatase III	295	0.53	2.44	0.22
4. Sephacryl S-200				
Phosphatase II	70	2.06	4.46	0.46
Phosphatase III	34	2.32	12.97	0.18
5. DEAE-Sepharose				
Phosphatase II	17	4.76	8.29	0.57
Phosphatase III	3	12.33	96.33	0.13

Comparison of the Distribution and Recovery of G-Substrate Phosphatase Activity From Cerebellar and Extra-Cerebellar Brain Tissue

Homogenization, ammonium sulfate fractionation, and DEAE-cellulose chromatography was carried out using cerebellum, and whole brain from which the cerebellum was removed (extra-cerebellum). The distribution of G-substrate phosphatase activity in the two preparations was then examined (Tables 6 & 7). For comparison, the activity using phosphorylase a (measured in the presence of $MnCl_2$) and p-NPP was also determined. The results indicate that G-substrate phosphatase activity was not specifically localized in the cerebellum (Table 6). However, when measured in the 12,000 x g supernatant, this activity was about 2-fold more concentrated in the cerebellum, a difference that seemed to reflect a relatively higher peak II phosphatase activity. In contrast, the activity measured towards phosphorylase a was about equally distributed in both preparations, although the proportion of peak II phosphatase activity measured towards this substrate was higher in the cerebellum as well. Peak I phosphatase (measured as a Mn^{2+}-stimulated activity) seemed to be somewhat more active in the extra-cerebellar preparation, whereas peak III phosphatase activity was about equally distributed.

A comparison of the recovery of the different phosphatase activities during each of the purification steps revealed additional differences between the cerebellar and extra-cerebellar preparations (Table 7). The stepwise recovery of the activity measured towards G-substrate from each of the first two purification steps was about equal for the two preparations. However, the stepwise yield of cerebellar phosphorylase phosphatase activity was about two to three-fold higher than that of the extra-cerebellar preparation. This difference may have reflected the loss of specific phosphorylase phosphatase activators from the extra-cerebel-

lar preparation during DEAE-cellulose chromatography.

The distribution of activity between peaks I, II, and III following DEAE-cellulose chromatography indicated that while cerebellar phosphatase II represented the major activity towards G-substrate (55% of the total activity recovered in peaks I, II, and III), the extra-cerebellar phosphatase II represented a much smaller proportion (28%) of this activity. Phosphatase II represented less of the total activity measured towards phosphorylase as well (12% extra-cerebellar compared with 53% cerebellar). In both cases, peak I seemed to account for most of this difference.

Table 6. Comparison of the Distribution of Phosphatases I, II, and III in Cerebellar and Extra-Cerebellar Brain Tissue.

Cerebellum (78 g) and whole brain from which the cerebellum was removed (739 g) were separately homogenized, centrifuged at 12,000 x g, precipitated with 55% $(NH_4)_2SO_4$, and fractionated by DEAE-cellulose chromatography as described under Experimental Procedures. The columns were developed using 0.12 M KCl in Buffer A to elute phosphatase I, followed by a non-linear KCl gradient (as shown in Figs. 3 & 7) to separate phosphatases II and III. Care was taken to pool corresponding peak fractions in a similar manner. Phosphatase activity was measured towards (^{32}P)G-substrate and (^{32}P)phosphorylase a in the presence of 2 mM and 0.5 mM $MnCl_2$, respectively, and towards p-NPP at pH 8.6 as described under Experimental Procedures. The ratio of cerebellar to extra-cerebellar activity is presented in two ways: Units/Gm represents the total measured activity per gram of starting material (wet weight); Specific Activity is presented in units of activity per mg protein for each individual fraction.

	Activity Ratio (Cerebellum : Extra-Cerebellum) Towards					
	G-Substrate		Phosphorylase a		p-NPP	
Fraction	Units /Gm	Specific Activity	Units /Gm	Specific Activity	Units /Gm	Specific Activity
12,000 x g Super.	1.9	2.3	0.9	1.3	1.7	2.4
55% $(NH_4)_2SO_4$	1.2	1.1	1.1	0.9	1.6	1.4
DEAE-Cellulose						
Peak I	0.5	0.4	0.3	0.3	1.0	0.8
Peak II	3.1	0.8	10.7	2.7	2.0	0.5
Peak III	1.2	0.6	1.8	0.9	1.8	0.9

Table 7. Comparison of the Recovery of Phosphatases I, II, and III from Cerebellar and Extra-Cerebellar Brain Tissue

Cerebellum and whole brain from which the cerebellum was removed were separately homogenized and fractionated as described in the legend to Table 6. Activity is expressed as total units (U) recovered in the pooled fractions. Percentages reflect the stepwise recovery of total activity (i.e., the combined activity of peaks I, II, and III) from the first and then the second stage of purification. Decimals (listed for peaks I, II, and III) represent the fractional recovery of activity in each of the peaks following DEAE-Cellulose chromatography.

A. Cerebellum

	Activity Towards		
Fraction	G-Substrate	Phosphorylase a	p-NPP
12,000 x g Super.	918 U (16%)	1378 U (13%)	65900 U (44%)
55% $(NH_4)_2SO_4$	473 U (32%)	868 U (20%)	57993 U (50%)
DEAE-Cellulose			
Peak I	7.8 U (0.05)	7.8 U (0.04)	6691 U (0.23)
Peak II	81.4 U (0.55)	93.6 U (0.53)	12291 U (0.42)
Peak III	60.0 U (0.40)	75.8 U (0.43)	10001 U (0.35)
Column Recovery	149.2 U (1.00)	177.2 U (1.00)	28984 U (1.00)

B. Whole Brain Minus Cerebellum

	Activity Towards		
Fraction	G-Substrate	Phosphorylase a	p-NPP
12,000 x g Super.	4642 U (19%)	13928 U (5%)	366781 U (49%)
55% $(NH_4)_2SO_4$	3881 U (23%)	7762 U (9%)	347353 U (52%)
DEAE-Cellulose			
Peak I	157 U (0.18)	220 U (0.31)	65626 U (0.36)
Peak II	250 U (0.28)	83 U (0.12)	59280 U (0.33)
Peak III	484 U (0.54)	406 U (0.57)	56160 U (0.31)
Column Recovery	891 U (1.00)	709 U (1.00)	181066 U (1.00)

Physical Properties of Phosphatases I, II, and III

Determination of Molecular Weight

Gel filtration of the purified cerebellar phosphatase I (Step 5, Table 4) on Sephacryl S-200 resulted in the elution of a single symmetrical peak of activity (Fig. 13). The apparent M_r of this peak was estimated to be 85,000 (Stokes radius 3.8 nm), a value that was significantly lower than that observed following gel filtration of the DEAE-cellulose fraction (Table 4, step 2) on Sephacryl S-200 (Fig. 4). A dissociation of phosphatase I during purification is the simplest explanation of this difference; this possibility was not investigated further. No change in the elution behavior of phosphatases II and III was observed when comparing the results of gel filtration following DEAE-cellulose chromatography (Figs. 9 & 10) or following the last purification step (not shown).

Sucrose gradient ultracentrifugation of cerebellar phosphatases I and III (Fig. 14a & d) resulted in the sedimentation of single peaks of activity ($s_{20,w}$ = 4.0 and 5.6, respectively). A single activity peak ($s_{20,w}$ = 3.7) also resulted from the sedimentation of phosphatase S, a low molecular weight phosphatase (apparent M_r 38,000) prepared by ethanol treatment of the crude cerebellar homogenate (Fig. 14c). In contrast, sedimentation of phosphatase II (Fig. 14b) resulted in two major activity peaks ($s_{20,w}$ = 6.4 and 8.4), as well as the variable appearance of additional minor activity peaks. A comparison of the sedimentation at three different pH levels (pH 6.0, 7.5, and 8.5) resulted in unchanged activity profiles for each of the major peaks of phosphatase activity (not shown). However, faster sedimenting ($s_{20,w}$ = 8.5 - 11) minor phosphatase II activity peaks were observed at the lower pH levels, but not at pH 8.5, suggesting that the appearance of these minor peaks may have been the result of protein aggregation.

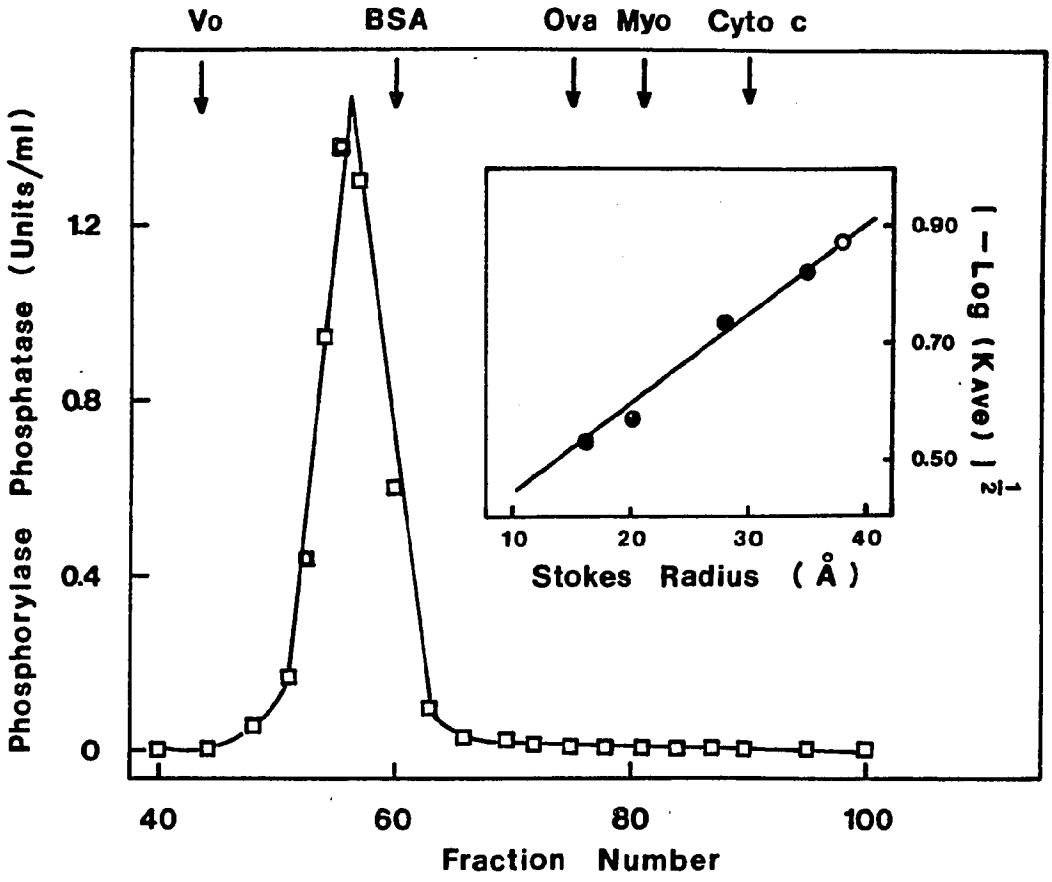


Figure 13. Gel Filtration of Purified Phosphatase I on Sephacryl S-200. 500 ug of cerebellar phosphatase I (Step 5, Table 4) was applied to a Sephacryl S-200 column (2.6 x 49 cm) equilibrated with Buffer B. The column was eluted at a flow rate of 3.0 mL-hr^{-1}-cm^{-2} while collecting 2.1 mL fractions. Activity towards (^{32}P)phosphorylase a was measured following preactivation with F_a + Mg·ATP as described under Experimental Procedures. The arrows mark the elution positions of the following standards (left to right): blue dextrin (V_o), BSA, ovalbumin, myoglobin, and cytochrome c. The inset is a replot of the data showing the elution volumes of the marker proteins (●) and of phosphatase I (O).

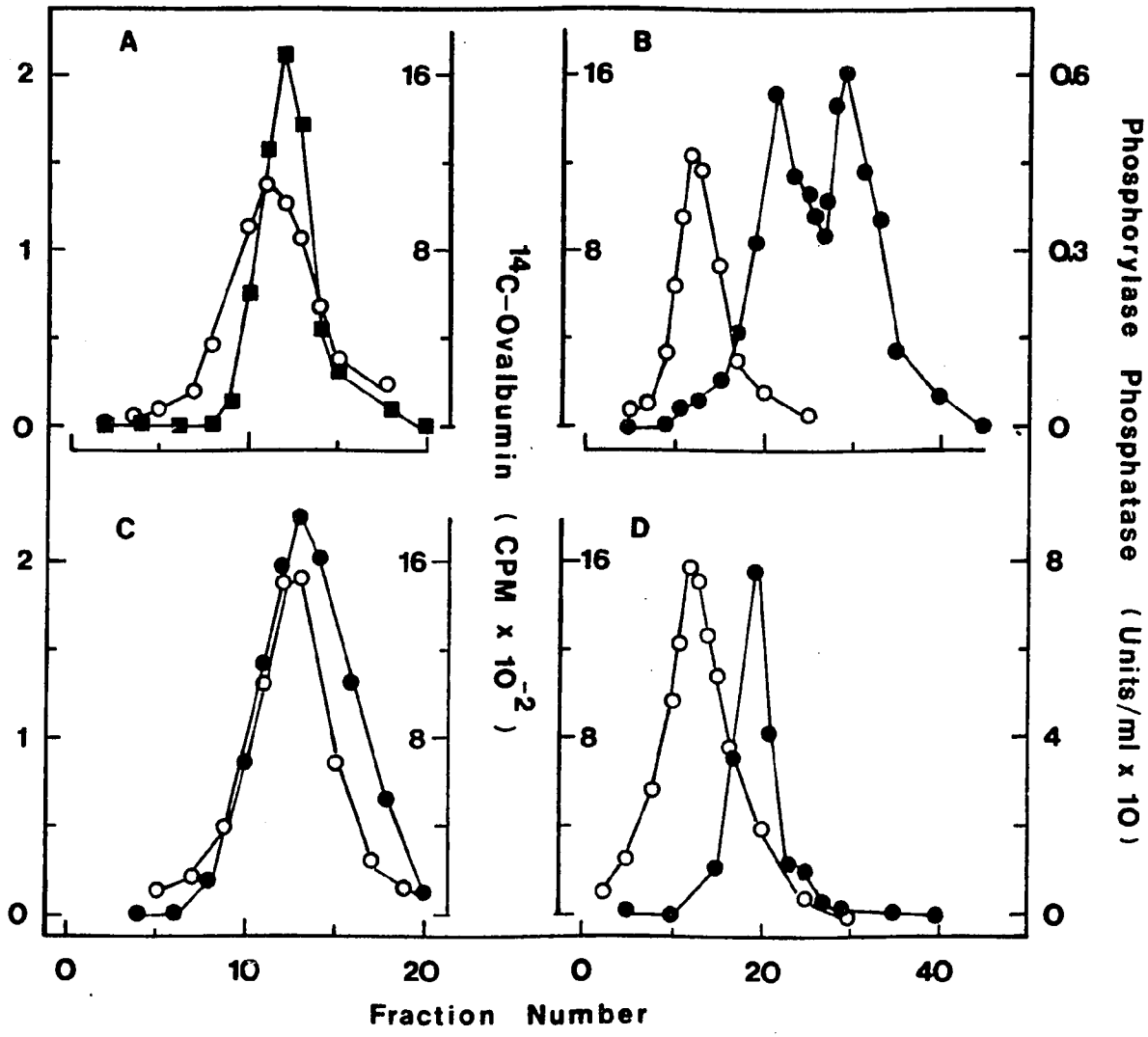


Figure 14. Sucrose Gradient Ultracentrifugation of Cerebellar Phosphatases I, II, III, and S. A. Phosphatase I (12 ug), B. phosphatase II (41 ug), C. phosphatase S (10 ug), and D. phosphatase III (10 ug), together with ^{14}C-ovalbumin (O—O) that was added as an internal standard, were centrifuged at 35,000 rpm for 18 hours using a linear 5 - 20% sucrose gradient. Following centrifugation, the tubes were fractionated, and phosphorylase a phosphatase activity was measured in the presence of 0.5 mM $MnCl_2$ (●—●) or following preactivation with F_a + Mg·ATP (■—■). Details of these procedures are described under Experimental Procedures.

The molecular weight for each of the cerebellar phosphatases was calculated using these $s_{20,w}$ values, the Stokes radii estimated by gel filtration, and an assumed partial specific volume of 0.725 cm^3/g. These data are summarized in Table 8. When calculating the molecular weight of phosphatase II, the faster sedimenting activity peak was assumed to correspond to gel filtration peak IIA (Fig. 9), and the slower sedimenting peak to peak IIB. (The sedimentation coefficient of phosphatase IIC was not determined.) The frictional (f/f_o) and axial ratios estimated for phosphatases I, II, and III were indicative of an asymmetric or elongated shape, whereas, phosphatase S, and the ethanol-treated phosphatases II and III (described below) seemed to be more globular.

The same physical parameters were determined for bovine cardiac phosphatases I, II, and III, and are summarized in Table 9. Cerebellar and cardiac phosphatases I, II, and III displayed several similar chromatographic and catalytic properties (described below). However, while the cerebellar phosphatases were resistant to extensive purification, the cardiac phosphatases were purified in this laboratory to near-homogeneity as part of a separate study (D. Tabarini, Ph. D. Dissertation, Mt. Sinai Sch. Med., 1984; and H.-C. Li, unpublished results). For these reasons, a comparison of the physical properties of the cerebellar and cardiac phosphatases was carried out. The molecular weight of phosphatases I and III isolated from the two tissues was in close agreement (Tables 8 & 9). Gel filtration of cardiac phosphatase II on Sephacryl S-200 resulted in the elution of a major peak of activity (Stokes radius = 5.4 nm) that displayed an apparent molecular weight similar to that of cerebellar phosphatase IIA. A single minor peak of activity (Stokes radius = 3.3 nm) was also observed. Like its cerebellar counterpart, cardiac phosphatase II displayed multiple peaks of activity following sucrose gradient ultracentrifugation ($s_{20,w}$ = 3.0, 5.2, 6.9, and 7.8). The major activity peak ($s_{20,w}$ = 6.9) was used in the calculation of molecular weight.

Table 8. Molecular Properties of Cerebellar Phosphatases I, II, III, and S

The molecular weight was calculated from the Stokes radius and $s_{20,w}$ values assuming a partial specific volume of 0.725 cm^3/g. The axial ratio was estimated assuming a shape of a prolate ellipsoid with the dimensions a > b = c. The apparent M_r was estimated by gel filtration on Sephacryl S-200. Phosphatase S was prepared by ethanol precipitation of the crude cerebellar homogenate, and phosphatases II (EtOH) and III (EtOH) were prepared by ethanol precipitation of the purified phosphatases II and III, respectively. Details of these procedures and calculations are described under Experimental Procedures.

Phosphatase	Stokes Radius (nm)	$s_{20,w}$	Molecular Weight	f/f_o	Axial Ratio	M_r(Apparent)
I	3.8	4.0	62,750	1.45	8	85,000
IIa	5.4	8.4	187,260	1.43	8	200,000
IIb	4.0	6.4	105,690	1.28	6	130,000
IIc	3.0	-	-	-	-	35,000
II (EtOH)	2.5	3.3	34,000	1.17	5	34,000
III	4.4	5.6	101,720	1.43	8	125,000
III (EtOH)	2.6	3.3	35,400	1.18	5	35,000
S	2.6	3.7	39,600	1.19	5	38,000

Table 9. Molecular Properties of Cardiac Phosphatases I, II, and III

Phosphatase	Stokes Radius (nm)	$s_{20,w}$	Molecular Weight	f/f_o	Axial Ratio	M_r(Apparent)
I	3.9	3.8	61,000	1.50	8	85,000
II	5.4	6.9	153,600	1.53	9	195,000
III	4.6	5.4	102,400	1.47	8	148,000

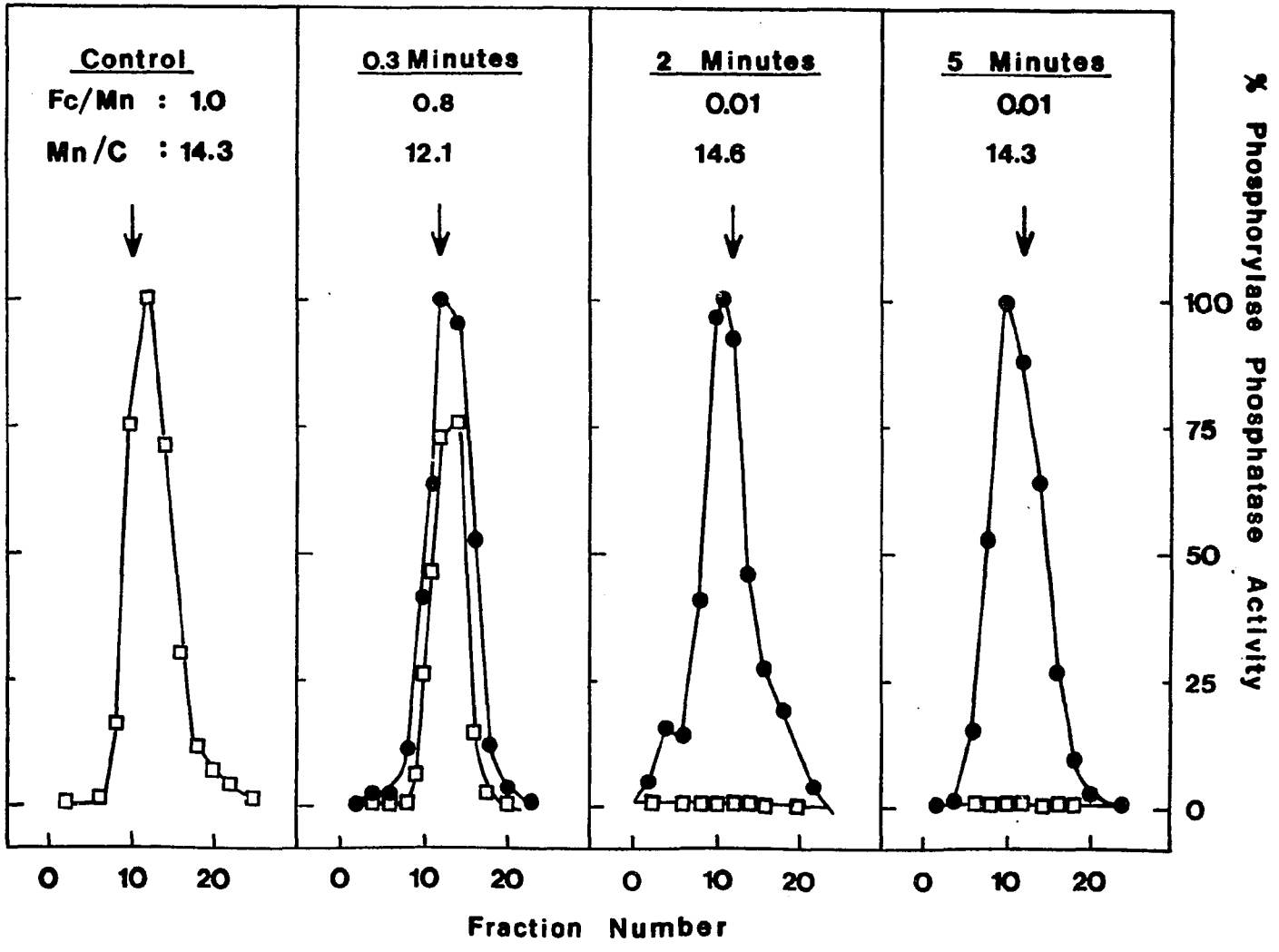
Preparation of Low Molecular Weight Phosphatases

Effect of Trypsin on Phosphatase I - Incubation of cerebellar phosphatase I with TPCK-treated trypsin resulted in a progressive reduction in molecular weight. When examined by sucrose gradient ultracentrifugation (Fig. 15), the sedimentation coefficient ($s_{20,w}$) of the peak activity decreased from 4.0 to 3.0 following the treatment with trypsin. The corresponding apparent molecular weight decreased from 54,000 to 35,000. The change in the molecular weight was concurrent with a change in the catalytic properties. The untreated phosphatase displayed, under these conditions, equivalent activity towards phosphorylase a whether measured in the presence of Mn^{2+}, or following preactivation with F_a + Mg·ATP (F_c/Mn = 1.0). The activity measured in the presence of Mn^{2+} was 14-fold greater than the spontaneous or control activity (Mn/C = 14.3). The lower molecular weight form that resulted from trypsin treatment was unresponsive to activation with F_a + Mg·ATP (F_c/Mn = 0.01), but a 14-fold activation by Mn^{2+} was still observed. However, the total activity measured in the presence of Mn^{2+} decreased from 0.6 units/mL at time = 0 min to 0.02 units/mL at time = 5 min.

