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PHOSPHATASES FROM CANINE HEART.

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PURIFICATION AND PROPERTIES OF
PHOSPHOPROTEIN PHOSPHATASES FROM CANINE HEART

by

KWANG-JEN HSIAO

A dissertation submitted to the
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of Doctor of Philosophy, The
City University of New York.

1978

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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This thesis is dedicated to

my wife, Yuh-Yeh

and

my son, Hann

ABSTRACT

PURIFICATION AND PROPERTIES OF PHOSPHOPROTEIN PHOSPHATASES FROM CANINE HEART

by

Kwang-Jen Hsiao

Advisor: Professor Heng-Chun Li

The enzymic interconversions of active and inactive forms of regulatory proteins and enzymes through cycles of phosphorylation and dephosphorylation represent an important mechanism of metabolic control. The effects of a number of hormones on these enzymic interconversions have been shown to be mediated by cyclic AMP and cyclic AMP-dependent protein kinases which catalyze the phosphorylation reactions. The corresponding dephosphorylation reactions, which are catalyzed by phosphoprotein phosphatases (phosphoprotein phosphohydrolase EC 3.1.3.16), also play a potential regulatory role in the mechanism of this metabolic control. The objective of this research was to study the molecular forms, substrate specificity, and mechanism of the regulation of phosphoprotein phosphatases.

Phosphoprotein phosphatase activities in canine heart extract were separated by ion-exchange and gel filtration chromatography into five isozyme species, namely phosphatase A

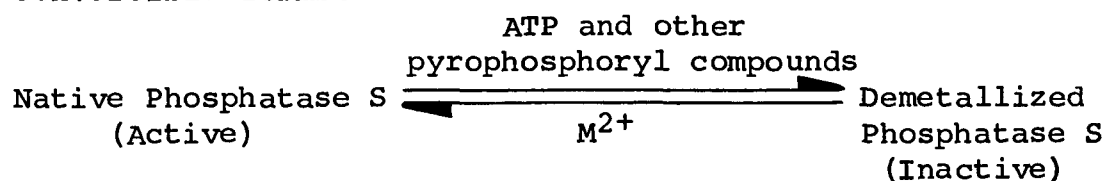
($M_r=156,000$), phosphatase B ($M_r=161,000$), phosphatase C ($M_r=95,000$), phosphatase U-1 ($M_r=61,000$), phosphatase U-2 ($M_r=49,000$). Based on their subunit composition, divalent cation dependency and substrate specificity, these five isozyme species may be classified into two classes. Class I isozymes, which include phosphatases A, B, and C, are divalent cation-independent, exhibit a broad substrate specificity and consist of a common catalytic subunit of $M_r=35,000$ (phosphatase S). This class of isozymes can be activated with concomitant dissociation into the smaller catalytic entity, which suggests that class I isozymes may exist in interconvertible forms: holoenzymes with low activity and catalytic subunit with high activity. Class II isozymes, which include phosphatases U-1 and U-2, are divalent cation-dependent, exhibit a narrow substrate specificity and consist of different catalytic entities from those of the class I isozymes (phosphatase S). Phosphatase U-1 is a Mn^{2+} -activated phosphohistone/phosphorylase phosphatase. Phosphatase U-2 is a Mg^{2+} -activated phosphocasein phosphatase.

Phosphatase S, the common catalytic subunit of class I isozymes, was purified to apparent homogeneity (10,000-fold purification with 30% yield of activity) by a procedure which involved treating the enzyme preparation with 80%