These results indicated that catalytically active fragments could be generated from phosphatase I by limited proteolysis. Catalytic activity seemed to be limited to a M_r 35,000 fragment: activity was not observed in fractions corresponding to a lower apparent molecular weight. The decrease of total activity in the M_r 35,000 fragment with increasing time of incubation with trypsin further supported this interpretation. These results suggested that phosphatase I might contain two or more functional domains or subunits. At least one would be catalytic and, apparently, could be activated by Mn^{2+}. Another domain or subunit would confer sensitivity to F_a + Mg·ATP.

Because a detailed investigation of the subunit structure and mechanism of

Figure 15. Sucrose Gradient Ultracentrifugation of Trypsin-Treated Phosphatase I. Cerebellar phosphatase I (12 ug) was incubated at 30°C in a volume of 50 uL that contained 10 mM Tris·HCl, pH 7.4, 1 mM DTT, 10% (v/v) glycerol, and 1.5 ug of TPCK-trypsin. In the control experiment, the phosphatase was incubated for 2 min in the absence of added trypsin. At the indicated times, 150 uL of the same buffer that contained 12.5 ug of soy trypsin inhibitor was added. After the further addition of 2 uL (10 nCi) of (^{14}C)ovalbumin, the mixture was applied to a 5 - 20% (w/v) linear sucrose gradient, and was centrifuged for 18 hours at 35,000 rpm. After fractionation, 5 uL aliquots were tested for activity towards (^{32}P)phosphorylase a under control conditions, following preactivation with F_a + Mg·ATP (□—□), or in the presence of 0.5 mM $MnCl_2$ (●—●). Details of the assay and ultracentrifugation procedures are described under Experimental Procedures. Fc/Mn is the ratio of activity of the peak fractions measured following activation with F_a + Mg·ATP to that measured in the presence of $MnCl_2$; Mn/C is the ratio of the activity measured in the presence of $MnCl_2$ to that measured under control conditions. The arrows indicate the sedimentation peak of the (^{14}C)ovalbumin. Phosphorylase phosphatase activity is expressed as a percentage of the maximum that was measured for a given sample.

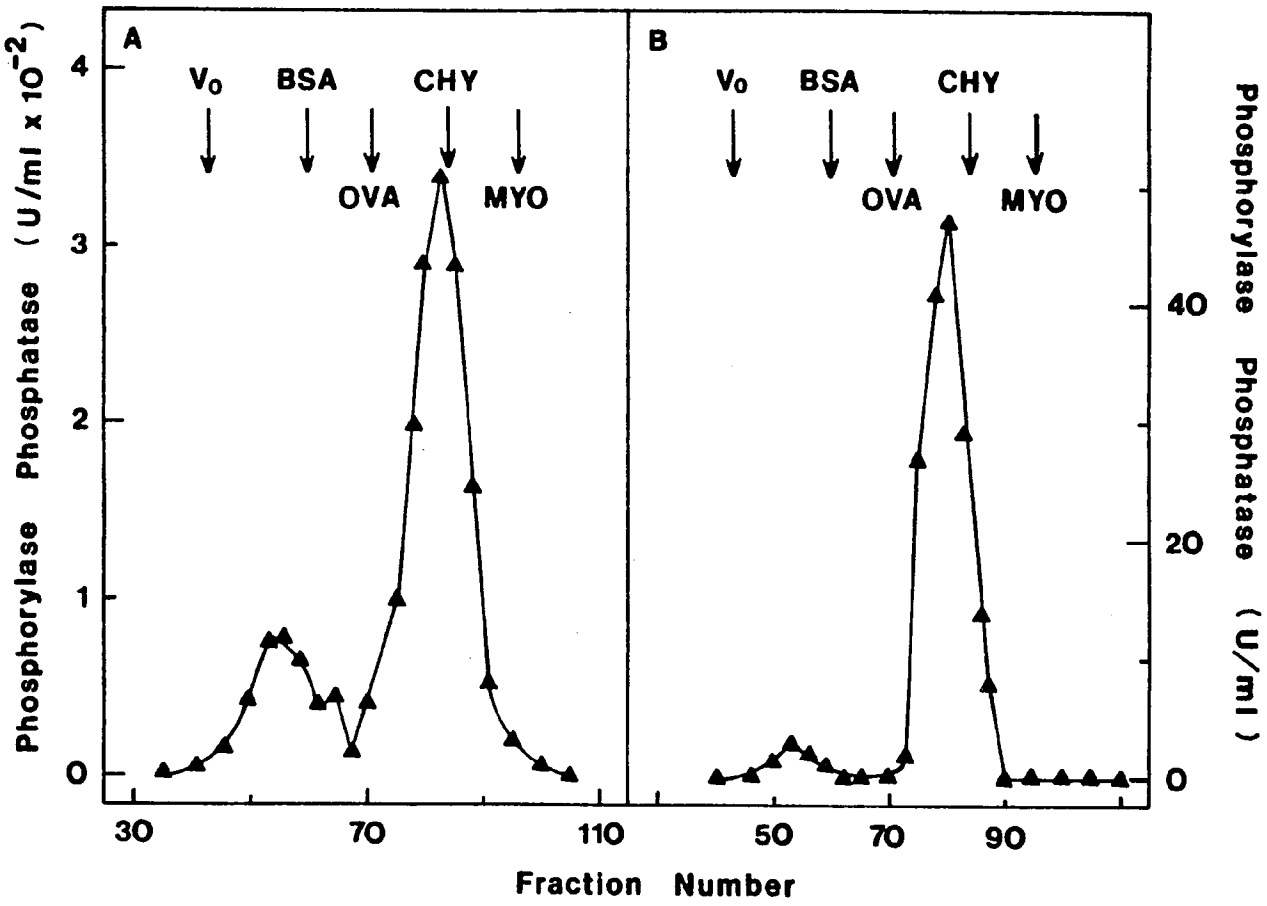

Control
Fc/Mn : 1.0
Mn/C : 14.3
0.3 Minutes
0.8
12.1
2 Minutes
0.01
14.6
5 Minutes
0.01
14.3
100
75
50
25
0
% Phosphorylase Phosphatase Activity
0
10
20
Fraction Number

activation of bovine heart phosphatase I was carried out in this laboratory as part of a separate study (D. Tabarini, Ph. D. Dissertation, Mt. Sinai Sch. Med., 1984), the subunit structure of cerebellar phosphatase I was not investigated further.

Effect of Ethanol Precipitation on Phosphatases II and III - Phosphatases II and III were both converted to lower molecular weight, catalytically active forms following precipitation with ethanol. Gel filtration (Fig. 16) and sucrose gradient ultracentrifugation (not illustrated) of the ethanol-treated phosphatases resulted in both cases in the appearance of a single major peak of activity. The Stokes radii were estimated to be 2.5 and 2.6 nm for phosphatases II and III, respectively, whereas a common sedimentation coefficient ($s_{20,w}$ = 3.3) was determined for both. Using these parameters, and an assumed partial specific volume of 0.725 cm^3/g, the calculated molecular weights of the ethanol-treated phosphatases were 34,000 (phosphatase II) and 35,400 (phosphatase III). The conversion was usually complete, although a secondary activity peak (apparent M_r 85,000) was occasionally observed. The appearance of this secondary peak varied with different phosphatase preparations, and possibly resulted from the cross-contamination with phosphatase I; the molecular weight of phosphatase I was apparently not altered by ethanol precipitation (not shown).

Precipitation with ethanol was concomitant with a high recovery of activity. In a typical experiment, the ethanol-treated phosphatases II and III displayed 90% and 107% of the pre-treatment activity measured towards phosphorylase a in the presence of 0.5 mM $MnCl_2$. These results demonstrated that phosphatases II and III could both be quantitatively converted to catalytically active, low molecular weight species following precipitation with ethanol. The results were also consistent with the possibility that the catalytic activity of phosphatases II and III was conferred by a subunit with an approximate molecular weight of 35,000. Catalytic properties of the ethanol-treated phosphatases II and III are detailed below.

Figure 16. Chromatography of Ethanol-Precipitated Phosphatases II and III on Sephadex G-100. A. Cerebellar phosphatase II (800 ug) and B. phosphatase III (200 ug) contained in 200 uL of Buffer A + 60% glycerol and 0.1 mM EDTA were mixed with 2 vol of 100% ethanol (4°C), and were immediately centrifuged at 10,000 x g for 10 min. The ethanol was decanted, and the precipitated protein was resuspended in Buffer B + 10 mM $MgCl_2$ and 2 mM EGTA. Following an additional centrifugation to clarify the extracted protein solution, the mixture was applied to a Sephadex G-100 column (2 x 100 cm) equilibrated with the same buffer. The column was eluted at a constant flow rate of 5.4 $mL \cdot hr^{-1} \cdot cm^{-2}$, and 2.1 mL fractions were collected. Arrows mark the elution volumes (left to right) of the following standards: blue dextrin (V_o), BSA, ovalbumin, chymotrypsinogen, and myoglobin. Enzymic activity was measured towards (^{32}P)phosphorylase a in the presence of 0.5 mM $MnCl_2$ as described under Experimental Procedures.

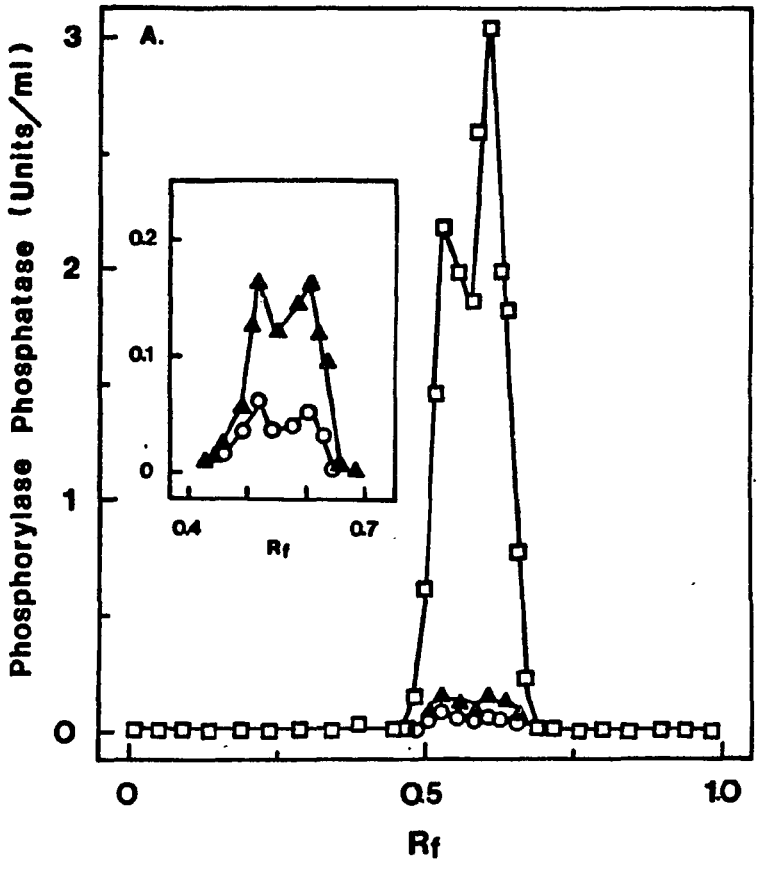

A
B
V_0
BSA
CHY
OVA
MYO
Phosphorylase Phosphatase (U/ml x 10^{-2})
Phosphorylase Phosphatase (U/ml)
0
1
2
3
4
0
20
40
30
70
110
50
70
90
Fraction Number

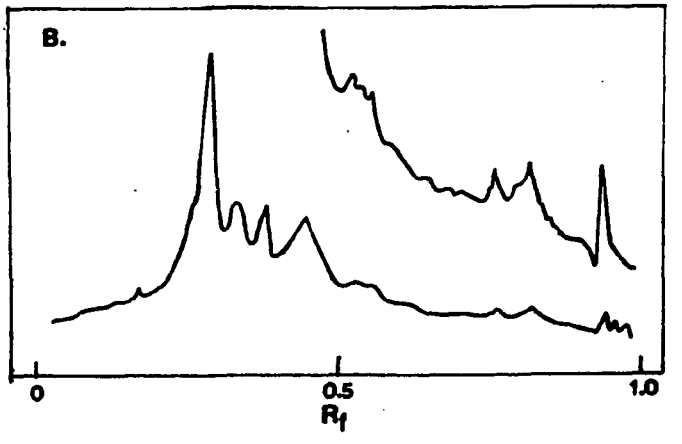
Non-Denaturing Polyacrylamide Gel Electrophoresis

Phosphatase I - Polyacrylamide gel electrophoresis of purified cerebellar phosphatase I resolved the enzymic activity into two closely spaced peaks with R_f values of 0.52 and 0.60 (Fig. 17a). Both peaks were dependent upon F_a + Mg·ATP for activity, could be partially activated by Mn^{2+} alone, and both were sensitive to inhibitor-1 and inhibitor-2. Coomassie brilliant blue staining of the non-denaturing gel showed several protein bands (Fig. 17b) indicating that the phosphatase I preparation was not homogeneous with respect to protein. However, the activity peaks of phosphatase I were distinctly separated from those of phosphatases II and III, as shown when electrophoresis of each of the phosphatases was carried out using adjacent lanes of the same gel (not illustrated).

The fractions that included the two activity peaks (Fig. 17, R_f 0.4 - 0.7) were re-electrophoresed in the presence of SDS. Several protein bands were apparent (not shown). However, phosphatase I activity correlated most closely with bands in the molecular weight range of 60,000 - 62,000, and 32,000 - 38,000. The M_{SDS} 60,000 - 62,000 bands were evenly represented in both activity peaks, whereas peak 1 contained relatively more of the M_{SDS} 38,000 protein bands, and peak 2 contained more of the M_{SDS} 32,000 bands.

Figure 17. Polyacrylamide Gel Electrophoresis of Phosphatase I. A. Cerebellar phosphatase I (75 ug) was electrophoresed in 7.5% acrylamide under non-denaturing conditions. At the completion of the run, the gel was sliced into 1 mm sections which were eluted for 18 hours at $4^{o}C$ into 100 uL of a buffer containing 20 mM Tris·HCl, pH 7.4, 1 mM DTT, and 10% glycerol. Aliquots from indicated fractions were tested for enzymic activity towards (^{32}P)phosphorylase a following preactivation with F_a + Mg·ATP (□—□). The inset shows a replot of the activity measured under control conditions (O—O) and in the presence of 0.5 mM $MnCl_2$ (▲—▲) using an expanded activity scale. B. An adjacent lane (25 ug phosphatase I) was stained for protein using Coomassie brilliant blue. A densitometric scan is shown at two levels of sensitivity. Details are described under Experimental Procedures.


A.
Phosphorylase Phosphatase (Units/ml)
3
2
1
0
0.2
0.1
0
0.4
0.7
Rf
0
0.5
1.0
Rf


B.
0
0.5
1.0
Rf

Phosphatases II and III - Polyacrylamide gel electrophoresis of cerebellar phosphatase II (Fig. 18) resulted in the separation of four active peaks (R_f = 0.32, 0.45, 0.52, and 0.60). Gel electrophoresis of phosphatase III resulted in the separation of a single major active peak (R_f = 0.60) and several minor peaks with lower mobility (Fig. 19). In contrast, ethanol precipitation of each phosphatase prior to electrophoresis resulted in the migration of single activity peaks (Fig. 20). Each of the activity peaks, whether derived from phosphatase II or III, dephosphorylated G-substrate, phosphorylase a (Figs. 18 & 19), and p-NPP (not illustrated). Coomassie brilliant blue staining of corresponding gels indicated that neither phosphatase preparation was homogeneous with respect to protein (not illustrated; see Fig. 25).

The activity of each of the phosphatase II peaks measured towards G-substrate and phosphorylase a was Mn^{2+}-dependent (Fig. 18). The magnitude of these two activities in each of the peaks was approximately equivalent. In contrast, the phosphorylase a phosphatase activity of the major and the minor phosphatase III peaks (Fig. 19) was stimulated less than two-fold by Mn^{2+}, whereas the activity measured towards G-substrate was totally dependent upon added Mn^{2+}. In addition, the activity of the phosphatase III-derived peaks measured towards phosphorylase a was more than five-fold higher than that measured towards G-substrate. These observations indicated that the relative substrate specificity and divalent cation dependency characteristic of phosphatases II and III were displayed both before and after non-denaturing gel electrophoresis.

Cardiac Phosphatases II and III - The electrophoretic activity profiles of cardiac phosphatases II and III (termed phosphatases II-H and III-H) were qualitatively similar to those prepared using the corresponding cerebellar phosphatases. Using (^{32}P)phosphorylase a as substrate, three main peaks of activity were separated

from phosphatase II-H (Fig. 21a, R_f 0.33, 0.50, and 0.66), and two from phosphatase III-H (Fig. 21b, R_f 0.53 and 0.69). Besides phosphorylase a, each of these peaks also displayed activity towards p-NPP (not shown).

The electrophoretic mobility of the cardiac and cerebellar phosphatases displayed a close correspondence. When each of the phosphatases was electrophoresed using adjacent lanes of the same gel, the three activity peaks of phosphatase II-H co-migrated with peaks 1, 2, and 4 of cerebellar phosphatase II, and the major peaks of the two phosphatase III preparations also co-migrated (not illustrated). The relative migration (R_f) of the activity peaks varied when comparing different electrophoretic runs, but the multiplicity and relative activity of the different peaks was reproducible.

Some differences between the cardiac and cerebellar phosphatases were observed when comparing the distribution of the recovered activity in each of the corresponding peaks. For example, the R_f 0.52 activity peak of cerebellar phosphatase II was quantitatively the major peak of activity (Fig. 20a). In contrast, the R_f 0.50 peak of cardiac phosphatase II-H represented only a minor amount of the total recovered activity (Fig. 21a). Similar differences were observed when comparing cerebellar and cardiac phosphatase III (Figs. 20b and 21b).

Coomassie brilliant blue staining of the two cardiac phosphatases (Fig. 22) demonstrated that each of the peaks of activity co-migrated with a band of protein. Typically, these bands were diffuse, and in the case of the fastest migrating peaks, several closely spaced protein bands were apparent. However, the most intensely stained protein bands of phosphatase II-H (R_f 0.26 and 0.43) did not co-migrate with any apparent enzymic activity. Nor did the slowest and fastest migrating bands (R_f 0.43 and 0.81, respectively) of phosphatase III-H. Each of these protein bands was analyzed by SDS-polyacrylamide gel electrophoresis as described below.

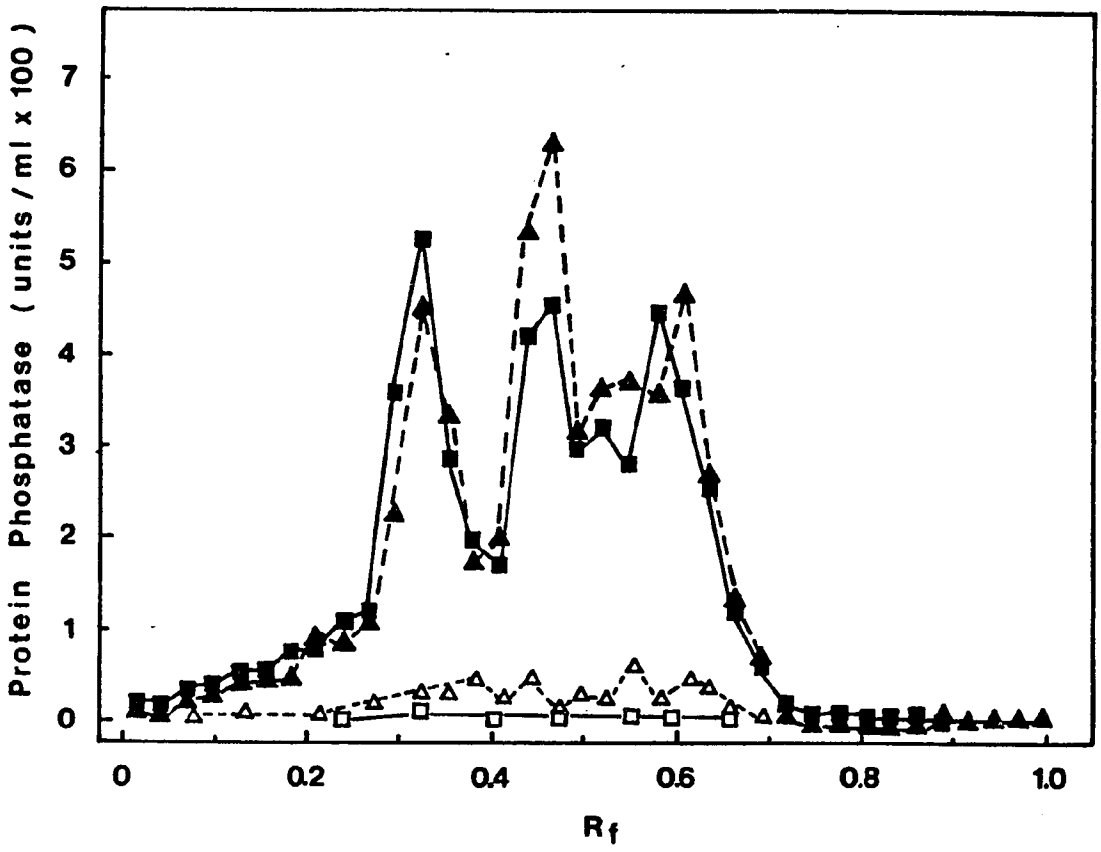


Figure 18. Polyacrylamide Gel Electrophoresis of Phosphatase II. Cerebellar phosphatase II (100 ug) purified through gel filtration (Step 4, Table 5) was electrophoresed in 7% polyacrylamide under non-denaturing conditions. Following the run the gels were sliced into 1 mm sections, and were then eluted at 4°C for 72 hours into a buffer containing 20 mM Tris·HCl, pH 7.4, 0.2 mg/mL BSA, and 10% (v/v) glycerol. Enzymic activity was measured in a 30 min assay towards (^{32}P)G-substrate (■—■ , + 2 mM $MnCl_2$; □—□ , + 2 mM EDTA) and (^{32}P)phosphorylase a (▲—▲ , + 0.5 mM $MnCl_2$; △—△ , + 2 mM EDTA). Details are described under Experimental Procedures.

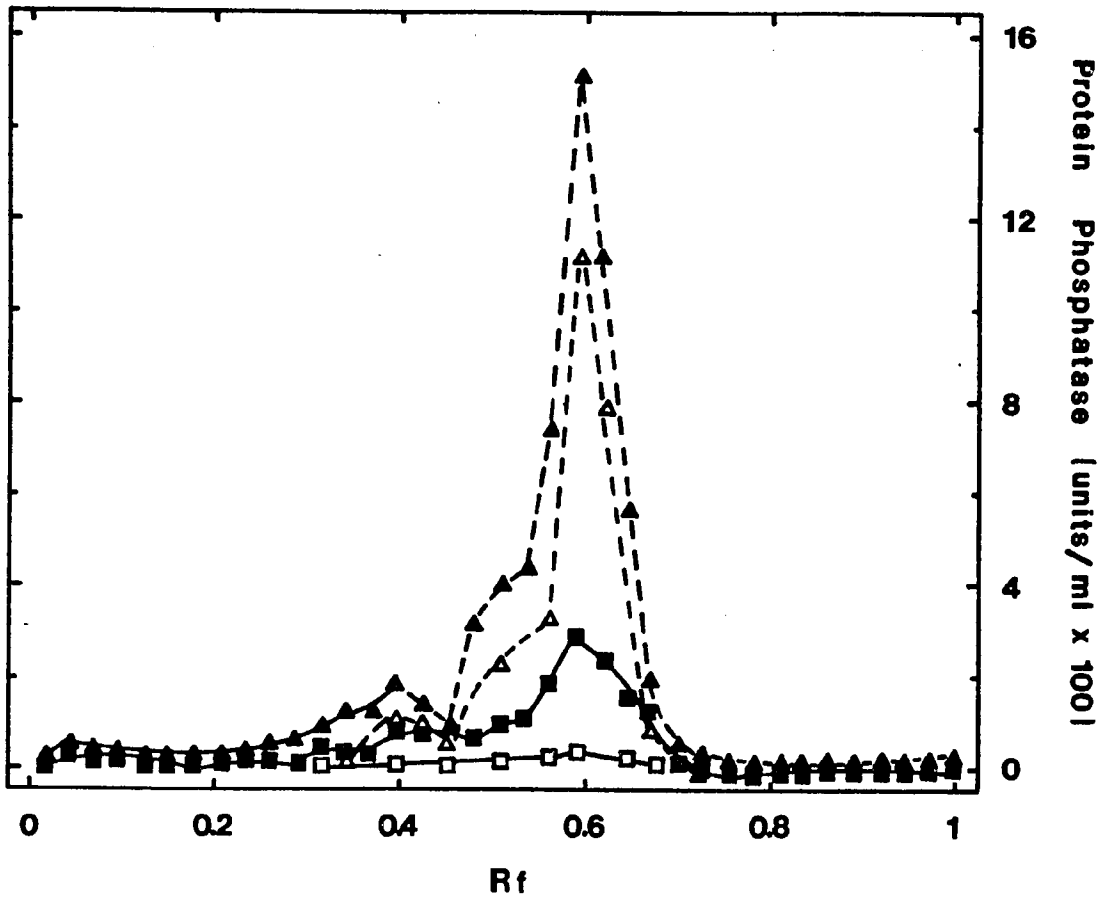


Figure 19. Polyacrylamide Gel Electrophoresis of Phosphatase III. Cerebellar phosphatase III (45 ug) purified through gel filtration (Step 4, Table 5) was electrophoresed in 7% polyacrylamide under non-denaturing conditions. Following the run the gels were sliced into 1 mm sections, and were then eluted at 4°C for 72 hours into a buffer containing 20 mM Tris·HCl, pH 7.4, 0.2 mg/mL BSA, and 10% (v/v) glycerol. Enzymic activity was measured in a 30 min assay towards (^{32}P)G-substrate (■—■ , + 2 mM $MnCl_2$, □—□ , + 2 mM EDTA) and (^{32}P)phosphorylase a (▲—▲ , + 0.5 mM $MnCl_2$; △—△ , + 2 mM EDTA). Details are described under Experimental Procedures.

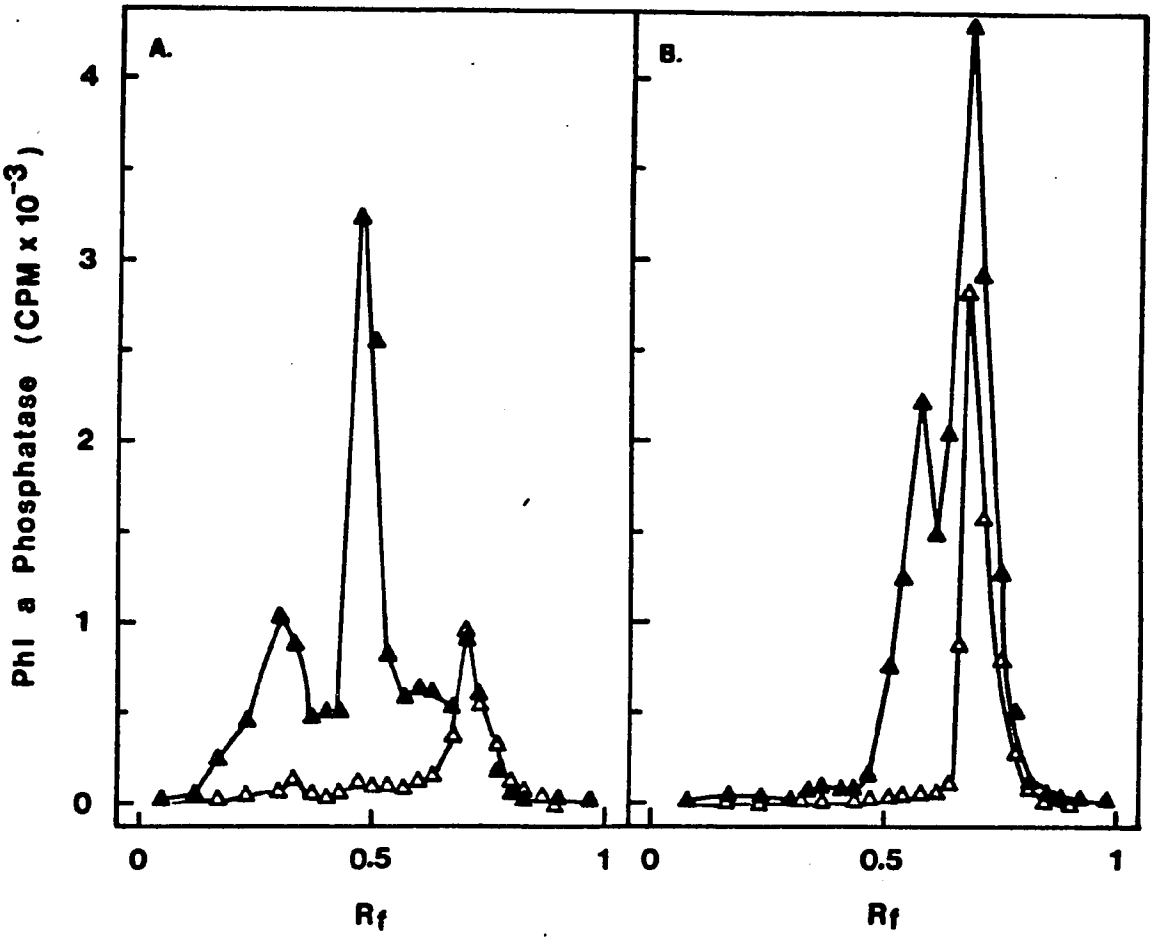
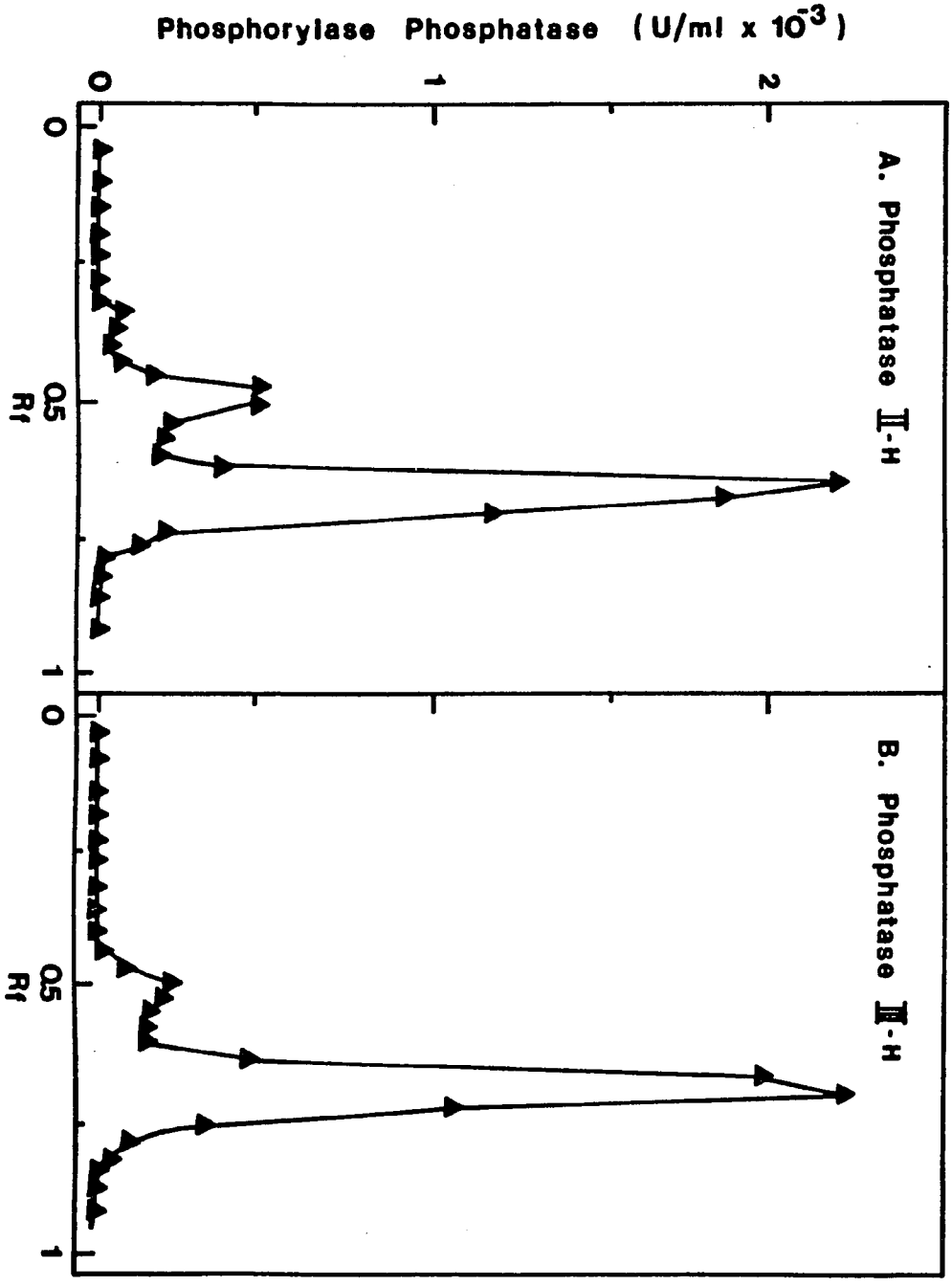


Figure 20. Polyacrylamide Gel Electrophoresis of Ethanol-Precipitated Cerebellar Phosphatases II and III. 100 uL of iced-cold 100% ethanol was added to A. phosphatase II (200 ug) and B. phosphatase III (50 ug) that were contained in 50 uL of Buffer A + 60% glycerol + 0.1 mM EDTA. The mixture was immediately centrifuged at 10,000 x g for 1.5 min, the ethanol decanted, and the precipitated protein was resuspended in 50 uL of a buffer that contained 20 mM Tris·HCl, pH 7.4, 10% glycerol, and 0.1 mM EDTA. In the control experiment, the same amounts of phosphatases II and III were processed in an identical manner, except for the addition of ethanol. Both the ethanol-treated (△—△) and untreated (▲—▲) phosphatases were then electrophoresed in 7% acrylamide under non-denaturing conditions using adjacent lanes of the same gel. Following the run, the gel was cut into 1 mm sections, and the slices were eluted for 24 hours as described in the legend to Fig. 17. Enzymic activity was measured towards (^{32}P)phosphorylase a in the presence of 0.5 mM $MnCl_2$. Details are described under Experimental Procedures.

Figure 21. Polyacrylamide Gel Electrophoresis of Cardiac Phosphatases II-H and III-H. Purified bovine heart phosphatase II (12 ug) and phosphatase III (20 ug) were electrophoresed in 7.5% acrylamide under non-denaturing conditions. Following the run, the gels were sliced into 1 mm sections, and were eluted for 24 hours at $4^{o}C$ into 100 uL of a buffer that contained 20 mM Tris·HCl, pH 7.5, 1 mM DTT, and 10% glycerol. Aliquots from the indicated fractions were tested for enzymic activity towards (^{32}P)phosphorylase a in the presence of 0.5 mM $MnCl_2$. Details are described under Experimental Procedures.


Phosphorylase Phosphatase (U/ml x 10^{-3})
0
1
2
A. Phosphatase II-H
B. Phosphatase III-H
0
0.5
1
Rf

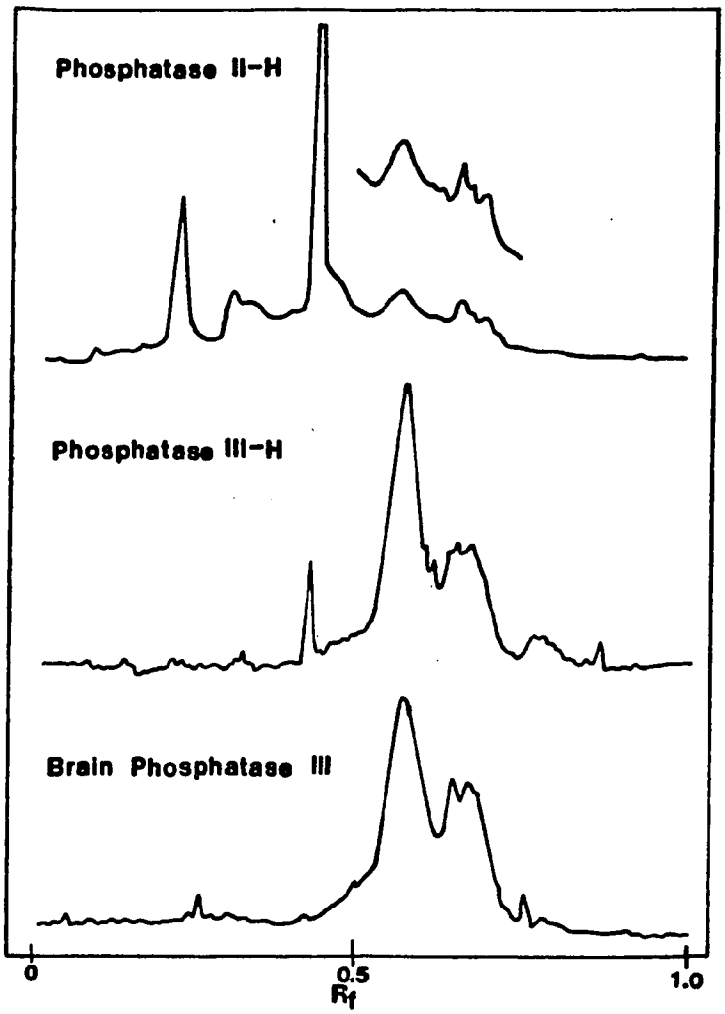


Figure 22. Polyacrylamide Gel Electrophoresis of Cardiac Phosphatases II-H and III-H, and Brain Phosphatase III: Protein Stain. Purified bovine heart phosphatases II-H (12 ug) and III-H (20 ug), and whole brain phosphatase III (22 ug) were electrophoresed in 7.5% acrylamide under non-denaturing conditions as described under Experimental Procedures. Following the run, the gel was stained for protein using Coomassie brilliant blue. Densitometric scans (at two levels of sensitivity for phosphatase II-H) are shown.

SDS-Polyacrylamide Gel Electrophoresis

Cardiac Phosphatases II-H and III-H - SDS-polyacrylamide gel electrophoresis resolved four major protein bands from phosphatase II-H, and two bands from phosphatase III-H (Fig. 23). The two protein bands of phosphatase III-H, termed alpha (A) and beta (B) in order of increasing molecular weight, co-migrated exactly with two of the four phosphatase II-H protein bands (Fig. 23c). The molecular weights calculated for these bands ($M_{SDS} \pm$ SEM, n = 6 separate gels) were 38,110 $\pm$ 450 (alpha) and 62,750 $\pm$ 350 (beta). The phosphatase II-H band migrating between the A and B protein bands was termed gamma (G), and had a molecular weight of 53,850 $\pm$ 490 (n = 5). The fourth protein band of phosphatase II-H had an estimated M_{SDS} = 92,500 (n = 2). As described below, each of the active fractions of phosphatases II-H and III-H that were separated by non-denaturing gel electrophoresis contained one or more of the alpha, beta, and gamma protein bands.

The molecular stoichiometry of phosphatase II-H (B : G : A) was estimated to be 1 : 1.06 $\pm$ 0.10 : 2.31 $\pm$ 0.12 ($\pm$ SEM, n = 7), and that of phosphatase III-H (B : A) was 1 : 1.11 $\pm$ 0.09 (n = 8). Assuming that these three proteins entirely accounted for the subunit composition of these phosphatases, the probable subunit stoichiometries suggested by these data were (B : G : A_2) and (B : A) for phosphatases II-H and III-H, respectively. The predicted molecular weight of the holoenzymic form of phosphatase II-H would then be 192,820, and that of phosphatase III-H would be 100,860. The latter value was in close agreement with the M_r of phosphatase III-H (102,400) that was calculated from the Stokes radius and $s_{20,w}$ (Table 9). The M_r of phosphatase II-H was calculated to be 153,600, a value more consistent with a subunit stoichiometry of (B : G : A) (predicted M_{SDS} = 154,700). However, minor activity peaks of higher apparent molecular weight were resolved following gel

filtration and sedimentation of phosphatase II-H (see Determination of Molecular Weight), suggesting that the major peak of activity may have represented a partially dissociated form of phosphatase II-H.

Brain Phosphatases I, II, and III - SDS-polyacrylamide gel electrophoresis of phosphatase III prepared from whole brain resolved five major protein bands (Fig. 24). Two of these bands co-migrated with the alpha and beta proteins of phosphatases II-H and III-H. The molecular stoichiometry of these two proteins (B : A) was calculated to be 1 : 1.22 $\pm$ 0.25 (n = 3), suggesting a subunit stoichiometry of (B : A). The predicted molecular weight (M_{SDS} = 100,360) of this complex was in close agreement with the M_r = 102,000 that was calculated from Stokes radius and $s_{20,w}$ data (not shown). The multiple bands of protein resolved by SDS-gel electrophoresis of cerebellar phosphatases I, II, and III (Fig. 25), and from whole brain phosphatase II (not illustrated) precluded an analysis of the subunit composition of these enzymes.

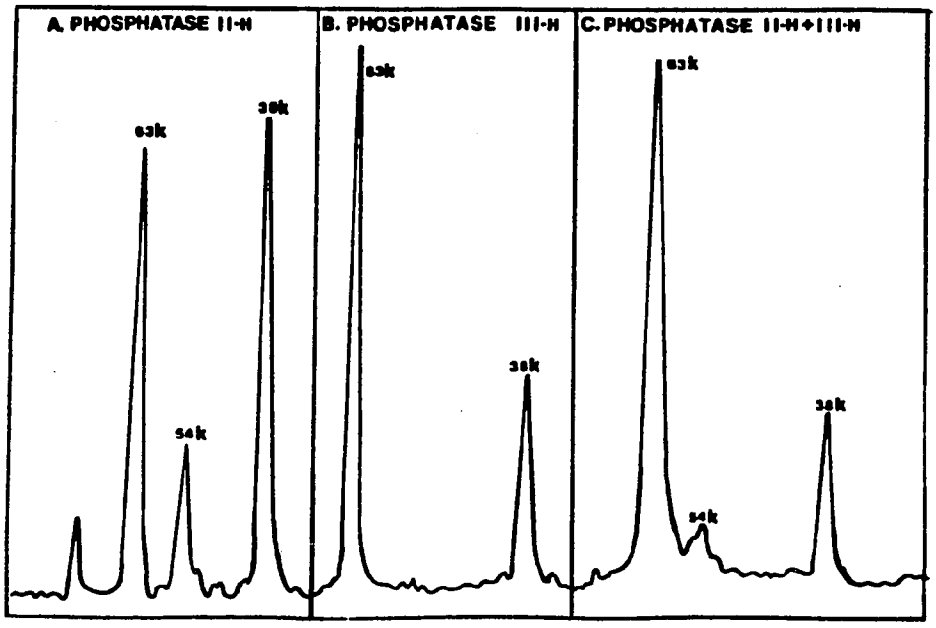


Figure 23. SDS-Polyacrylamide Gel Electrophoresis of Cardiac Phosphatases II-H and III-H. A. Purified bovine heart phosphatase II-H (2 ug), B. phosphatase III-H (1.2 ug), and C. phosphatase II-H (2 ug) + phosphatase III-H (1.2 ug) were electrophoresed in 10% acrylamide in the presence of SDS. Following the run, the gels were stained with Coomassie brilliant blue, and were scanned densitometrically. Details are described under Experimental Procedures.

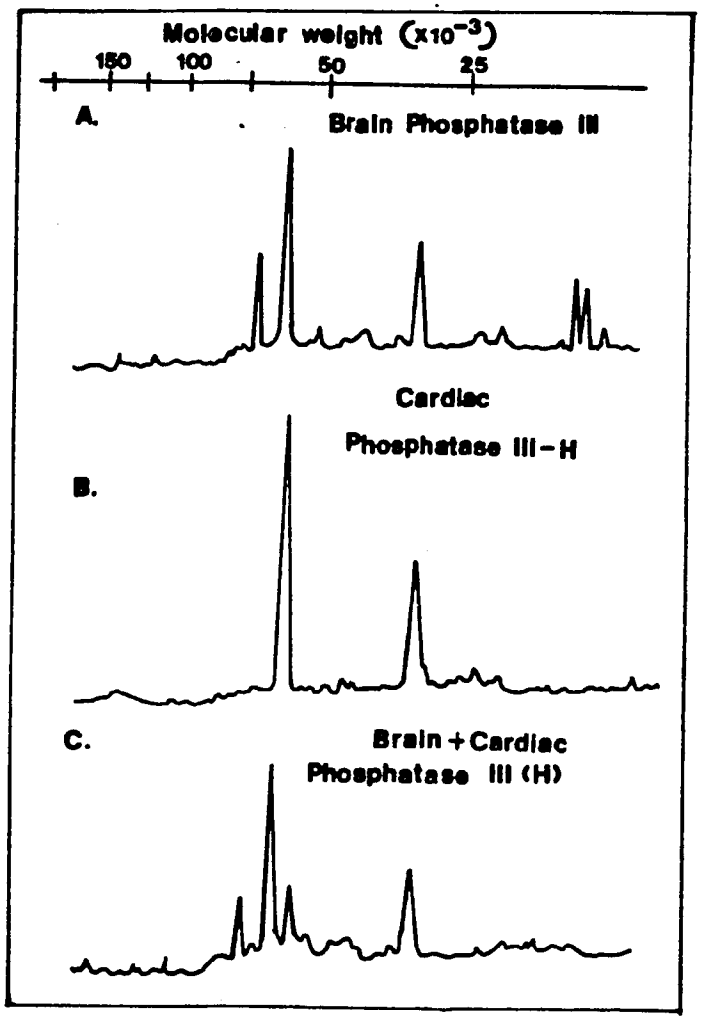


Figure 24. SDS-Polyacrylamide Gel Electrophoresis of Cardiac Phosphatase III-H and Brain Phosphatase III. A. Whole brain phosphatase III (2 ug), B. purified bovine heart phosphatase III-H (2.4 ug), and C. phosphatase III (2ug) + phosphatase III-H (2 ug) were electrophoresed in 10% acrylamide in the presence of SDS. Following the run, the gels were stained with Coomassie brilliant blue, and were scanned densitometrically. Details are described under Experimental Procedures.

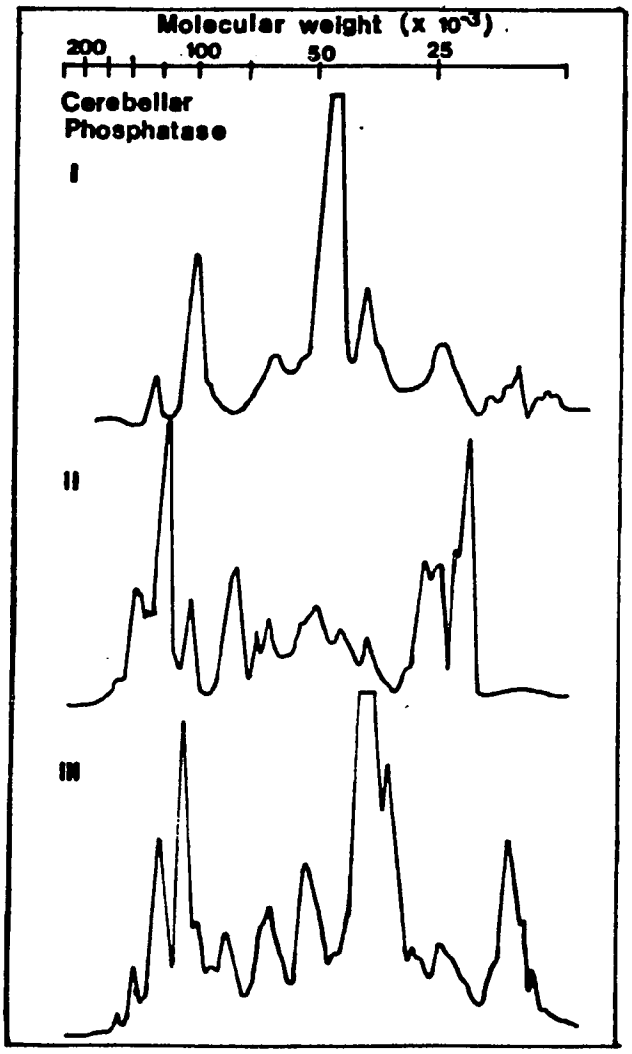


Figure 25. SDS-Polyacrylamide Gel Electrophoresis of Cerebellar Phosphatases I, II, and III. Cerebellar phosphatase I (20 ug), II (27 ug), and III (18 ug) were electrophoresed in 10% acrylamide in the presence of SDS. Details are described under Experimental Procedures. Densitometric scans of the Coomassie-stained gels are shown.