ethanol at room temperature. The pure enzyme consists of a single polypeptide chain with molecular weight of about 35,000 as determined by polyacrylamide gel electrophoresis, gel filtration on Sephadex G-100 and sucrose density gradient ultracentrifugation. Phosphatase S exhibits a broad substrate specificity and is active toward phosphorylase a, phosphorylated histone, casein, cyclic AMP-dependent protein kinase, and myosin light chain. The K_m values of phosphatase S for phosphorylase a, phosphorylated histone and casein are 4, 27, and 16 μM , respectively. Phosphatase S shows an optimum at pH 6.5-8 and is inhibited by ATP, other nucleoside phosphates, P_i , PP_i , or NaF , but not inhibited by EDTA or EGTA. Cyclic AMP and cyclic GMP show no effect on phosphatase S activity toward all phosphoproteins tested except cyclic AMP-dependent protein kinase. When phosphorylated cyclic AMP-dependent protein kinase was used as a substrate, cyclic AMP greatly stimulated the dephosphorylation reaction catalyzed by phosphatase S.

Studies on the mechanism of ATP inactivation on phosphatase S indicate that phosphatase S may be a metallo-enzyme which contains a tightly bound divalent cation(s), probably Mn^{2+} or/and Co^{2+} , in the phosphoryl moiety binding site of the enzyme. ATP, PP_i and other pyrophosphoryl

compounds, due to their structural similarity to the phospho-protein substrate and their ability to complex divalent cations, can readily enter the active site to complex and remove the essential divalent cation(s). The demetallized-phosphatase S, which has the same size as the original phosphatase S, was isolated and can be reactivated by pre-incubating with either Mn^{2+} or Co^{2+} but not Mg^{2+} , Ca^{2+} , Zn^{2+} or several other divalent cations examined. These results indicate that phosphatase S can exist in two inter-convertible forms:



ATP, other pyrophosphoryl compounds, and divalent cation(s) may play an important role in the regulation of this class of phosphatases in vivo.

PUBLICATIONS RESULTING FROM THIS EXPERIMENTAL WORK ARE:

1. Kwang-Jen Hsiao and Heng-Chun Li
The Dissociation of A Nucleoside Phosphate-Stimulated
Histone Phosphatase from Canine Heart.
Fed. Proc. 36, 778 (1977)
2. Heng-Chun Li and Kwang-Jen Hsiao
Purification of Protein Phosphatases from Canine Heart.
Fed. Proc. 36, 778 (1977)
3. Kwang-Jen Hsiao; Wanda W.S. Chan and Heng-Chun Li
Dissociation of Phosphohistone Phosphatases from Canine
Heart.
Biochim. Biophys. Acta, 483, 337-347 (1977)
4. Kwang-Jen Hsiao; Wanda W.S. Chan and Heng-Chun Li
Evidence for a Protein Phosphatase of $M_r=35,000$ as a
Metalloenzyme.
Third International Conference on Cyclic Nucleotides.
New Orleans, Louisiana, U.S.A. (July, 1977)
Advances in Cyclic Nucleotide Research, Vol. 2, 750 (1978)
5. Heng-Chun Li; Kwang-Jen Hsiao and Wanda W.S. Chan
Purification and Properties of Phosphoprotein Phosphatases
with Different Substrate and Cation Specificities from
Canine Heart.
Eur. J. Biochem. 84, 215-225 (1978)
6. Kwang-Jen Hsiao; Alan R. Sandberg and Heng-Chun Li
The Role of ATP and Divalent Cations in the Regulation of
a Cardiac Phosphorylase Phosphatase (Phosphoprotein
Phosphatase) of $M_r=35,000$.
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ABBREVIATIONS

Abbreviations follow the "Collected Tentative Rules and Recommendation of the Commission on Biochemical Nomenclature UIPAC-IUB and Related Documents", 2nd ed. (1975), American Society of Biological Chemists, Bethesda, Maryland.

Cyclic AMP:	Adenosine 3',5'-monophosphate
Cyclic GMP:	Guanosine 3',5'-monophosphate
Cyclic CMP:	Cytosine 3',5'-monophosphate
AMP(CH ₂)P:	(α,β -methylene)adenosine 5'-diphosphate
AMP(CH ₂)PP:	(α,β -methylene)adenosine 5'-triphosphate
AMP-P(CH ₂)P:	(β,γ -methylene)adenosine 5'-triphosphate
AMP-P(NH)P:	(β,γ -imido)adenosine 5'-triphosphate
SDS:	Sodium dodecyl sulfate
TNI:	Inhibitory subunit of troponin
TNT:	Tropomyosin-binding subunit of troponin
TNC:	Ca ²⁺ -binding subunit of troponin
P _i :	inorganic phosphate
PP _i :	inorganic pyrophosphate
Ser-P:	Phosphoserine
Thr-P:	Phosphothreonine

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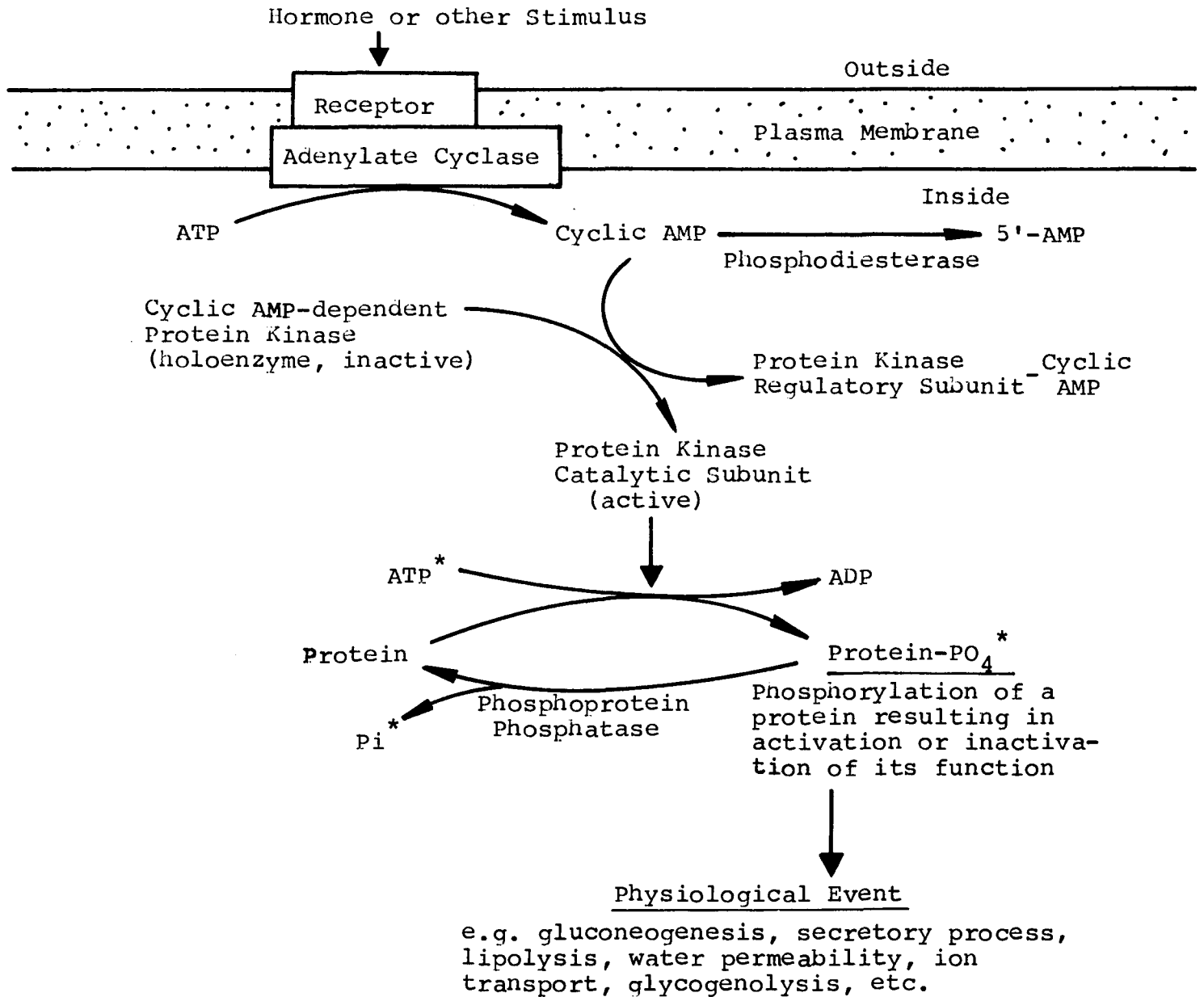
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INTRODUCTION