Cardiac Phosphatases II and III: Re-Electrophoresis of the Non-Denaturing Gel Fractions - Non-denaturing gel electrophoresis resolved three peaks of activity from phosphatases II-H, and two activity peaks from phosphatase III-H (Fig. 21). Further analysis of these peak fractions by SDS-polyacrylamide gel electrophoresis indicated that each contained proteins with molecular weights of 38K and 63K (Figs. 26 & 27). The peak fractions derived from phosphatase II-H also contained a 54K protein band (Fig. 26). (A trace amount of this protein was also detected in the phosphatase III-H fractions.) Together, these three proteins represented the most intensely stained proteins recovered from each of the peak fractions.

The magnitude of the enzymic activity seemed to be correlated with the intensity of the 38K protein band. The fastest migrating activity peak of both phosphatases displayed the highest enzymic activity (Fig. 21), and contained the greatest amount of the 38K protein (Figs. 26 & 27). (Note that each of the gels were scanned using a constant level of sensitivity so that the intensity of the component protein bands could be directly compared.) Furthermore, the intensity of the 38K protein band decreased in the immediately adjacent lanes in relation to the decrease in enzymic activity (not illustrated). The 38K protein band was not recovered from any of the fractions that did not display enzymic activity, with the exception of the slowest migrating band (R_f 0.26) of phosphatase II-H. This fraction contained almost exclusively an intensely stained 38K protein band (Fig. 28).

A second apparently inactive protein band was separated from phosphatase II-H by gel electrophoresis (Fig. 22). This protein (R_f 0.43) co-migrated with an apparently inactive phosphatase III-H protein band. SDS-gel electrophoresis of these fractions indicated that both primarily contained a 63K protein band (Fig.

28). Other apparently inactive fractions also contained a 63K protein band; typically, these fractions migrated between the different activity peaks (not illustrated). These inactive fractions also contained a 54K protein band (in the case of phosphatase II-H), but were apparently devoid of any 38K protein.

These results suggested that each of the active fractions of phosphatase II-H and phosphatase III-H contained the same alpha (38K), beta (63K), and gamma (54K) proteins as those already described above. This observation would seem to indicate that the multiplicity of peaks following non-denaturing gel electrophoresis is not explainable in terms of a simple dissociation of the alpha, beta, and gamma subunits from the holoenzyme. Rather, the relative proportions of these three proteins varied in each of the peak fractions. Peak 1 of both phosphatases contained the 63K protein in excess of the 38K protein, while the converse was apparent for peaks 3 and 2 of phosphatases II-H and III-H, respectively (Figs. 26 & 27). Peak 2 of phosphatase II-H displayed an intermediate ratio of these two proteins (Fig. 26). The proportion of gamma protein varied as well, with the highest relative concentration apparent in peak 2 of phosphatase II-H. (The variable background intensity of the silver-stained gels precluded an exact quantitation of these ratios.) Thus, the different electrophoretic mobilities of the peak fractions may have been related to the relative proportion of the alpha, beta, and gamma proteins.

Cerebellar Phosphatases II and III: Re-Electrophoresis of the Non-Denaturing Gel Fractions - The activity peaks of cerebellar phosphatases II and III (Fig. 20) were also analyzed by SDS-polyacrylamide gel electrophoresis (Figs. 29 & 30). The large number of protein bands contained in these fractions complicated the analysis, but at least three similarities were apparent when comparing the protein composition of the cerebellar fractions with that already detailed for the cardiac phosphatases. Each of the cerebellar peak fractions contained a 38K and a 63K

protein band, and each of the phosphatase II peak fractions contained, in addition, a 54K protein band (Fig. 29). Re-electrophoseris of the ethanol-precipitated phosphatases demonstrated a relative increase in the intensity of the 38K protein bands, although 63K and 54K protein bands were also apparent (not illustrated).

The magnitude of the recovered enzymic activity seemed to be correlated with the intensity of the 38K protein band in the peak fractions of phosphatase III (Fig. 30). However, this relationship was not as apparent in the phosphatase II fractions, particularly with respect to activity peak 2. Finally, the relative composition of the 38K, 54K, and 63K proteins varied in the different peak fractions. A comparison of the four activity peaks of phosphatase II indicated the following approximate molar ratios (A = 38K, B = 63K, and G = 54K): peak 1, (B : G : $A_{0.5}$); peak 2, (B : G_3 : $A_{0.5}$); peak 3, (B : $G_{0.1}$: A_2); and peak 4, (B : $G_{0.1}$: A). A similar analysis of the peak fractions of phosphatase III indicated molar ratios of (B : A) and (B : A_2) for peaks 1 and 2, respectively. Thus, the multiplicity of activity peaks following non-denaturing gel electrophoresis of both the cardiac and cerebellar phosphatases seemed to be related to differences in the relative proportion of the 38K, 54K, and 63K proteins.

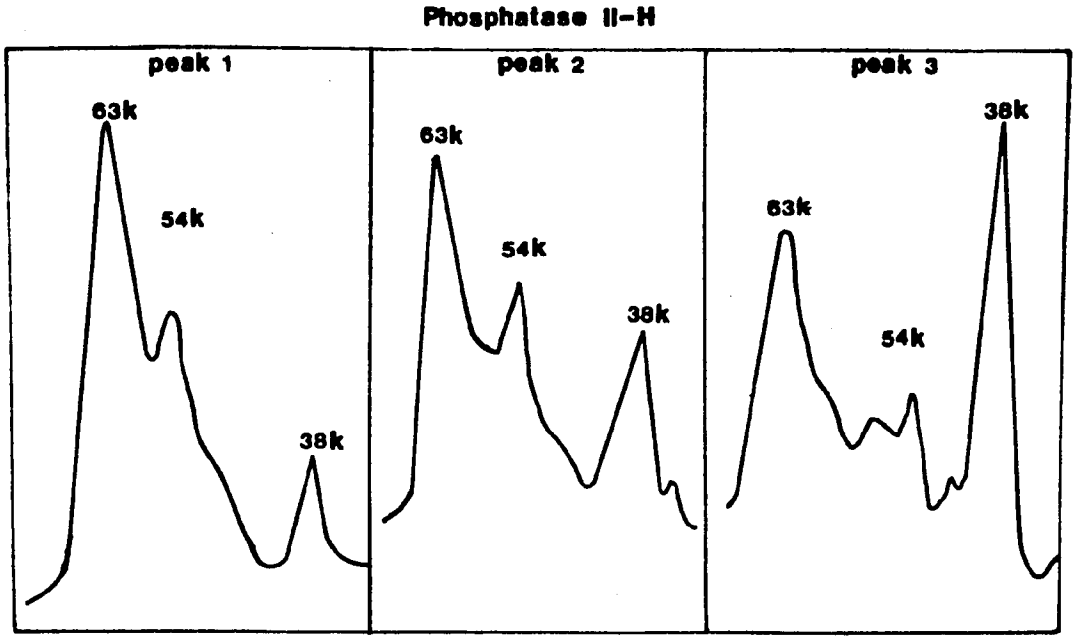


Figure 26. SDS-Polyacrylamide Gel Electrophoresis of the Peak Fractions of Cardiac Phosphatase II-H. Bovine heart phosphatase II-H was electrophoresed under non-denaturing conditions as described in the legend to Fig. 21. The peak fractions 1 (R_f 0.33), 2 (R_f 0.50), and 3 (R_f 0.66) were re-electrophoresed in 10% acrylamide in the presence of SDS as described under Experimental Procedures. A densitometric scan (using a fixed level of sensitivity) of the silver-stained gel is shown.

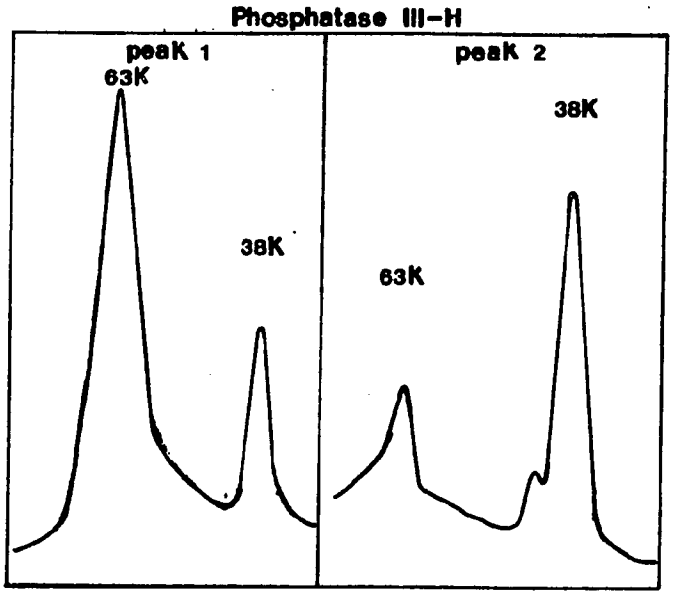


Fig. 27. SDS-Polyacrylamide Gel Electrophoresis of the Peak Fractions of Cardiac Phosphatase III-H. Bovine heart phosphatase III-H was electrophoresed under non-denaturing conditions as described in the legend to Fig. 21. The peak fractions 1 (R_f 0.53) and 2 (R_f 0.69) were re-electrophoresed in 10% acrylamide in the presence of SDS as described under Experimental Procedures. A densitometric scan (using a fixed level of sensitivity) of the silver-stained gel is shown.

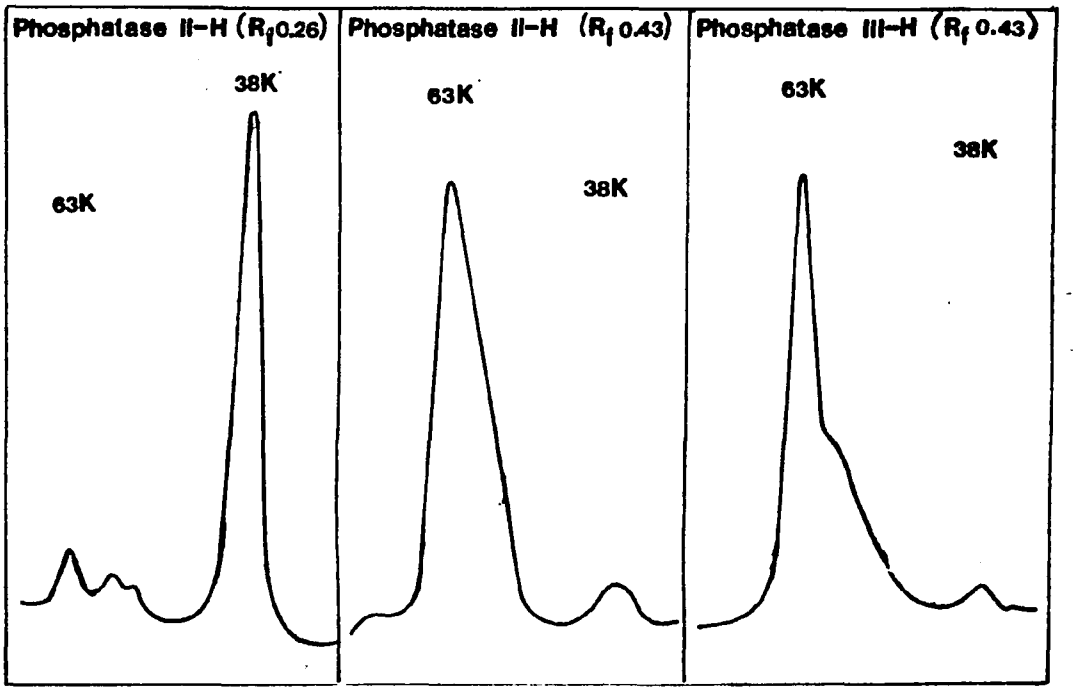


Figure 28. SDS-Polyacrylamide Gel Electrophoresis of the Inactive Protein Bands of Cardiac Phosphatases II-H and III-H. Bovine heart phosphatases II-H and III-H were electrophoresed under non-denaturing conditions as described in the legend to Fig. 21. Two protein bands of phosphatase II (R_f 0.26 and 0.43), and one of the phosphatase III protein bands (R_f 0.43) were not correlated with enzymic activity (Fig. 22). The fractions containing these bands were re-electrophoresed in 10% acrylamide in the presence of SDS as described under Experimental Procedures. A densitometric scan of the silver-stained gel is shown.

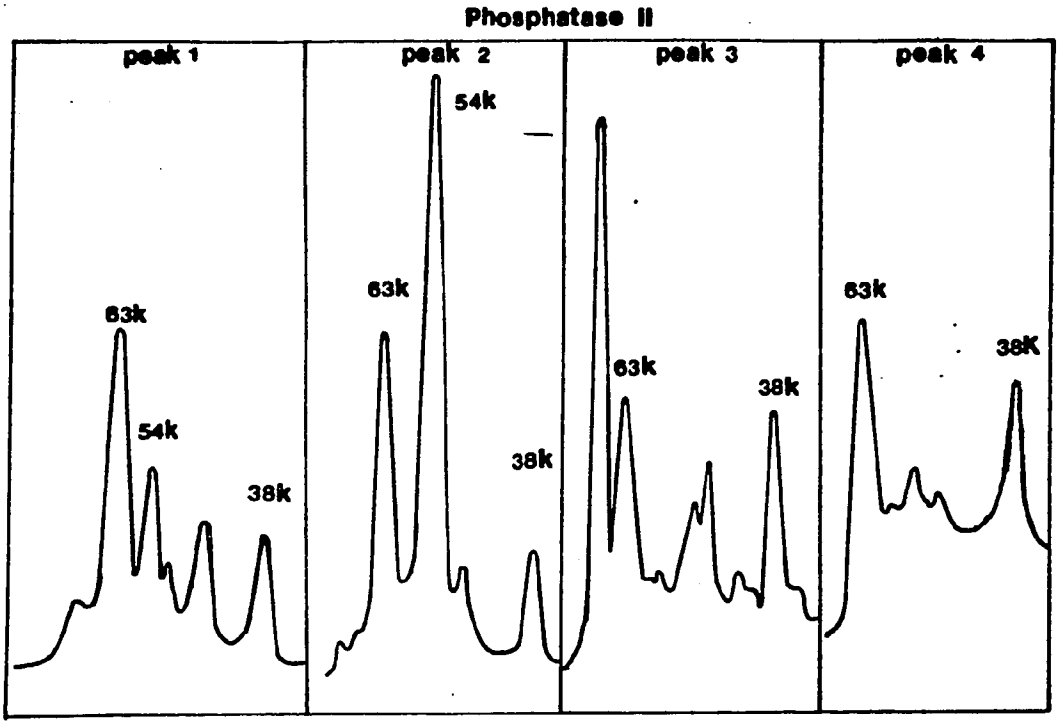


Figure 29. SDS-Polyacrylamide Gel Electrophoresis of the Peak Fractions of Cerebellar Phosphatase II. Cerebellar phosphatase II was electrophoresed under non-denaturing conditions as described in the legend to Fig. 20. Peak fractions 1 (R_f 0.30), 2 (R_f 0.47), 3 (R_f 0.60), and 4 (R_f 0.70) were re-electrophoresed in the presence of SDS (10% acrylamide) as described under Experimental Procedures. A densitometric scan of the Coomassie-stained gel is shown.

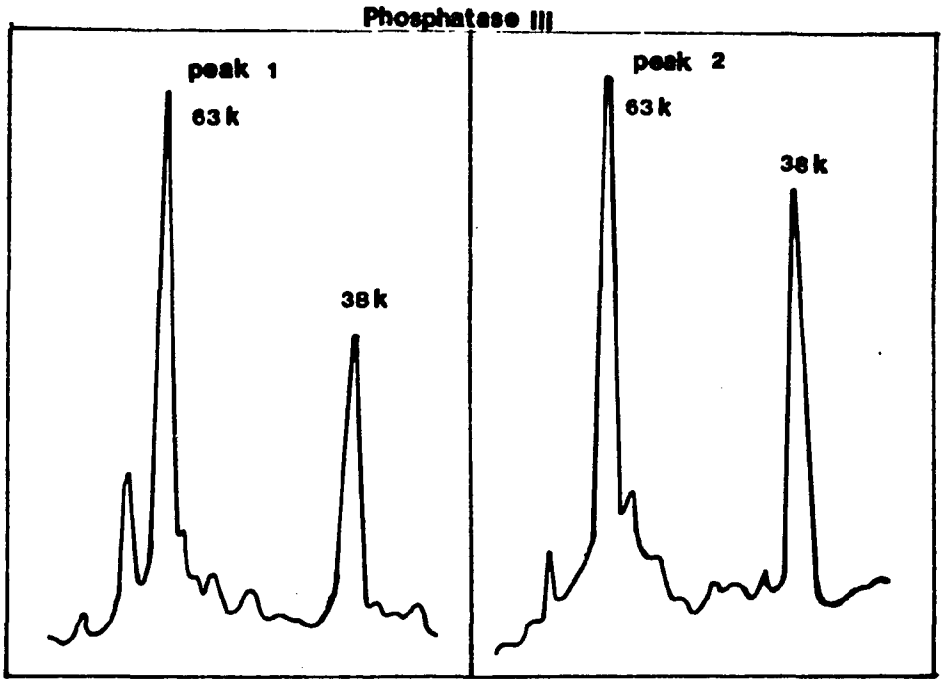


Figure 30. SDS-Polyacrylamide Gel Electrophoresis of the Peak Fractions of Cerebellar Phosphatase III. Cerebellar phosphatase III was electrophoresed under non-denaturing conditions as described in the legend to Fig. 20. Peak fractions 1 (R_f 0.58), and 2 (R_f 0.68) were re-electrophoresed in the presence of SDS (10% acrylamide) as described under Experimental Procedures. A densitometric scan of the Coomassie-stained gel is shown.

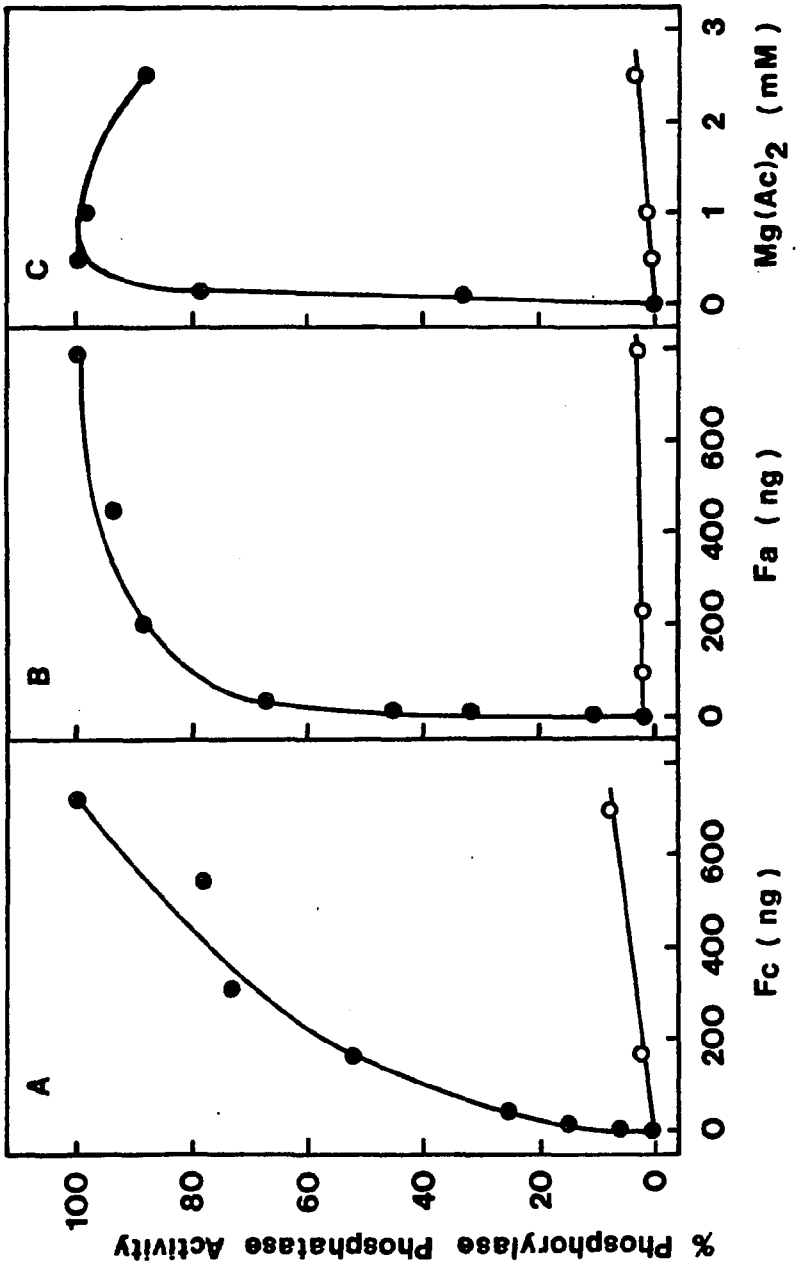
Catalytic Properties of Phosphatases I, II, and III

Effect of Divalent Metal Ions, ATP, and pH

Phosphatase I - Complete activation of cerebellar phosphatase I required the simultaneous presence of ATP, Mg^{2+}, and F_a (Fig. 31). The extent of the reaction was proportional to the amount of added phosphatase I (Fig. 31a), and at a constant concentration of Mg·ATP, activation of phosphatase I was dependent upon F_a concentration (Fig. 31b). The optimal concentration of Mg^{2+} (expressed as the final assay concentration) was 0.5 mM (Fig. 31c) when measured at 0.1 mM ATP; the optimal concentration of ATP, measured at 0.5 mM $Mg(CH_3COO)_2$, was 0.1 mM (not illustrated). The activation process was time-dependent, with maximal activation being reached after 10 minutes incubation (data not shown). The activation was not dependent upon the continued presence during the phosphorylase phosphatase reaction of optimal concentrations of F_a and Mg·ATP since the activation persisted even after a substantial dilution of the activated phosphatase (not shown).

In contrast to the results obtained with F_a + Mg·ATP, activation of phosphatase I by Mn^{2+} was instantaneous, since no difference was observed whether or not the phosphatase was preincubated with 0.6 mM $MnCl_2$ for 10 minutes prior to the assay (data not shown). When phosphorylase a was used as the substrate, the optimal concentration of $MnCl_2$ was found to be in the range of 0.2 - 0.5 mM. Higher concentrations of $MnCl_2$ resulted in a progressive inhibition of activity (Fig. 32). This inhibition by Mn^{2+} might have been a substrate-directed effect since it was not observed when inhibitor-1 (Fig. 32) or G-substrate (not illustrated) was used as substrate. The dephosphorylation of inhibitor-1 (or G-substrate) displayed a hyperbolic dependence on Mn^{2+} with no apparent

Figure 31. Activation of Phosphatase I by Fa, Mg·Acetate, and ATP. A. Enzymic activity was measured using (^{32}P)phosphorylase a as substrate as described under Experimental Procedures in the presence of a fixed amount of F_a (41 ng), and in the presence (●—●) or absence (○—○) of 0.5 mM $Mg(CH_3COO)_2$ + 0.1 mM ATP. The amount of cerebellar phosphatase I added to the reaction mixture was varied as indicated. B. Activity was measured in the presence of a fixed amount of phosphatase I (38 ng) in the presence (●—●) or absence (○—○) of 0.5 mM $Mg(CH_3COO)_2$ + 0.1 mM ATP, and a varied amount of F_a, as indicated. C. Activity was measured using a fixed amount of F_a (41 ng) and phosphatase I (29 ng), and a varied concentration (expressed as the final assay concentration) of $Mg(CH_3COO)_2$ plus (●—●) or minus (○—○) 0.1 mM ATP.


% Phosphorylase Phosphatase Activity
100
80
60
40
20
0
A
B
C
0
200
400
600
Fc (ng)
Fa (ng)
0
1
2
3
Mg(Ac)2 (mM)

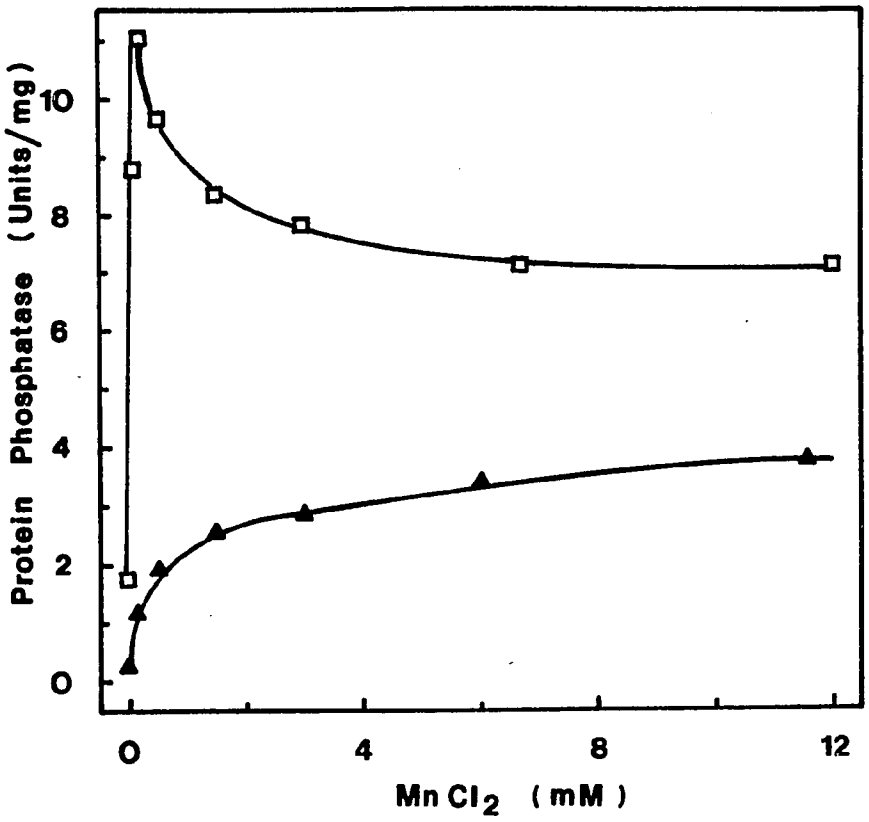


Figure 32. Effect of Varying $MnCl_2$ Concentration on the Activity of Phosphatase I. The activity of cerebellar phosphatase I (190 ng) towards (^{32}P)phosphorylase a (□—□, 10 uM), and (^{32}P)inhibitor-1 (▲—▲ , 1 uM) was measured as described under Experimental Procedures, with the exceptions that the F_a was not added, and the control activity (i.e., no added $MnCl_2$) was measured in the presence of 2 mM EDTA. $MnCl_2$ was added to a final assay concentration as indicated.

inhibition at concentrations of $MnCl_2$ up to 12 mM. With respect to inhibitor-1, the K_a value for $MnCl_2$ was estimated to be 1.4 mM.

Effect of pH on G-Substrate Phosphatase Activity - The effect of varying pH on the activity of cerebellar phosphatases II and III measured towards G-substrate is shown in Figure 33. Both phosphatases displayed a single pH optimum of approximately 7.5. Similar results were obtained whether Tris or imidazole buffers were used.

Activation of Phosphatases II and III By Divalent Metal Ions - Phosphatases II and III displayed little activity towards G-substrate either in the absence of an added divalent cation, or in the presence of EDTA (Table 10). Of the metals examined, only Mn^{2+} and Co^{2+} could effectively stimulate activity towards G-substrate, and both of these metals (at a concentration of 4 mM) had a roughly equivalent effect. Phosphatase II activity measured towards phosphorylase a was also stimulated by these two metals, although in this case, Mn^{2+} was more than twice as effective as Co^{2+}. In contrast, phosphatase III exibited substantial activity towards phosphorylase a in the absence of any divalent cation; this activity was not inhibited by EDTA. The activity of phosphatase III towards phosphorylase a could, however, be stimulated about two-fold by the addition of Mn^{2+} (but not Co^{2+}) to the assay mixture.

Zn^{2+} generally acted as a strong inhibitor of these phosphatase activities, an effect observed whether measured in the absence or presence of added Mn^{2+} (data not shown). Calcium was without effect, whether added alone, or in combination with calmodulin (not shown). Neither magnesium, nor a preincubation with F_a + Mg^{2+}·ATP (Table 10) had an appreciable effect on the activity of phosphatases II and III.

The effect of varying Mn^{2+} concentration on the activity of phosphatases II and III was investigated (Fig. 34). Both phosphatases displayed similar hyperbolic dependencies on $MnCl_2$ when catalyzing the dephosphorylation of either phosphorylase *a* or G-substrate. The activation constants for Mn^{2+} - K_a (Mn^{2+}) - were estimated from double-reciprocal plots of these data (not illustrated). The double-reciprocal plot of the Mn^{2+} activation of phosphatase II activity towards G-substrate resulted in a biphasic curve with two apparent $K_a(Mn^{2+})$ values (0.2 and 0.8 mM). Only a single $K_a(Mn^{2+})$ value (0.2 mM) was apparent when phosphorylase *a* was used as the substrate. There were only single $K_a(Mn^{2+})$ values apparent for phosphatase III-catalyzed dephosphorylation of either substrate, but these were different from one another (0.2 and 0.05 mM for G-substrate and phosphorylase *a*, respectively). These results indicated that the concentration of Mn^{2+} resulting in optimal phosphatase activity varied depending upon which substrate was used, suggesting that at least part of the effect of Mn^{2+} was substrate-directed.

Table 10. Effect of Divalent Cations on the Activity of Phosphatases II and III.

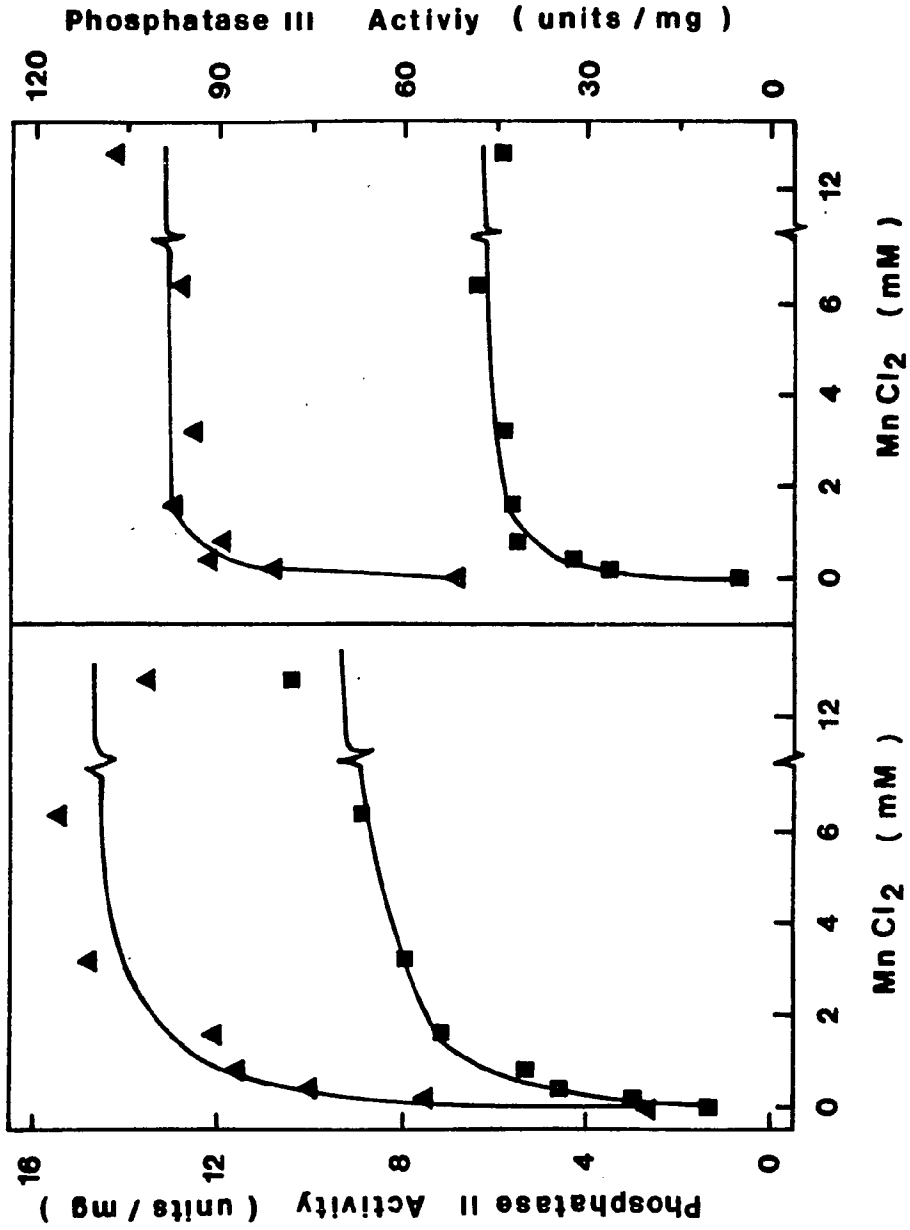
Cerebellar phosphatases II and III were assayed at 30°C for 10 min in the presence of 4 mM metal salt or 2 mM EDTA, as indicated. F_a + Mg·ATP activity was measured following preincubation with 0.1 mM ATP, 0.5 mM Mg $(CH_3COO)_2$, and 90 ng of Fa. Details of the assay are described under Experimental Procedures. Activity is expressed in Units/mg protein.

	G-Substrate		Phosphorylase a	
Addition	P'tase II	P'tase III	P'tase II	P'tase III
NONE	0.9	3.6	1.5	29.5
EDTA	0.7	2.5	1.3	30.2
$CaCl_2$	0.5	2.5	1.5	29.6
$CoCl_2$	6.2	37.6	4.9	33.9
$MgCl_2$	0.4	4.0	1.7	31.8
$MnCl_2$	7.5	35.2	11.5	62.4
$ZnCl_2$	0.1	3.2	0.2	1.2
F_a + Mg·ATP	1.3	3.9	1.8	32.4

Figure 33. Effect of Varying pH on the Activity of Phosphatases II and III Towards G-Substrate. Whole brain phosphatase II (A, 11 ng) and phosphatase III (B, 2 ng) were tested for activity towards (^{32}P)G-substrate (1 uM) at 30°C in a reaction volume of 22 uL that contained 1 mM DTT, 4 mM $MnCl_2$, and 50 mM of one of the following buffers: Tris, pH 7 - 9.5 (●—●), or imidazole, pH 5 - 8.5 (□—□). The reaction was initiated by the addition of enzyme, and was terminated after 10 min by the addition of 20 uL of 8% (w/v) TCA. Other details of the assay are described under Experimental Procedures.

Figure 34. Effect of Varying $MnCl_2$ Concentration on the Activity of Phosphatases II and III. The activity of cerebellar phosphatase II (left) and phosphatase III (right) was measured towards (^{32}P)G-substrate (■—■) and (^{32}P)phosphorylase a (▲—▲) in the presence of a varied concentration of $MnCl_2$, as indicated. Control activity (i.e., no added $MnCl_2$) was measured in the presence of 2 mM EDTA. Details of the assay are described under Experimental Procedures.

G-Substrate Phosphatase (Units/mg)
0
10
20
30
A
B
pH
5
6
7
8
9
0
30
60
90
120
150
G-Substrate Phosphatase (Units/mg)


Phosphatase III Activiy (units / mg)
120
90
60
30
0
0
2
4
6
12
Mn Cl2 (mM)
Phosphatase II Activity (units / mg)
16
12
8
4
0

Kinetics of Dephosphorylation

Site-Specific Dephosphorylation of G-Substrate - Cyclic GMP-dependent protein kinase phosphorylates two threonine residues in G-substrate (sites 1 and 2; see Table 2) at almost identical rates. However, the rate of phosphorylation of site 1 is four times that of site 2 when catalyzed by cyclic AMP-dependent protein kinase (Aitken et al, 1981). The following experiment was carried out to investigate whether the two sites were equivalent with respect to the rate of dephosphorylation when catalyzed by phosphatase II and phosphatase III.

G-substrate could be completely dephosphorylated by either phosphatase; the linearity of the dephosphorylation reaction (Fig. 35) suggested that no gross difference was apparent when comparing the two sites. Analysis of the phosphate content of the two sites during the course of dephosphorylation supported this observation. Aliquots withdrawn from the dephosphorylation reaction mixture were subjected to partial thermolytic digestion and two-dimensional peptide mapping (Fig. 36). The result indicated that the ratio of phosphate in the two sites did not significantly change during the dephosphorylation reaction. Therefore sites 1 and 2 seemed to be equivalent whether dephosphorylation was catalyzed by phosphatase II or phosphatase III. However, the possibility that one site was rapidly dephosphorylated following the dephosphorylation of the other site could not be ruled out by these experiments.

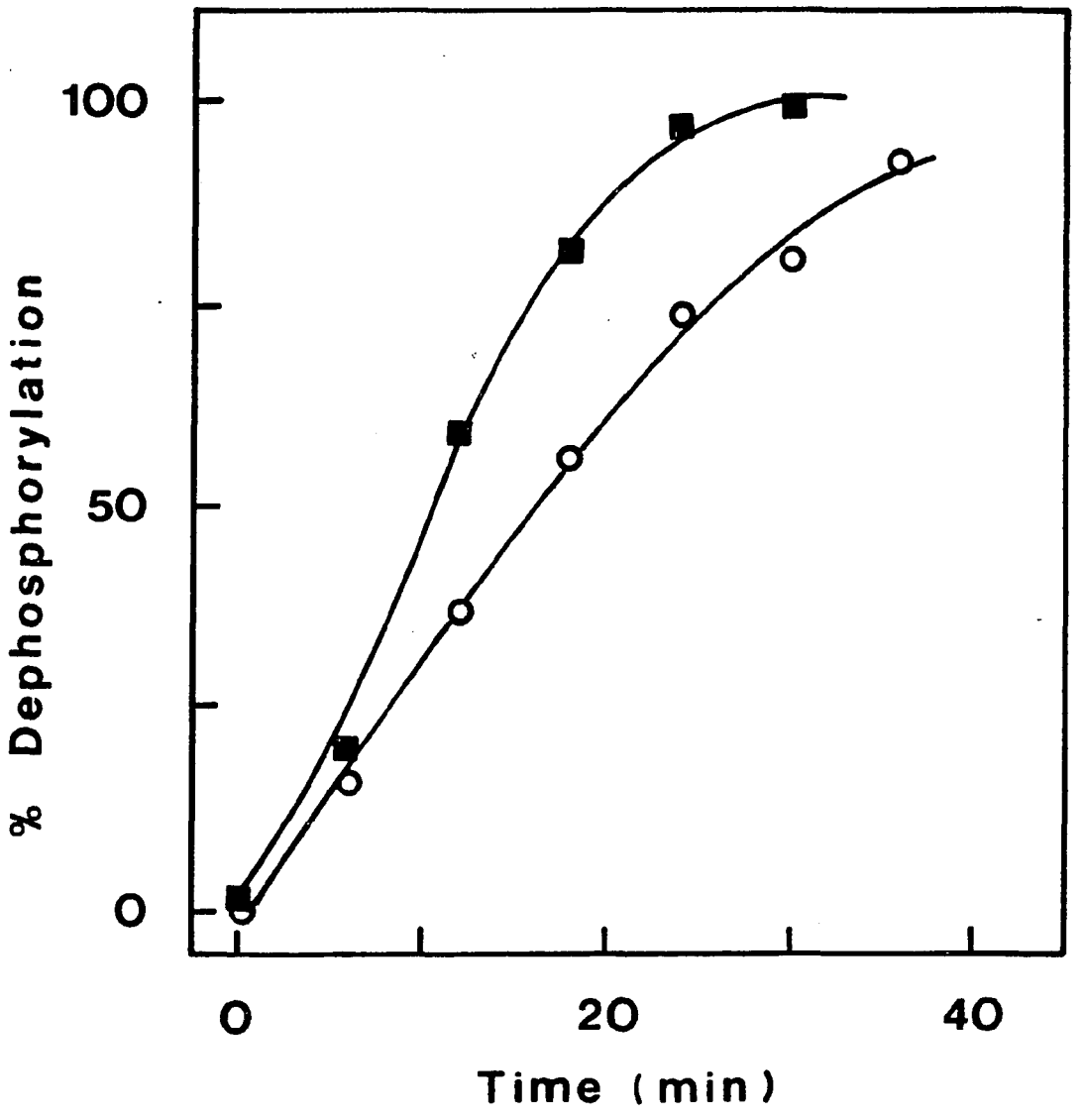


Figure 35. Time-Course of G-Substrate Dephosphorylation by Cerebellar Phosphatases II and III. (^{32}P)G-substrate (30 uM) was incubated at 30°C in a volume of 200 uL that contained 50 mM Tris·HCl, pH 7.4, 1 mM DTT, 2 mM $MnCl_2$, and either cerebellar phosphatase II (1 ug, ■—■) or phosphatase III (0.5 ug, O—O). At the indicated times, 2 uL aliquots were removed from the reaction mixture in order to measure the extent of dephosphorylation as described under Experimental Procedures.

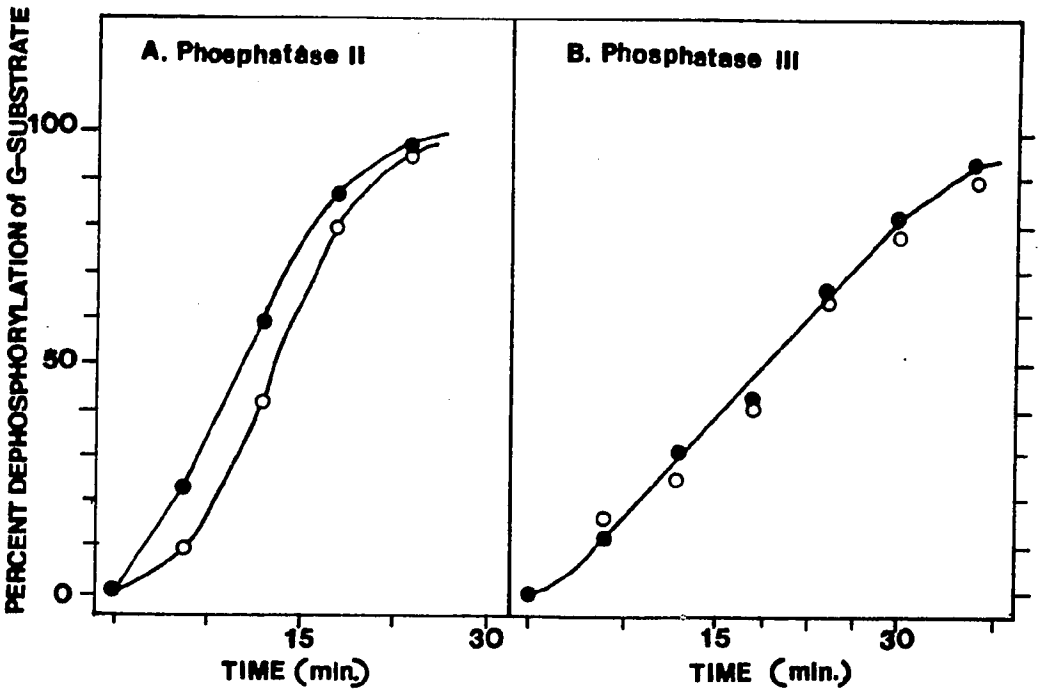


Figure 36. Time Course of the Site-Specific Dephosphorylation of G-Substrate. (^{32}P)G-substrate was dephosphorylated by A. cerebellar phosphatase II and B. phosphatase III as described in the legend to Fig. 35. At the indicated times, 10 uL aliquots were removed, and were then subjected to SDS-polyacrylamide gel electrophoresis, thermolytic digestion, and two-dimensional peptide mapping as described under Experimental Procedures. The peptides corresponding to site 1 (●—●) and site 2 (O—O) were localized by autoradiography. Those parts of the thin layer plate were then excised, and were quantitated by liquid scintillation counting. Results are expressed as a percentage of the total counts that were applied to the thin layer plate.

Substrate Kinetics - The dephosphorylation of phosphorylase a by phosphatase I followed Michaelis-Menten kinetics (Fig. 37). The kinetic parameters for this reaction were determined using a double-reciprocal plot of the same data (Fig. 37, inset). The K_m for phosphorylase a was 15 uM, and the maximal velocity (V_{max}) was 149 units/mg protein.

Phosphatases II and III also followed Michaelis-Menten kinetics when catalyzing the dephosphorylation of either G-substrate or phosphorylase a (Figs. 38 & 39). The K_m and V_{max} values were determined from double-reciprocal plots of these data (Figs. 38 & 39, insets), and are summarized in Table 11. Both phosphatases exibited similar K_m values for G-substrate (0.2 uM) which were significantly lower than the K_m values measured using phosphorylase a (4.5 and 17 uM for phosphatases II and III, respectively). The ratio of V_{max} values using G-substrate and phosphorylase a as substrate (3:1 for phosphatase II, and 1:5 for phosphatase III) suggested that, at saturating concentrations of both substrates, phosphatase II preferentially dephosphorylated G-substrate, and phosphatase III preferentially dephosphorylated phosphorylase a.

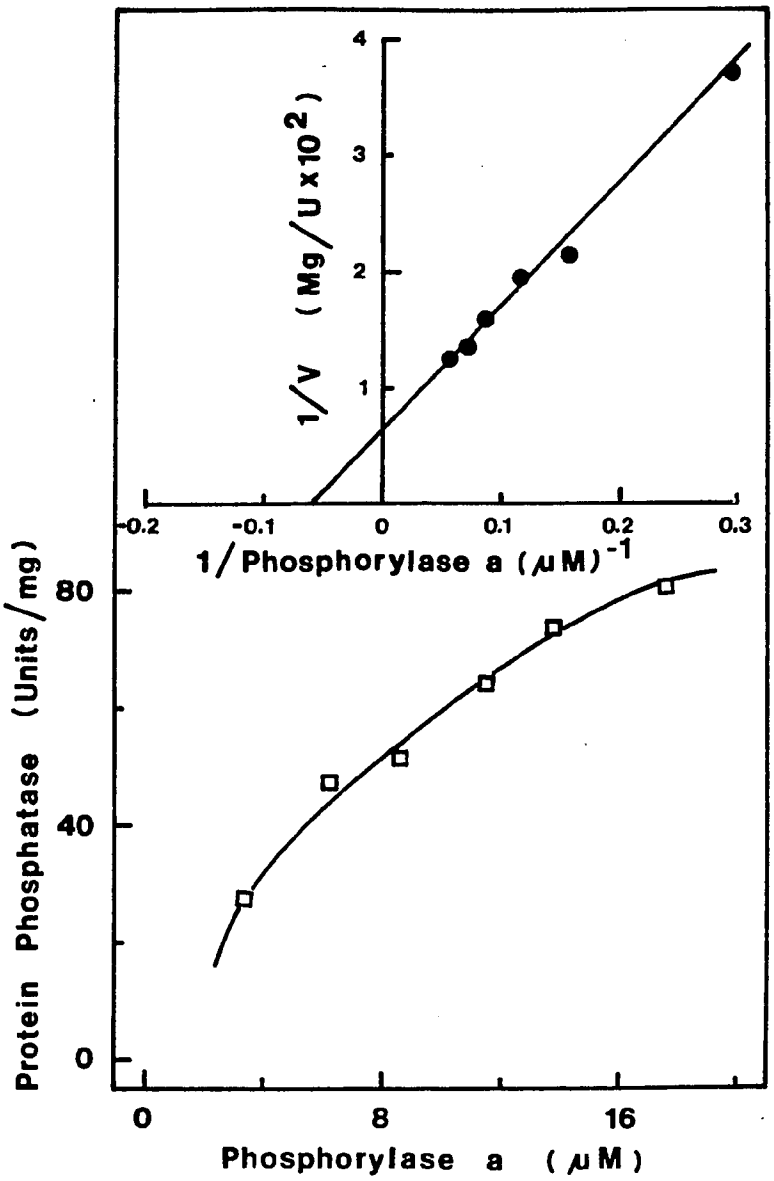
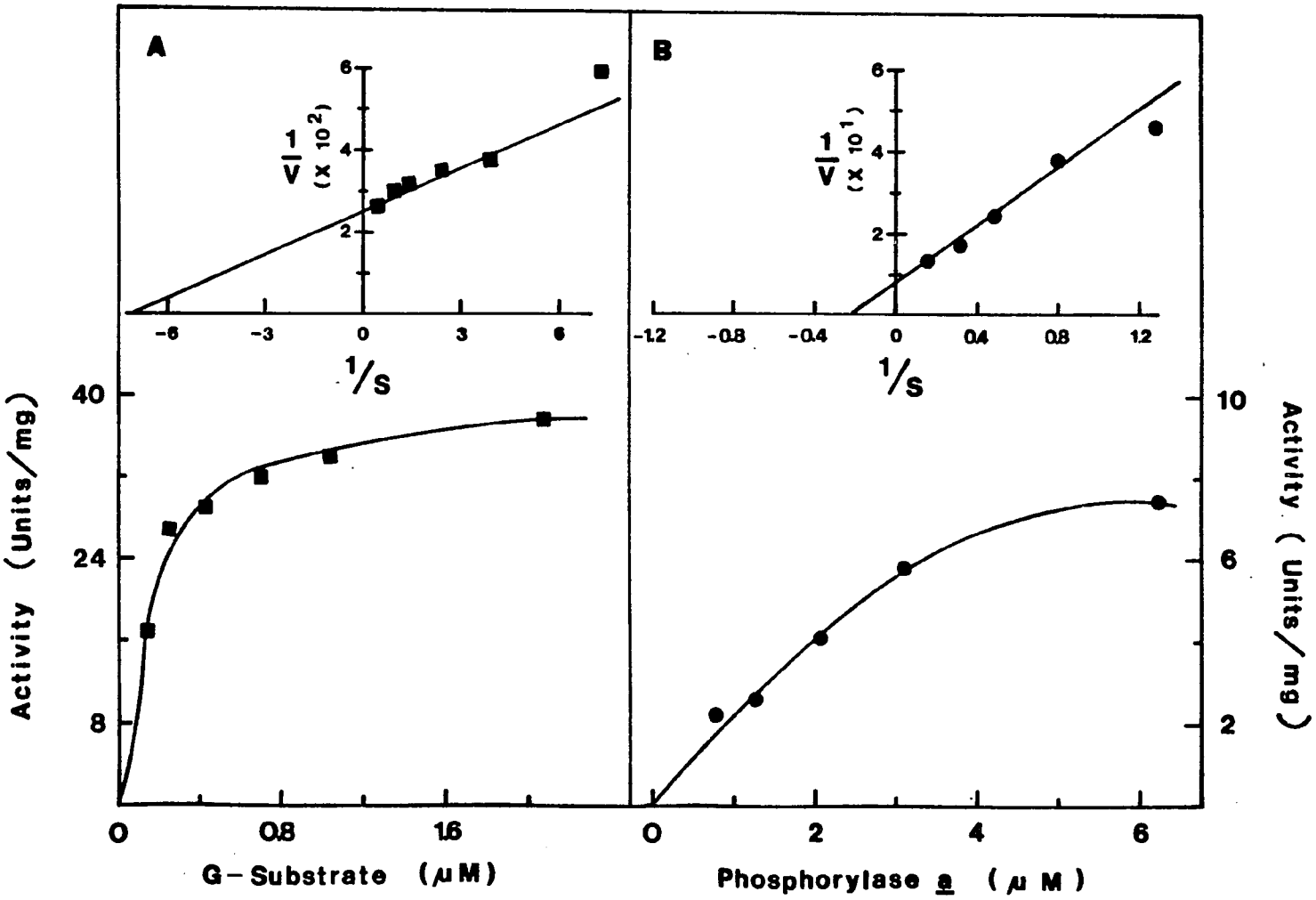


Figure 37. Effect of Varying Phosphorylase a Concentration on the Activity of Phosphatase I. Cerebellar phosphatase I (38 ng) was assayed following pre-activation with F_a + Mg·ATP as described under Experimental Procedures. The concentration of (^{32}P)phosphorylase a was varied as indicated. The inset shows a double-reciprocal plot of the same data.

Figure 38. Effect of Varying Substrate Concentration on the Activity of Phosphatase II. The activity of whole brain phosphatase II was measured at 30°C in a volume of 20 uL containing 50 mM Tris·HCl, pH 7.4, 1 mM DTT, and either A. 1 uM (^{32}P)G-substrate + 2 mM $MnCl_2$ (■—■), or B. 10 uM (^{32}P)phosphorylase a + 0.5 mM $MnCl_2$ (●—●). Other details of the assay are described under Experimental Procedures. The insets show double-reciprocal plots of the same data.

Figure 39. Effect of Varying Substrate Concentration on the Activity of Phosphatase III. Whole brain phosphatase III was assayed for activity towards A. (^{32}P)G-substrate (■—■) and B. (^{32}P)phosphorylase a (●—●) as described in the legend to Fig. 38. The insets show double-reciprocal plots of the same data.


A
B
1/V (x 10²)
1/V (x 10⁻¹)
1/S
1/S
Activity (Units/mg)
Activity (Units/mg)
G-Substrate (μM)
Phosphorylase a (μM)

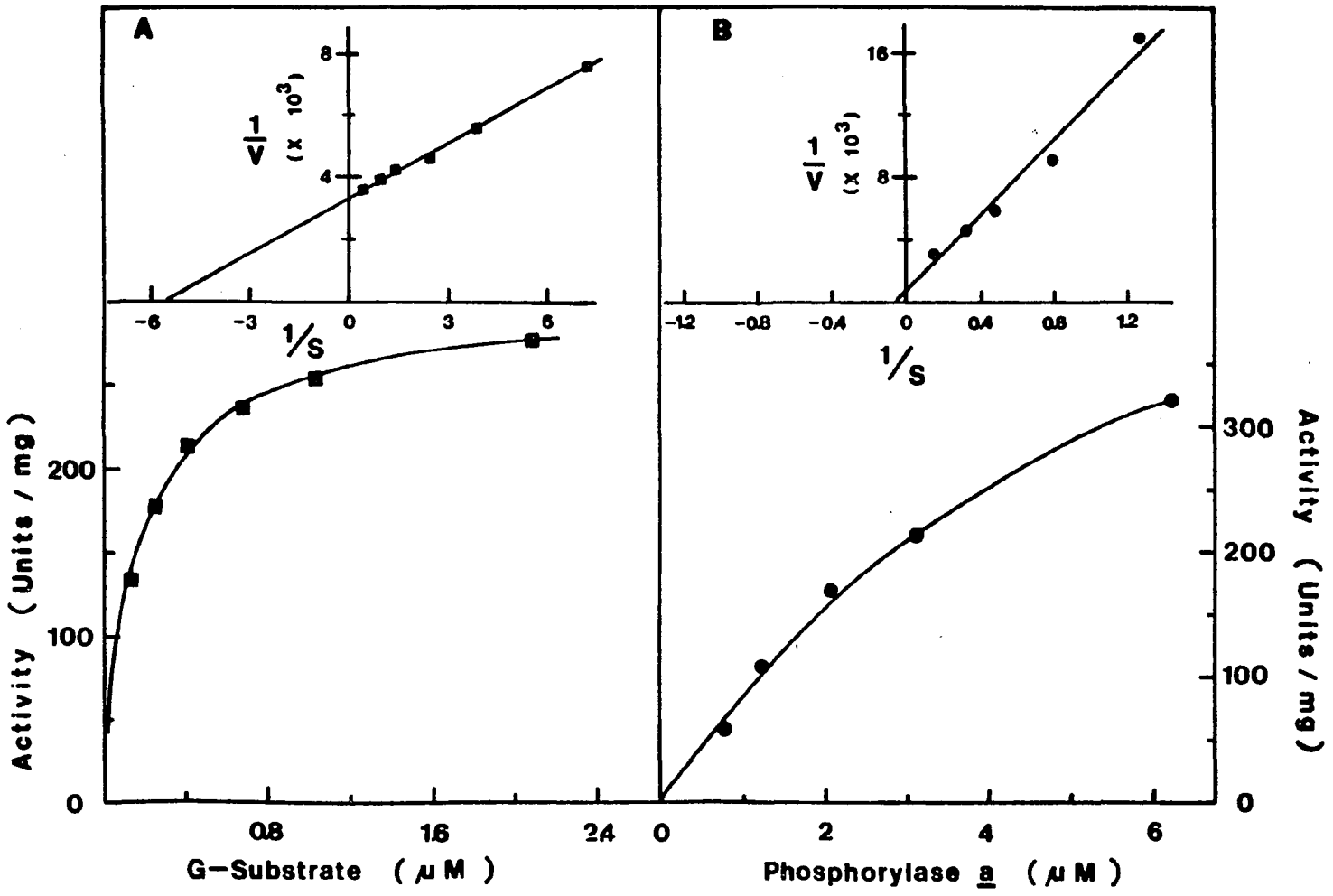

A
B
1/V (x 10^3)
1/S
Activity (Units / mg)
G—Substrate (μM)
Phosphorylase a (μM)
0
100
200
300
0.8
1.6
2.4
2
4
6

Table 11. Summary of Kinetic Parameters for Phosphatases II and III

The K_m and V_{max} values measured using G-substrate and phosphorylase a as substrates were determined from the double-reciprocal plots shown in Figures 38 and 39. The K_A values for Mn^{2+} were determined from double-reciprocal plots of the data illustrated in Fig. 34.

Parameter	Substrate	Phosphatase II	Phosphatase III
K_m	G-Substrate	0.2 uM	0.2 uM
K_m	Phosphorylase a	4.5 uM	17.0 uM
V_{max}	G-Substrate	40 U/mg	300 U/mg
V_{max}	Phosphorylase a	13 U/mg	1500 U/mg
$K_a(Mn^{2+})$	G-Substrate	0.2 & 0.8 mM	0.2 mM
$K_a(Mn^{2+})$	Phosphorylase a	0.2 mM	0.05 mM

Substrate Specificity

Comparison of the Substrate Specificity of Phosphatases I, II, and III - The activity of cerebellar phosphatases I, II, and III was compared using four different phosphoprotein substrates (Table 12). The results illustrate the differential effects of added Mn^{2+}. The activity of phosphatases I and II measured towards phosphorylase a was activated by Mn^{2+} from 6 to 9-fold, whereas the activity of phosphatase III was activated less than two-fold. A three-fold stimulation of the activity of phosphatases I and II measured towards phosphocasein was observed, though Mn^{2+} had no apparent effect on the activity of phosphatase III measured using this substrate. Each of the phosphatases was, however, dependent upon Mn^{2+} when catalyzing G-substrate and inhibitor-1 dephosphorylation (10 to 20-fold activation).

None of the phosphatases was specifically active towards a single substrate, but differences in the pattern of substrate specificity were observed. Phosphatase I was relatively specific for phosphorylase a; when measured following activation with F_a + Mg·ATP, phosphatase I catalyzed phosphorylase a dephosphorylation at a rate more than 200-fold faster than that measured using the other substrates. When measured instead in the presence of Mn^{2+}, a condition allowing optimal expression of activity towards G-substrate, inhibitor-1, and phosphocasein (but not phosphorylase a), phosphatase I catalyzed phosphorylase a dephosphorylation 10-fold faster than the other substrates.

In contrast to phosphatase I, phosphatases II and III displayed broader substrate specificity. Like phosphatase I, phosphatases II and III also displayed the highest activity towards phosphorylase a. However, phosphatase III displayed comparable activity towards phosphocasein, and phosphatase II displayed similar rates of activity using each of the four substrates. Thus, in comparison with

Table 12. Substrate Specificity of Phosphatases I, II, AND III.

The activities of cerebellar phosphatases I, II, and III were measured as described under Experimental Procedures. The final concentration of (^{32}P)phosphorylase a and (^{32})phosphocasein was 10 uM, and that of (^{32}P)inhibitor-1 and (^{32}P)G-substrate was 1 uM. Specific activities measured towards (^{32}P)phosphorylase a in the presence of $MnCl_2$ (equated to 100% for each phosphatase) were 19 units/mg (I), 6 units/mg (II), and 119 units/mg (III).

		Relative Specific Activity		
Substrate	Addition	Phosphatase I	Phosphatase II	Phosphatase III
Phosphorylase a	None	11	16	57
	$MnCl_2$ (0.5 mM)	100	100	100
	Fa + ATP·Mg	643	28	62
G-Substrate	None	1	5	2
	$MnCl_2$ (2 mM)	9	83	26
	Fa + ATP·Mg	3	6	2
Inhibitor-1	None	1	3	2
	$MnCl_2$ (2 mM)	13	52	14
	Fa + ATP·Mg	3	2	1
Phosphocasein	None	2	16	48
	$MnCl_2$ (2 mM)	5	55	47
	Fa + ATP·Mg	2	19	49

phosphatases I and III, phosphatase II displayed the highest specificity for G-substrate and inhibitor-1.

Activation with F_a + Mg·ATP seemed to convert phosphatase I to a form that was relatively specific for phosphorylase a. F_a + Mg·ATP activation resulted in a 60-fold increase of phosphatase I activity measured towards phosphorylase a, but stimulated the activity measured towards G-substrate and inhibitor-1 only 3-fold, and did not stimulate the activity towards phosphocasein at all. Phosphatase II activity measured towards phosphorylase a was also slightly stimulated by F_a + Mg·ATP. However, this effect was not noted in every preparation of phosphatase II, and consequently was thought to represent a partial contamination of this preparation with phosphatase I.

Effect of Ethanol Precipitation on the Activity of Phosphatases II and III - Ethanol precipitation of phosphatases II and III resulted in the isolation of catalytically active, lower molecular weight (M_r 35,000) phosphatases that displayed similar physical properties and electrophoretic mobility (see Table 8 and Fig. 20). However, when the substrate specificity of the ethanol-treated phosphatases II and III were compared, some differences were apparent. The results presented in Table 13 compare the activity recovered in the peak fractions of phosphatases II and III following non-denaturing gel electrophoresis. The ratio comparing the rates of inhibitor-1 and G-substrate dephosphorylation were approximately equal for the two ethanol-treated phosphatases (in both cases, 2 to 3-fold faster inhibitor-1 dephosphorylation). In contrast, a 7-fold difference was observed when comparing the rates of activity measured towards phosphorylase a and G-substrate; phosphorylase a was dephosphorylated 7.5-fold faster by the ethanol-treated phosphatase II, and 48.9-fold faster by the ethanol-treated phosphatase III.

Similar differences were observed when the activity towards G-substrate and phosphorylase a were compared directly following ethanol-precipitation

(Table 14). Under the conditions employed in this experiment, the ratio of activity measured towards G-substrate prior to ethanol precipitation (phosphatase II : phosphatase III) was 0.91. Ethanol-precipitation increased this ratio to 2.11. In contrast, the activity ratio of the two phosphatases using phosphorylase a as substrate displayed little change following ethanol precipitation (0.47 pre-treatment compared with 0.40 post-treatment). This difference was reflected in the recovery of activity measured using the two substrates. When measured towards phosphorylase a, phosphatase II displayed 90% and phosphatase III 107% of the pretreatment activity. When measured instead using G-substrate, phosphatase II displayed 47%, and phosphatase III displayed only 19% of the activity measured before precipitation with ethanol. Similar results were obtained whether ethanol precipitation was carried out at 4°C (Table 14) or 25°C (not illustrated). Similar results were also obtained when enzymic activity was measured following gel filtration of the ethanol-treated phosphatases (not illustrated).

Differences were also apparent when the effects of Mn^{2+} on the rate of dephosphorylation were compared. The peak fraction of the ethanol-treated phosphatase II was completely dependent upon Mn^{2+} when catalyzing the dephosphorylation of G-substrate (Table 13) or inhibitor-1 (not illustrated). (Nearly identical results were obtained when comparing the effects of Mn^{2+} on inhibitor-1 and G-substrate dephosphorylation by each of the peak fractions recovered following gel electrophoresis.) In contrast, although the peak fraction of the ethanol-treated phosphatase III was stimulated approximately three-fold by Mn^{2+} when catalyzing G-substrate and inhibitor-1 dephosphorylation, appreciable activity was measured towards these two substrates in the presence of EDTA. A differential effect of Mn^{2+} was also observed when comparing the activity of the two ethanol-treated phosphatases using phosphorylase a as substrate. Ethanol-treated phosphatase II was stimulated five-fold by added Mn^{2+} (Table 13), while the ethanol treated phosphatase III was slightly inhibited by the addition of Mn^{2+}.

Table 13. Substrate Specificity of Phosphatase II and III Peak Fractions Following Polyacrylamide Gel Electrophoresis.

Cerebellar phosphatases II and III were electrophoresed in 7% acrylamide as described in the legend to Fig. 20. Phosphatase II activity peaks 1 (R_f 0.30), 2 (0.47), 3 (0.60), and 4 (0.70), and phosphatase III activity peaks 1 (R_f 0.58) and 2 (0.68) were tested for enzymic activity measured towards (^{32}P)G-substrate (G-sub, 0.5 uM + 2 mM $MnCl_2$), (^{32}P)inhibitor-1 (Inh-1, 0.5 uM + 2 mM $MnCl_2$), and (^{32}P)phosphorylase a (Phl a, 8.8 uM + 0.5 mM $MnCl_2$) using a 5 uL undiluted aliquot as described under Experimental Procedures. The peak fractions from the ethanol-treated phosphatases II and III were also tested. The activity ratio is defined as the ratio of activity measured in the presence of 2 mM EDTA to that measured in the presence of $MnCl_2$.

		Relative Activity		Activity Ratio (EDTA/Mn)	
Phosphatase	Peak	Inh-1 : G-sub	Phl a : G-sub	G-sub	Phl a
II	1	1.1	2.6	0.02	0.06
II	2	0.8	3.9	0.01	0.02
II	3	1.6	5.5	0.01	0.18
II	4	1.8	5.2	0.02	0.06
II	(Ethanol Ppt.)	2.0	7.5	0.01	0.20
III	1	2.0	16.7	0.05	0.72
III	2	2.0	22.6	0.11	0.72
III	(Ethanol Ppt.)	2.9	48.9	0.31	1.17

Table 14. Effect of Ethanol Precipitation on the Activity of Cerebellar Phosphatases II and III.

100 uL of iced-cold ethanol (100%) was added to cerebellar phosphatase II (200 ug) or phosphatase III (50 ug) that was contained in 55 uL of Buffer A + 60% glycerol + 0.1 mM EDTA. The mixture was immediately centrifuged at 10,000 x g for 1.5 min, the ethanol decanted, and the precipitated protein was resuspended in 50 uL of a buffer containing 20 mM Tris·HCl, pH 7.4, 10% (v/v) glycerol, and 0.1 mM EDTA. 5 uL aliquots of the ethanol-treated and untreated phosphatases (both diluted 1:20 in the same buffer) were then used to measure phosphatase activity at 30°C for 10 min in a reaction volume of 25 uL that contained 50 mM Tris·HCl, pH 7.4, 0.5 mM DTT, 0.2% (w/v) BSA, and either 0.5 uM (^{32}P)G-substrate + 2 mM $MnCl_2$, or 10 uM (^{32}P)phosphorylase a + 5 mM theophylline + 0.5 mM $MnCl_2$. Alternatively, 2 mM EDTA was substituted for the $MnCl_2$. Other details of the assay are described under Experimental Procedures.

		G-Substrate		Phosphorylase a	
Phosphatase	EtOH	Spec. Act. (U/mL)	EDTA/Mn^{2+}	Spec. Act. (U/mL)	EDTA/Mn^{2+}
II	-	4.0	0.14	12.6	0.18
II	+	1.9	0.13	10.8	0.68
III	-	4.4	0.17	25.4	0.51
III	+	0.9	0.34	27.2	1.12

Effect of Protein Inhibitors

Inhibitor-1 and Inhibitor-2 - The activity of phosphatase I was completely inhibited by low concentrations of inhibitor-1 or inhibitor-2 (Fig. 40). When activity was measured using phosphorylase a as substrate, the inhibitor concentrations resulting in 50% inhibition (IC_{50}) were 0.7 nM and 1.9 ug/mL for inhibitor-1 and inhibitor-2, respectively. In this experiment, the inhibitor was added to the preincubation mixture, and so was included in both the F_a + Mg·ATP preactivation step, as well as in the assay mixture per se. However, both inhibitors were effective at similar concentrations when added solely to the assay mixture (data not shown). In either case, only the phosphorylated form of inhibitor-1 was effective as an inhibitor (not illustrated).

Inhibitor-1 and inhibitor-2 were much less effective as inhibitors of phosphatase II and phosphatase III activity (Fig. 41). When comparing the effects of the inhibitors, the amount of phosphatase added to the assay mixture was maintained at a constant level (1 - 3 milliunits of activity measured maximally); preliminary experiments (not shown) indicated that the degree of inhibition observed at a given concentration of inhibitor was inversely related to the amount of added phosphatase. Furthermore, in this set of experiments, the effect of inhibitor-1 on phosphorylase a dephosphorylation was measured in the presence of EDTA to preclude dephosphorylation of the inhibitor during the course of the reaction. The effect of inhibitor-2 was measured in the presence of $MnCl_2$, as usual, to allow optimal expression of phosphatase activity. Under these conditions, the IC_{50} of inhibitor-1 was 1.5 uM for phosphatase II, and 4 uM for phosphatase III. And the IC_{50} of inhibitor-2 was 200 ug/mL and 750 ug/mL for phosphatases II and III, respectively.

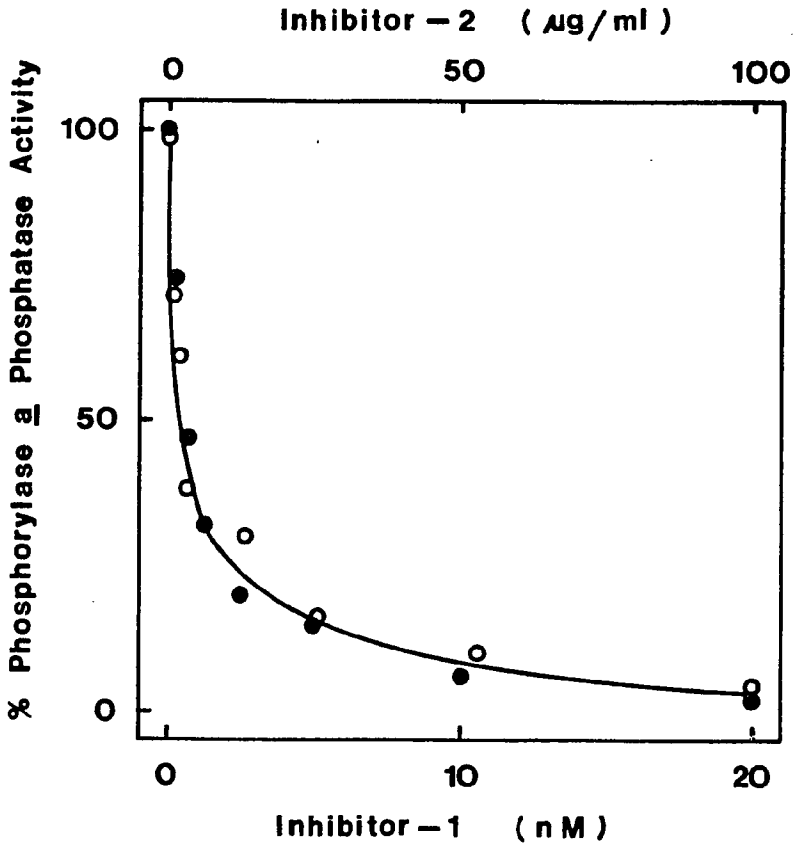


Figure 40. Effect of Inhibitor-1 and Inhibitor-2 on the Activity of Phosphatase I. The activity of cerebellar phosphatase I (2.1 milliunits) towards (^{32}P)phosphorylase a was measured following pre-activation with F_a + Mg$^{\cdot}$ATP as described under Experimental Procedures. Phosphorylated inhibitor-1 (●—●) or inhibitor-2 (O--O) were added to the preactivation mixture such that the final assay concentration was as indicated.

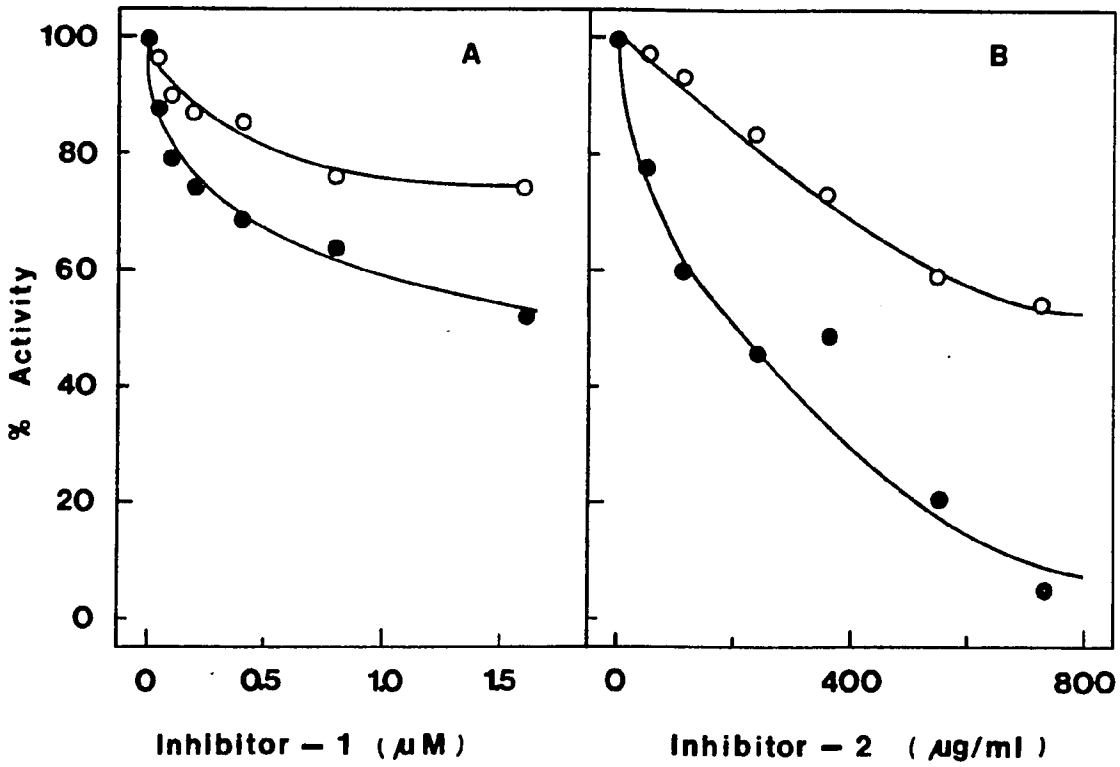


Figure 41. Effect of Inhibitor-1 and Inhibitor-2 on the Activity of Phosphatases II and III. The activity of cerebellar phosphatase II (●—●) and phosphatase III (O--O) was measured towards (^{32}P)phosphorylase a in the presence of A. 2 mM EDTA and a varied concentration of inhibitor-1, and B. in the presence of 0.5 mM $MnCl_2$ and a varied concentration of inhibitor-2. Maximal activity for phosphatases II and III were 1.1 and 1.4 milliunits (A), and 1.2 and 1.6 milliunits (B), respectively. Details are described under Experimental Procedures.

The activity of phosphatase II seemed to be more sensitive than the activity of phosphatase III to the two inhibitors (Fig. 41). In addition, the activity of phosphatase III was only inhibited partially: a maximum of approximately 50% inhibition was observed using either inhibitor. In contrast, the activity of phosphatase II measured towards phosphorylase a (Fig. 41b) and G-substrate (not illustrated) was completely inhibited by inhibitor-2, although the addition of inhibitor-1 only partially inhibited of phosphatase II activity, as well. (It was not possible to test the effects of higher concentrations of the two inhibitors). Thus, the addition of either inhibitor resulted in the inhibition of the activity of phosphatase II and phosphatase III. However, the IC_{50} of inhibitor-1 was more the 2000-fold greater, and the IC_{50} of inhibitor-2 was more than 100-fold greater than that measured against phosphatase I activity.

G-Substrate - The similarity of the structural and physical properties of inhibitor-1 and G-substrate (see Table 2) raised the possibility that G-substrate might also function as an inhibitor of protein phosphatase activity. When tested against phosphatase I, the activity measured towards phosphorylase a was inhibited by the addition of G-substrate in a concentration-dependent manner (Fig. 42a). The IC_{50} of G-substrate was estimated to be 1.5 uM, a value approximately 2100-fold greater than that measured for inhibitor-1. When tested at a concentration of 20 nM, inhibitor-1 completely inhibited phosphatase I activity (Fig. 40), but G-substrate had no apparent effect (not illustrated). Thus, in comparison with inhibitor-1, G-substrate was far less effective as an inhibitor of phosphatase I activity.

G-substrate was more effective than inhibitor-1 as an inhibitor of phosphatase III activity. (Again, the inhibitory effects were assayed in the presence of EDTA; under these conditions, less than 1% of the G-substrate was dephosphorylated during the 10 minute incubation period.) Using phosphorylase a, phosphohis-

tone, and phosphocasein as substrates, the activity of phosphatase III was progressively inhibited by increasing concentrations of G-substrate (Fig. 43). The IC_{50} of G-substrate was 0.2 uM using phosphorylase a as substrate, and 0.07 and 0.35 uM using phosphohistone and phosphocasein, respectively. At a 2 uM concentration of phospho-G-substrate, the enzymic activity measured towards each substrate was inhibited more than 80%.

The observation that phosphatase I activity was not inhibited by dephosphorylated inhibitor-1 suggested that the inhibitory effect of G-substrate might be related to its state of phosphorylation as well. Progressive dephosphorylation of G-substrate resulted in a parallel decrease in the inhibitory effect on phosphatase III activity (Fig. 44). When the concentration of phospho-G-substrate was reduced from 1.10 to 0.10 uM (i.e., 90% dephosphorylation), the inhibitory effect was totally lost. In these experiments, phospho-G-substrate was incubated with phosphatase II in the presence of Mn^{2+}. (Phosphatase II was used to dephosphorylate G-substrate because, as described above, phosphatase II seemed to be relatively specific for G-substrate, whereas phosphatase III seemed to be more specific for other substrates such as phosphorylase a.) At various time intervals, aliquots were withdrawn and diluted into a buffer containing EDTA to inhibit phosphatase II activity. These aliquots were then tested directly for the effect on phosphatase III activity. In a separate experiment, G-substrate was completely dephosphorylated using phosphatase II, and then the reaction was terminated by the addition of H_2SO_4 to completely destroy any residual phosphatase II activity. The neutralized and dialyzed dephospho-G-substrate was found to be completely ineffective as an inhibitor of phosphatase III activity, even at concentrations of dephospho-G-substrate ranging up to 1.3 uM. These results suggested that only the phosphorylated form of G-substrate was inhibitory.

The inhibitory effect of the dephosphorylated G-substrate could be restored

by rephosphorylation (Fig. 45). In this experiment, dephospho-G-substrate was prepared as described above, and then was rephosphorylated using cyclic GMP-dependent protein kinase. Aliquots taken from the reaction mixture at various times of incubation were tested in parallel for both the incorporation of $^{32}P_i$ into the G-substrate (Fig. 45a), and for the effect on phosphatase III activity measured towards phosphorylase a (Fig. 45b). In a separate experiment, the phosphorylation reaction was terminated by the addition of H_2SO_4 to destroy any residual kinase activity, and then the rephosphorylated G-substrate was analyzed for its inhibitory effect following extensive dialysis. This step ensured that the observed inhibitory effect was not artifactually related to the addition of any other component of the rephosphorylation reaction mixture. No difference was detected in the level of phosphatase III inhibition that was produced by either the rephosphorylated G-substrate, or a similar concentration of phospho-G-substrate that was not previously incubated with phosphatase II (not shown).

The possibility that a molecular change other than dephosphorylation was responsible for the loss of the inhibitory effect of G-substrate following the incubation with phosphatase II was investigated in the following way. Aliquots withdrawn from the rephosphorylation reaction mixture (Fig. 45) were analyzed by polyacrylamide gel electrophoresis carried out in the presence of SDS. Autoradiography of the Coomassie-stained gel was then used to locate the presence of radiolabeled protein bands (Fig. 46). $^{32}P_i$ was incorporated in a time-dependent manner into a single protein band of M_{SDS} 23,400 (Fig. 46, lanes 1 -7). No apparent difference in molecular weight was observed when phospho-G-substrate (Fig. 46, lane 8), dephosphorylated G-substrate (Coomassie stain not shown), and G-substrate that was subjected to a cycle of phosphorylation and rephosphorylation. These results suggested that dephosphorylation and rephosphorylation of G-substrate catalyzed by phosphatase II and cyclic GMP-dependent protein kinase,

respectively, could completely account for the changes in the inhibitory effect of G-substrate.

Phospho-G-substrate inhibited phosphatase III activity towards phosphorylase a in a non-competitive manner (Fig. 47). The inhibitory constant K_i for this reaction was estimated to be 0.15 uM. In contrast, G-substrate apparently acted as a mixed-competitive type of inhibitor of phosphatase I activity (Fig. 42b). In order to investigate whether the inhibitory effect of G-substrate was reversible, the following experiment was conducted (Fig. 48). Phosphatase III and G-substrate were incubated together in the presence of EDTA. A series of dilutions of this mixture were then tested for activity measured towards phosphorylase a. Such dilution resulted in the lowering of the concentrations of both the phospho-G-substrate and the phosphatase III, but the ratio of the concentrations of these two proteins remained constant. If phosphatase III and the phospho-G-substrate formed a tight or irreversible complex during this incubation, then a serial dilution of this mixture would have resulted in a constant level of inhibition that was independent of dilution. Therefore, the decreased inhibition that was observed with increasing dilution demonstrated that the inhibition of phosphatase III by phospho-G-substrate was reversible (Fig. 48).

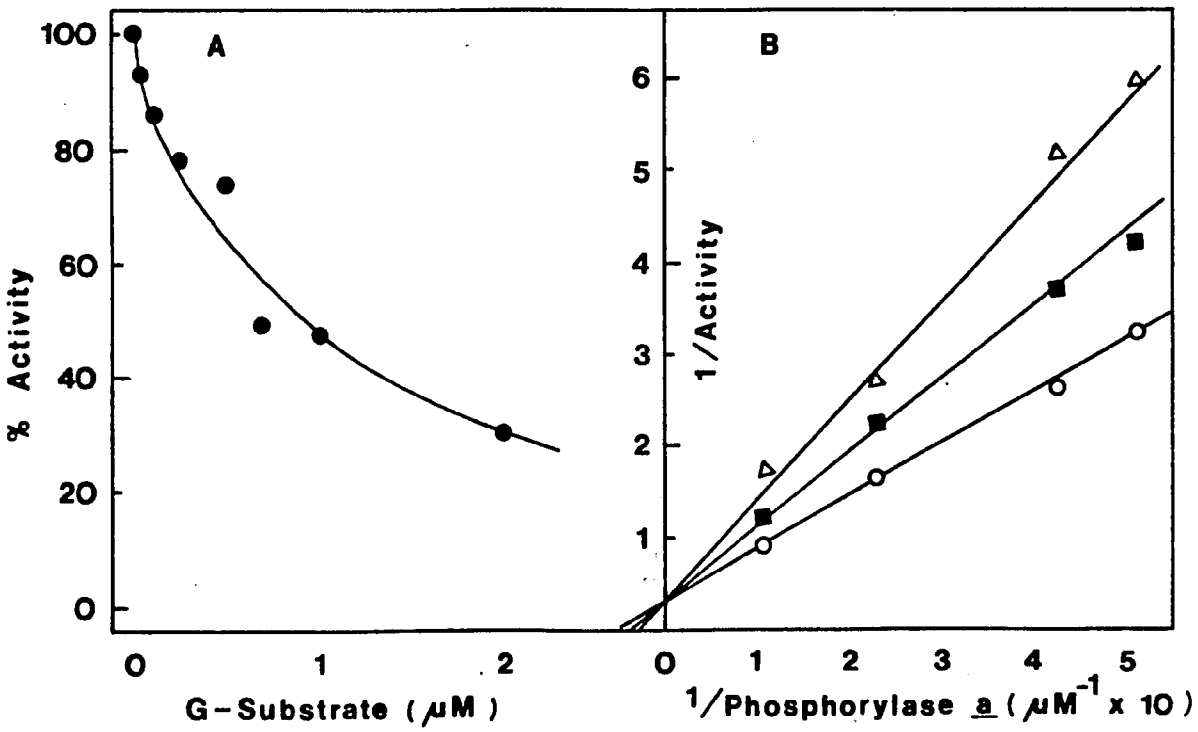


Figure 42. Effect of G-Substrate on the Activity of Phosphatase I. A. The activity of cerebellar phosphatase I (2.1 milliunits) towards (^{32}P)phosphorylase a was measured following pre-activation with F_a + Mg·ATP as described under Experimental Procedures. Phosphorylated G-substrate was added to the pre-activation mixture such that the final assay concentration (expressed in terms of protein) was as indicated. B. Phosphatase I activity towards (^{32}P)phosphorylase a was measured in the presence of different fixed concentrations of phospho-G-substrate. The activity was measured as described in A. with the exception that the G-substrate was added to the assay mixture following the preactivation of phosphatase I with F_a + Mg·ATP. The figure shows a double-reciprocal plot of the activity measured in the absence of added G-substrate (○—○), and at concentrations of G-substrate of 0.6 uM (■—■) and 1.4 uM (△—△).

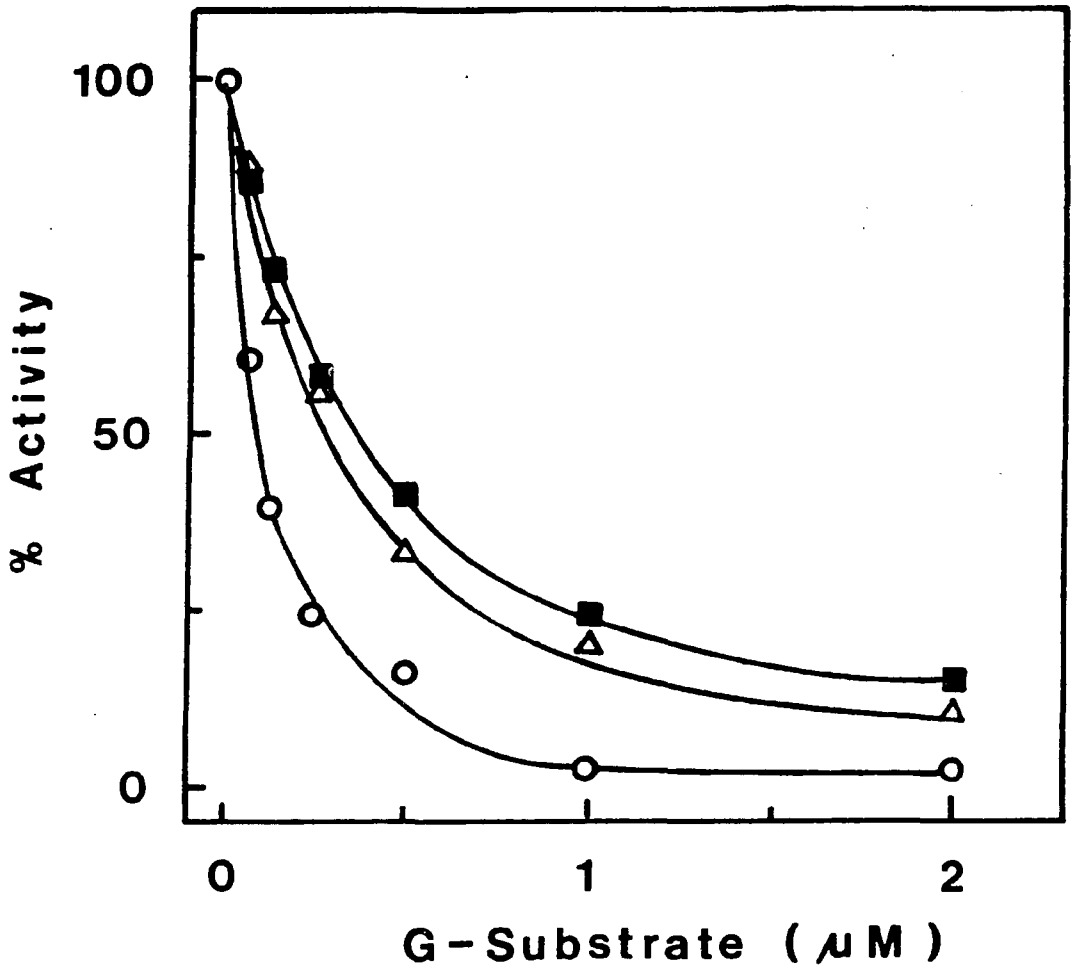


Figure 43. Effect of G-Substrate on the Activity of Phosphatase III. The activity of whole brain phosphatase III (a maximum of 2 milliunits measured towards phosphorylase a) was measured in the presence of 2 mM EDTA as described under Experimental Procedures, with the exception that the phosphatase was preincubated with phospho-G-substrate for 5 minutes at 30°C in a volume of 40 uL that contained all of the usual reaction components except the substrate. The reaction was then initiated by the 10 uL addition of ^{32}P-labeled phosphorylase a (△—△), phosphohistone (O—O), or phosphocasein (■—■). The amount of G-substrate (expressed in terms of protein) was varied such that the final assay concentration was as indicated.

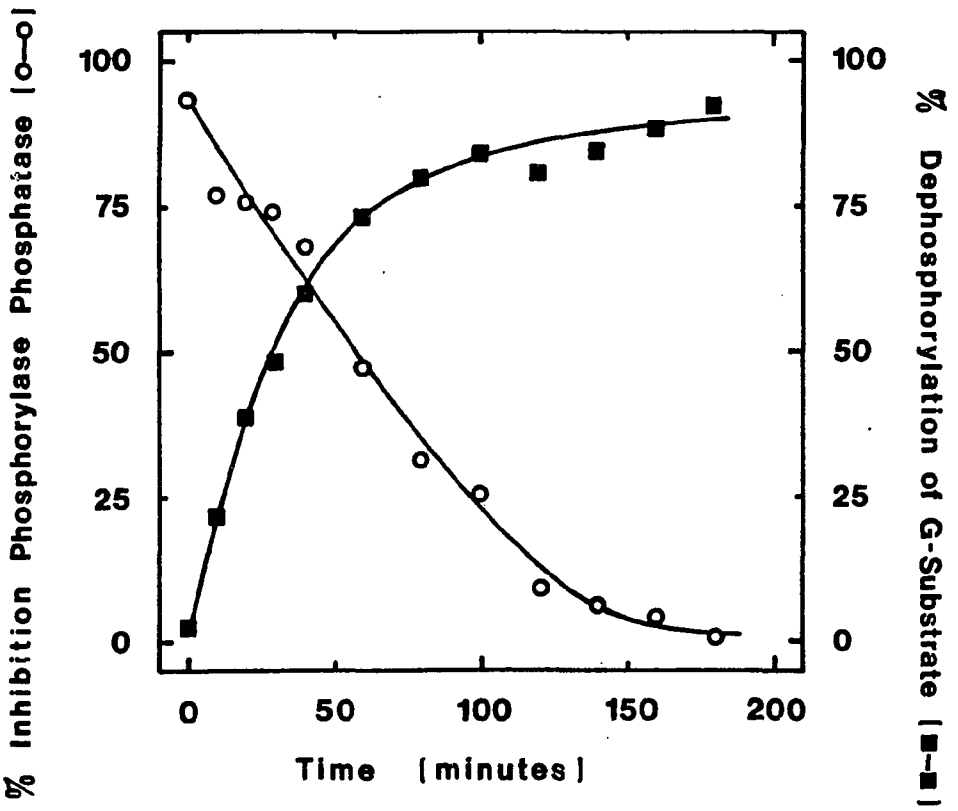


Figure 44. Time Course of Dephosphorylation and Inactivation of Phospho-G-Substrate by Phosphatase II. The dephosphorylation of (^{32}P)G-substrate was carried out using phosphatase II (75 milliunits) as described under Experimental Procedures. At the indicated time intervals, 40 uL aliquots were withdrawn and mixed with 200 uL of cold Buffer A + 2 mM EDTA to terminate the reaction. A 50 uL sample of this dilution was then used to determine the extent of dephosphorylation of the (^{32}P)G-substrate (■—■). Another 10 uL sample, containing 0.055 nmol (^{32}P)G-substrate at zero time, was used for measuring the inhibitory effect of G-substrate on whole brain phosphatase III activity towards phosphorylase a. The results are expressed as a percentage of the inhibition of phosphatase III (2 milliunits) that is produced by 1.1 uM phospho-G-substrate (O—-O). Details on the measurement of the extent of (^{32}P)G-substrate dephosphorylation and the activity of Phosphatase III are described under Experimental Procedures.

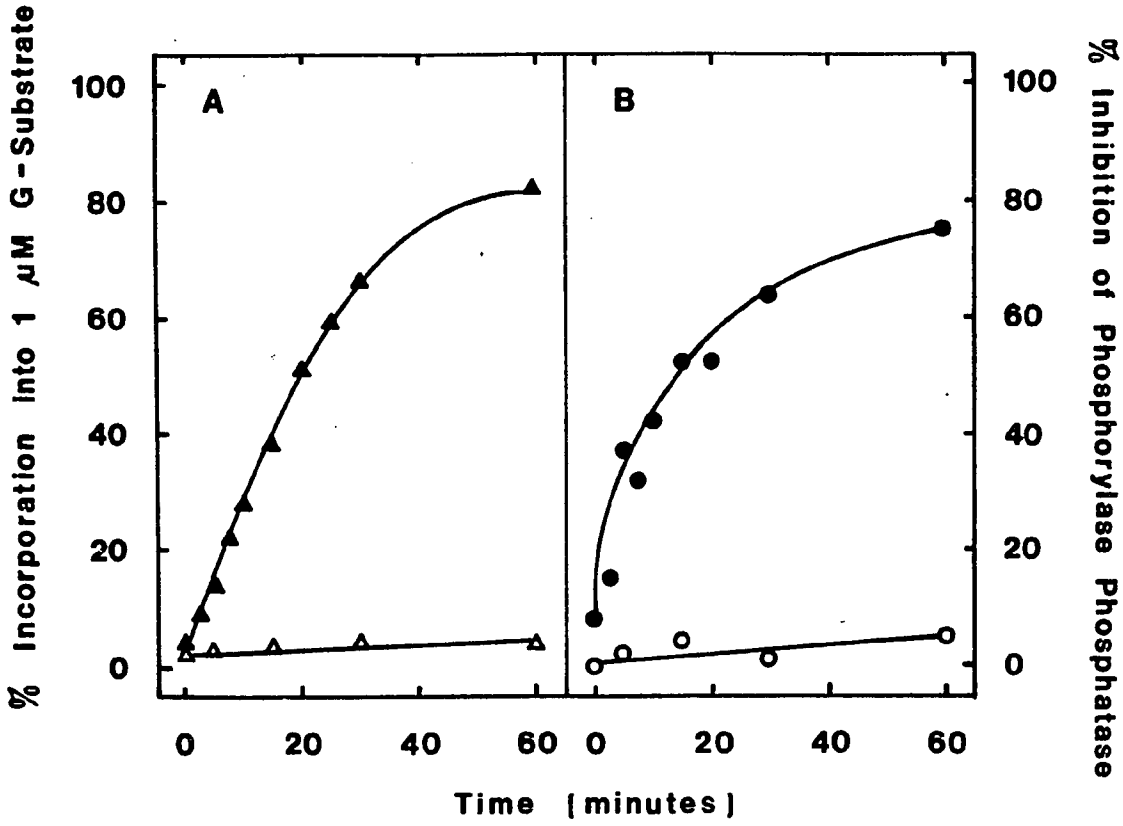
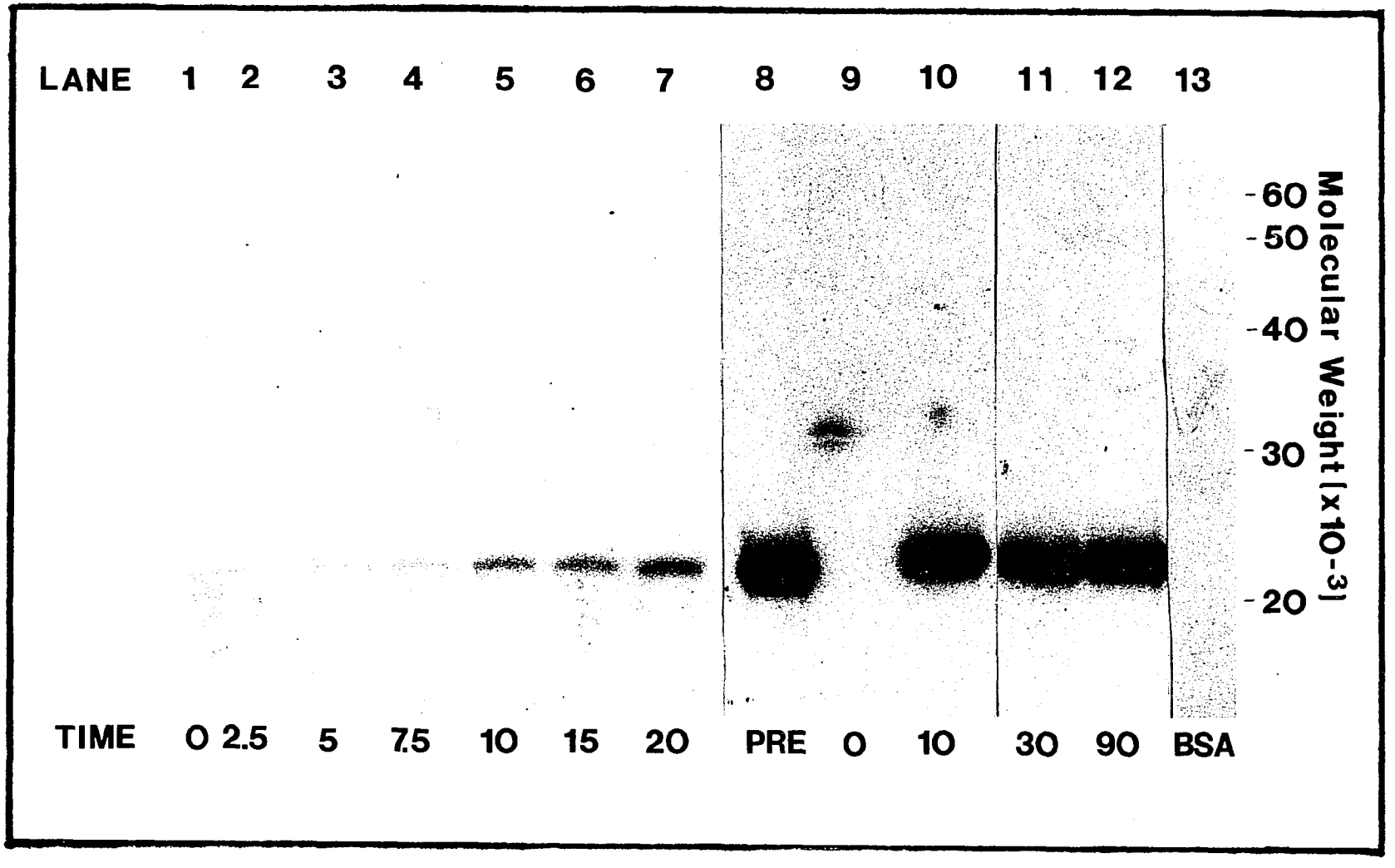


Figure 45. Time Course of Phosphorylation and Reactivation of Dephosphorylated G-substrate by Cyclic GMP-dependent Protein Kinase. A. Dephosphorylated G-substrate (prepared using phosphatase II) was rephosphorylated using cyclic GMP-dependent protein kinase and (γ-^{32}P)ATP. At the times indicated, 2 uL aliquots were removed and mixed with 23 uL of cold buffer A + 4 mM EDTA to stop the reaction. The results are expressed as percent $^{32}P_i$ incorporation. B. An identical phosphorylation experiment using non-radiolabeled ATP. 12 uL aliquots were diluted to give a final assay concentration of 0.8 uM G-substrate, and were then tested for the ability to inhibit whole brain phosphatase III activity (2 milliunits) measured in the presence of 2 mM EDTA towards phosphorylase a. The results are expressed as a percent of the inhibition caused by 0.8 uM phospho-G-substrate. Details of the dephosphorylation, rephosphorylation, and assay procedures are described under Experimental Procedures. ▲—▲, ●—●: G-substrate. △—△, O—O: BSA controls.

Figure 46. SDS-Polyacrylamide Gel Electrophoresis and Autoradiography of G-substrate Before and After a Cycle of Dephosphorylation and Rephosphorylation. Dephosphorylated G-substrate was rephosphorylated using ($-^{32}$P)ATP and cyclic GMP-dependent protein kinase as described in the legend to Fig. 45. At the indicated times, 10 uL aliquots were removed and added to 90 uL of Buffer C. Sample preparation, electrophoresis, and autoradiography were carried out as described under Experimental Procedures. (left) Lanes 1-7 show samples removed at times 0-20 minutes, as indicated. (right) Results of a similar experiment (50-fold more kinase) showing (^{32}P)G-substrate before dephosphorylation (lane 8), and rephosphorylated at times 0 (lane 9), and 10 - 90 minutes (lanes 10 - 12). Lane 13 shows a control (time = 30 min) in which BSA was substituted for G-substrate.


LANE 1 2 3 4 5 6 7 8 9 10 11 12 13
TIME 0 2.5 5 7.5 10 15 20 PRE 0 10 30 90 BSA
60
50
40
30
20
Molecular Weight (x10-3)

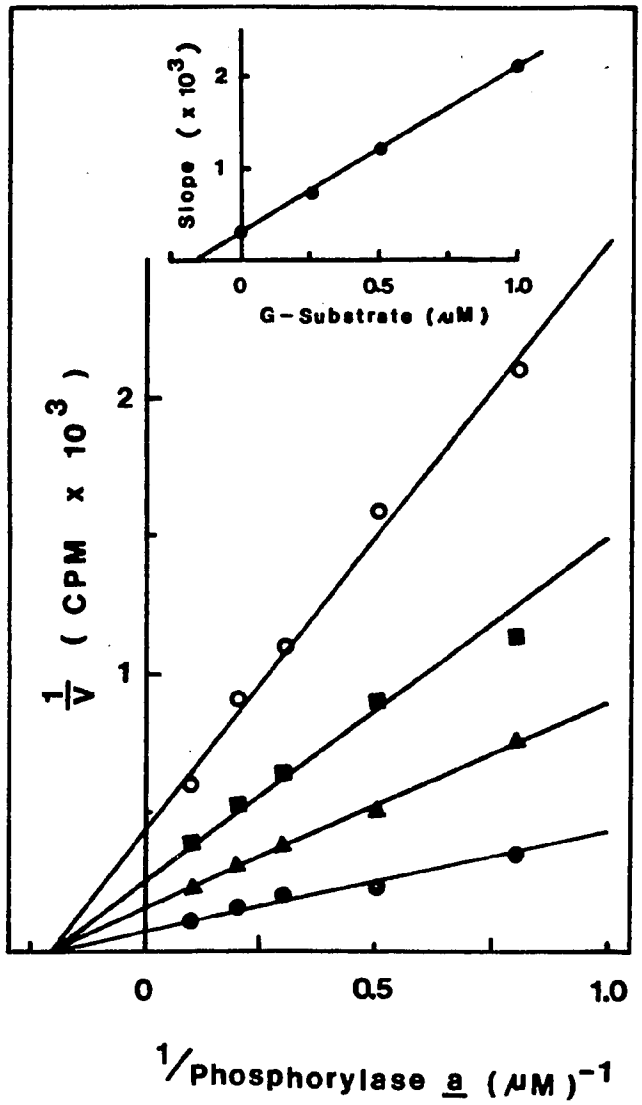


Figure 47. Double-Reciprocal Plot of Phosphatase III Activity Towards Phosphorylase a at Different Concentrations of Phospho-G-substrate. The activity of whole brain phosphatase III was measured in the presence of 2 mM EDTA and 0.0 (●—●), 0.25 (▲—▲), 0.5 (■—■), and 1.0 (O—O) uM phospho-G-substrate at various concentrations of phosphorylase a, as indicated. The inset shows a replot of the slopes derived from the double reciprocal plot versus the concentration of added G-substrate. Details of the assay are described under Experimental Procedures.

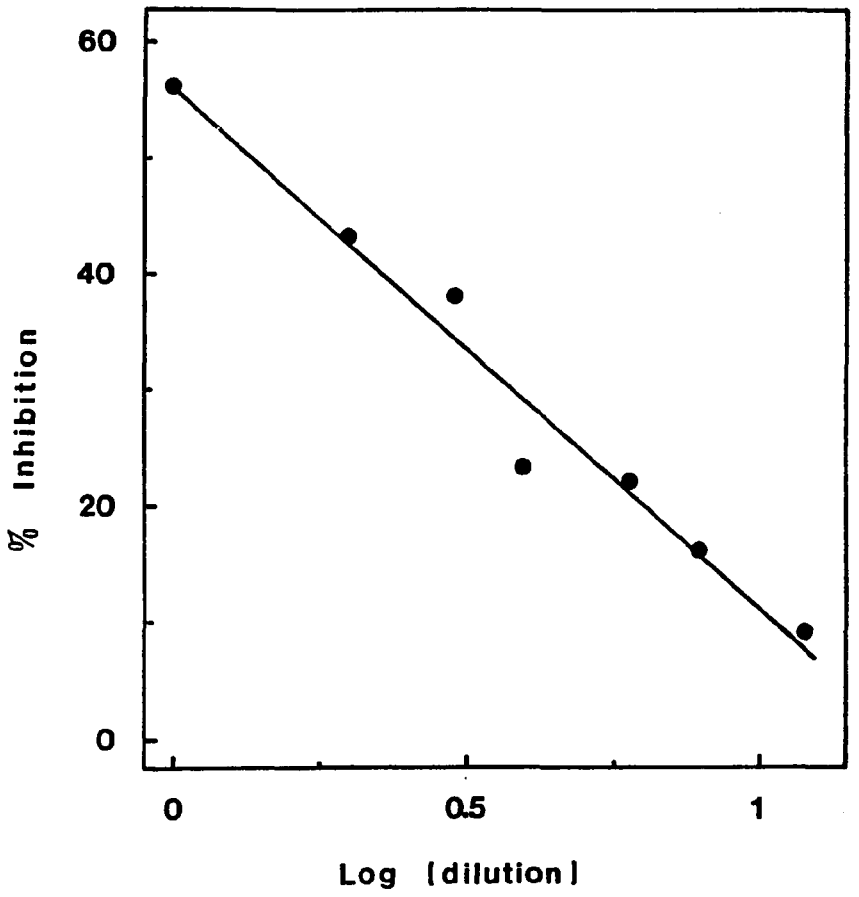


Figure 48. Effect of Dilution on the Inhibition of Phosphatase III by Phospho-G-substrate. Phosphatase III (1 ug) was incubated at 30°C in 500 uL of Buffer A containing 2 mM EDTA, and 5.5 uM phospho-G-substrate (sample), or an equivalent amount of BSA (control). After 10 minutes, aliquots were withdrawn from each reaction mixture, and were diluted with cold buffer A + 2 mM EDTA to varying extents, as indicated. 10 uL aliquots of the diluted mixtures were then used for the determination of phosphatase activity towards phosphorylase a as described under Experimental Procedures. Percent inhibition was calculated based on the ratio of activity measured using the diluted sample solution containing G-substrate and the corresponding control solution.

Discussion

The present results demonstrate that cerebellar tissue contains multiple protein phosphatase activities that can catalyze G-substrate dephosphorylation. At least four phosphatases could be differentiated on the basis of several physical and catalytic properties, including molecular weight, metal ion dependency, and the response to specific activators and inhibitors. Each of these phosphatases was active towards other substrates as well, suggesting that they might be generally involved in the regulation of cerebellar phosphorylation-dephosphorylation systems. In addition, phosphatases with similar properties were isolated from brain tissue other than cerebellum, and each displayed a close correspondence to phosphatases found in other tissues, including liver, heart, and skeletal muscle. These observations suggest that the major protein phosphatases potentially active in G-substrate dephosphorylation are the same or very similar to those involved in the regulation of a wide variety of cellular processes both within and outside of the nervous system.

Phosphatase I was found to be very similar to canine heart phosphatase U-1 (Li & Hsiao, 1977a) and skeletal muscle F_c (Yang et al, 1980a), and consequently is identified as a type 1 protein phosphatase. Like phosphatase U-1 and F_c, phosphatase I could be activated by F_a in a Mg^{2+}·ATP-dependent reaction. And like F_c, phosphatase I was completely inhibited by low concentrations of inhibitor-1 and inhibitor-2; the concentration of inhibitor-1 that resulted in half-maximal inhibition on phosphatase I activity (0.7 nM) is in very close agreement with that reported for other, non-brain type 1 protein phosphatases (Nimmo & Cohen, 1978b; Stewart et al, 1981; Foulkes et al, 1983c). While the molecular weight determined for phosphatase I (M_r 62,750) is close to that of phosphatase U-1 and

cardiac phosphatase II (M_r 61,000), it is somewhat less than that reported for skeletal muscle F_c (M_r 70,000: Yang et al, 1980a; Vandenheede et al, 1981b). However, Stewart et al (1981) reported that the molecular weight of protein phosphatase-1A (a type 1 protein phosphatase) is 62,000. And Jurgensen et al (1984) reported that purified skeletal muscle F_c contains three protein bands. The molecular weight of the largest (M_{SDS} 62,000-63,000) is in close agreement with the results presented here. The separation of two smaller bands (M_{SDS} 37,000-38,000, and 31,000) is similar to the results obtained following re-electrophoresis of the phosphatase I peak fractions in the presence of SDS.

The activation of phosphatase I by F_a is consistent with a mechanism involving phosphorylation. Activation was time-dependent, was strictly dependent on the simultaneous presence of Mg^{2+} and ATP, and persisted following substantial dilution of the activation mixture. Phosphatase I could also be activated by Mn^{2+}, but the activity measured towards phosphorylase a was stimulated to a far greater extent by F_a. The observation that Mn^{2+} could stimulate the activity towards both G-substrate and inhibitor-1 to a greater extent than F_a + Mg^{2+}·ATP suggests the possibility that the metal ion-stimulated activity was due to the contamination of the phosphatase I preparation with phosphatase II, -2, or perhaps an unidentified Mn^{2+}-dependent activity. However, several observations indicate that the Mn^{2+}-dependent activity was intrinsic to phosphatase I: Mn^{2+}- and F_a-stimulated activity co-purified through several purification steps, including non-denaturing gel electrophoresis; the electrophoretic migration of phosphatase I was different than that of phosphatase-2 (not shown) and phosphatases II and III; the protein bands contained in the most active fractions of phosphatases II, III, and -2 were found to have different molecular weights from those contained in the phosphatase I fractions; and in a preliminary experiment, inhibitor-2 could inhibit the activity of phosphatase I measured towards G-substrate either in the presence

of Mn^{2+} or following F_a + Mg^{2+}·ATP-activation.

A strict Mn^{2+}-dependence has been described for the catalysis of inhibitor-1 dephosphorylation by both protein phosphatase-1 and F_c (Goris et al, 1978; Nimmo & Cohen, 1978b; Foulkes et al, 1983c). In at least one tissue (dog liver), this requirement was abolished by the presence of a low molecular weight (M_r 9,000), heat stable factor termed deinhibitor protein (Defreyn et al, 1977; Goris et al, 1981). It has been suggested that the function of this protein, which itself can apparently be regulated by a cycle of phosphorylation-dephosphorylation (Goris et al, 1984), is to stabilize the enzyme in an active conformation which is insensitive to the effects of inhibitor-1 (Goris et al, 1983). Why Mn^{2+} - at seemingly nonphysiologic concentrations - would be required by the deinhibitor-free enzyme isn't known. In a related finding, Grankowski et al (1980a, 1980b) reported the purification of two protein activators of a Mn^{2+}-dependent phosphatase. The effect of these activators was strictly dependent on the presence of Mn^{2+}. Interestingly, one of the effects of these activators was to lower the Mn^{2+}-requirement of the phosphatase approximately 10-fold. These findings suggest that specific phosphatase modulators could in some cases lower the concentration requirements for Mn^{2+} to a level found under cellular conditions.

Preliminary studies of cerebellar F_a indicated that it is very similar to F_a purified from rabbit skeletal muscle (Vandenheede et al, 1980) and bovine heart (D. Tabarini, Ph. D. dissertation, Mt. Sinai Sch. Med., 1984). The chromatographic properties and molecular weight (M_r 50,000) of the muscle and brain forms of F_a are very similar. And like F_a purified from muscle, cerebellar F_a displayed protein kinase activity using glycogen synthase as a substrate. Furthermore, cerebellar F_a could activate cardiac muscle F_c. Conversely, cardiac F_a could activate cerebellar phosphatase I (not shown). Together with the observation that rabbit skeletal muscle inhibitors-1 and -2 were equally effective in the inhibition

of muscle and cerebellar F_c activity, these findings indicate that a close homology exists between the regulatory system of cerebellar phosphatase I and that of other type 1 protein phosphatases.

Phosphatase-2 was activated by calcium-calmodulin, and therefore was identified as a type 2 protein phosphatase. In separate studies (Chernoff et al, 1984; Li, 1984), phosphatase-2 was found to be identical to calcineurin on the basis of molecular weight, subunit composition, and immunological cross-reactivity (H.-C. Li, unpublished results). Recently, King et al (1984) demonstrated that calcineurin could dephosphorylate several brain-specific phosphoproteins, including G-substrate. However, the K_m measured for G-substrate (3.8 uM) was considerably higher than that reported here for either phosphatase II or phosphatase III (Table 11). Also, in contrast to the results depicted in Figure 36, calcineurin was reported to be highly specific for site 1 of G-substrate; site 2 was dephosphorylated at a rate approximately 20-fold slower than site 1. In light of these findings, it will be of interest to determine if the dephosphorylation of site 1 alone is sufficient to inactivate the inhibitory effects of G-substrate.

As part of the same study, DARPP-32, a neuron-specific phosphoprotein distributed primarily in the neostriatum and neocortex (Walaas & Greengard, 1981; Walaas et al, 1983; Oimet et al,1984), was found to be a substrate for calcineurin. DARPP-32, like G-substrate, shares several properties in common with inhibitor-1 (Hemmings et al, 1984b). Recently, it was found that in its phosphorylated form (catalyzed by cyclic AMP-dependent protein kinase), DARPP-32 is a potent and specific inhibitor of type 1 protein phosphatase activity (IC_{50} 10^{-9}M). Because the distribution of DARPP-32 parallels that of calcineurin (Wallace et al, 1980; Walaas et al, 1983; Oimet et al, 1984), it was proposed that calcineurin may be involved in the regulation of expression of this inhibitor (Hemmings et al, 1984b).

The possibility that calcineurin might also regulate the state of G-substrate phosphorylation in vivo is attractive because it would provide a mechanism by which calcium could influence the effects of a cyclic GMP-dependent reaction. Interactions between cyclic nucleotides and calcium are thought to be effected in a number of regulatory systems (Forn, 1984; Berridge, 1984). Recent findings by Ingebritsen et al (1983c) indicate that such a system may be operative in skeletal muscle. They observed that as the concentration of Ca^{2+} was raised from negligible levels to 3 uM (simulating "contracting muscle"), the proportion of the total potential inhibitor-1 phosphatase activity represented by calcineurin was increased from 0% to 65%. These authors reasoned that calcineurin-catalyzed inhibitor-1 dephosphorylation might therefore provide a mechanism by which calcium could attenuate the effects of cyclic AMP (Ingebritsen & Cohen 1983a). Nevertheless, the finding that phosphatase II and III together represented more than 90% of the recovered cerebellar G-substrate phosphatase activity, the lack of specificity for either of the two sites of G-substrate phosphorylation, and the 20-fold lower K_m in comparison with that reported for calcineurin together suggest that the type 3 protein phosphatases may be important in the dephosphorylation of G-substrate catalyzed in vivo.

The molecular weight, chromatographic and catalytic properties, and the apparent subunit composition of phosphatases II and III are consistent with their classification as type 3B and 3C protein phosphatases, respectively. In a result similar to that reported for pig heart protein phosphatases (Imaoka et al, 1980; Imazu et al, 1981; Imaoka et al, 1983), phosphatase II was found to contain alpha, beta, and gamma protein bands, whereas phosphatase III contained only the alpha and beta proteins. Moreover, the bands shared in common displayed the same molecular weight indicating that phosphatases II and III may be inter-related. In this regard, it was observed that the activity of of phosphatase II measured

towards phosphorylase a was less both in relative and absolute terms than that measured using phosphatase III. A similar result was obtained when comparing the activity of pig heart phosphatases reconstituted from alpha, beta, and gamma components (Imaoka et al, 1983; see Table 1). It was concluded that one of the effects of the gamma component was to lower the activity measured towards phosphorylase a.

Different peak fractions of each electrophoresed phosphatase contained the same component proteins as the non-electrophoresed enzyme, but in different stoichiometric proportions. The differences in substrate specificity displayed by these peaks could then be explained by these differences in relative subunit composition. In this respect, it was observed that the fractions containing the highest relative amount of the alpha protein also displayed the greatest relative activity towards phosphorylase a. A similar finding was noted when the specificity for phosphorylase a in comparison with inhibitor-1 was compared using rabbit skeletal muscle type 3B and 3C protein phosphatases that had been dissociated with 2-mercaptoethanol (Ingebritsen et al, 1983a). In both cases, dissociation resulted in a relatively greater activity measured towards phosphorylase a.

One implication of these findings is that the activity of type 3 phosphatases could be modulated by altering the composition of the components present in the phosphatase complex. How this type of system would be regulated physiologically is a matter for speculation. However, a more trivial implication is one of procedural importance. The observation that the composition and catalytic properties of phosphatases II and III (and other type 3 phosphatases: see Table 1) could be changed by steps in their purification indicates that the observed properties of a given type 3 phosphatase may be influenced by the nature of the substrate used as a probe in their purification. (Differences in stoichiometric

proportion were also observed in different high molecular weight peaks of cardiac phosphatase II following gel filtration indicating that these effects are probably not artifactually related to gel electrophoretic procedures.)

The results obtained when comparing the substrate specificity and metal ion dependency of the dissociated forms of phosphatases II and III are hard to reconcile with the existence of a common catalytic component. It has been suggested that differences in the catalytic properties and metal ion dependency displayed by different low molecular weight (dissociated) forms of the type 3 phosphatases are due to proteolysis, the effects of chelators, or perhaps to other factors related to enzyme purification (MacKenzie et al, 1980; Resink et al, 1983; Ingebritsen et al, 1983c). If the subunit composition of the holoenzymic forms of the two phosphatases is in fact different, then one of the two following possibilities could explain the persistence of differences in catalytic properties that are displayed by the dissociated enzymes: the component(s) not shared in common must selectively associate with an altered form of the catalytic subunit; or the catalytic subunit is more labile to alteration either in the presence or absence of these non-identical components. Alternatively, phosphatases II and III may contain catalytic components that are unique to the respective enzymes. Further work will be needed to clarify this point.

The selective loss of activators specific for phosphorylase phosphatase activity might provide an explanation for the relatively low recovery of this activity in comparison with that measured towards either G-substrate or p-nitrophenyl phosphate (Table 7). There have been a few reports of activators specific for histone phosphatase activity (Khandelwal et al, 1981; Knight & Teal, 1980; Knight & Skala, 1982). Typically, these have been low molecular weight, heat-stable and protease-sensitive proteins. However, the effect of these activators may be an alteration of the conformational state of histone, thereby

facilitating the interaction of this substrate with the phosphatase (Li & Hsiao, 1977b; Li, 1982). Recently, an activator specific for phosphorylase phosphatase activity has been purified from rabbit kidney (Wilson et al, 1982). Also a heat-stable protein (M_r 26,000), the main effect of this activator was to decrease the K_m for phosphorylase a approximately 20-fold. Interestingly, the K_m was reduced from 4.1 to 0.2 uM. These values are similar to those reported in this study for the dephosphorylation of phosphorylase a and G-substrate, respectively, catalyzed by either phosphatase II or III. (These authors indicated that a similar activator could be found in brain.)

The factors responsible for the regulation of type 3 protein phosphatase activity in vivo are not yet understood. The finding that G-substrate can reversibly modulate the activity of phosphatase III provides the first reported evidence that type 3 phosphatase activity may, at least in some situations, be regulated by a system analogous to that thought to participate in the regulation of type 1 protein phosphatase activity. A cycle can be envisioned in which cyclic GMP activates its target protein kinase, and coordinately inactivates phosphatase III activity by means of G-substrate-dependent inhibition. The inhibition of phosphatase III could then be reversed by dephosphorylation catalyzed by phosphatase II. It remains to be demonstrated whether phosphorylated G-substrate can effect an increase in the level of phosphorylation in Purkinje cell phosphoproteins. In addition, the factors regulating phosphatase II activity need to be further clarified.

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