At the turn of the twentieth century, proteins containing covalent-bound phosphate groups were first isolated and identified from egg yolk and milk (for review, see ref. 34). In the late forties and early fifties, it was found that the protein-bound phosphate in cells was involved in dynamic metabolic turnover. Since then, several enzymes have been found to be phosphorylated during their catalytic action in phosphate transfer reactions. In the mid-fifties, it was demonstrated that glycogen phosphorylase could be interconverted between an active (a) and inactive (b) form by phosphorylation-dephosphorylation reactions (352-357, 462-466). However, it is the discovery of the cyclic AMP (12,14,15) and cyclic AMP-dependent protein kinase (13,494) that linked the protein phosphorylation to hormonal actions and different cellular functions. This important discovery has changed the character of phosphoprotein research and has brought it into an entirely different new era during the past decade (1-10,12).

In 1968, a cyclic AMP-dependent protein kinase was discovered in skeletal muscle (494). Evidence indicated that protein kinase mediated the effects of cyclic AMP

in causing the breakdown of glycogen. Subsequently, Greengard and colleagues (13) proposed that regulation of protein kinases may be a general mechanism through which the diverse biological effects of cyclic AMP are mediated. Much evidence has now accumulated that regulation of protein kinase activity is involved in many of the actions of cyclic AMP in eukaryotic cells (7-9) (The criteria for mediation of an effect of cyclic AMP by phosphorylation of a protein were established by Krebs (553) and refined by Nimmo and Cohen (8)). A widely accepted model for the mediation of hormonal functions by cyclic AMP through protein phosphorylation is described in Scheme 1. Furthermore, recent studies indicate that phosphorylated proteins may play a much broader role and may act as general physiological effectors for many classes of regulatory agents in addition to those whose effects are mediated through cyclic AMP (10). These regulatory agents include cyclic GMP (504), steroid hormones (165,561), insulin (555,559,560), calcium (220,221,547,549, 550), interferon (554), thyroid hormone (556), vaccine virus (557), hemin (317-319,558), and light (136-139). The present studies indicate that a diverse group of regulatory agents, including those agents acting through cyclic AMP, may achieve certain of their biological actions through

SCHEME 1An Extended Second Messenger Model

effects on phosphorylation of specific proteins. Thus, it is clear that protein phosphorylation-dephosphorylation reactions play a central role in the control of diverse biological processes.

In this review I will discuss (I) the nature of the protein-phosphate linkage, (II) phosphoproteins and the possible regulatory function of their phosphorylation, (III) protein kinases which catalyze the phosphorylation reaction, and finally (IV) phosphoprotein phosphatases which catalyze the corresponding dephosphorylation reaction.

I. Protein-Phosphate Linkages

It is important to understand the nature of the protein-phosphate linkage for studying phosphoproteins and their metabolism. Both -O-P- and -N-P- are possible covalent linkages between protein and phosphorus. There is no apparent evidence for a -S-P- linkage, although it may occur, theoretically, in the form of a phosphocysteine bond. Different covalent linkages besides phosphoester bonds, which are formed and hydrolyzed by protein kinases and phosphoprotein phosphatases respectively, have been found in biological systems. It appears that the phosphoester bond is a stable and probably the major linkage between protein and phosphate as far as it is known. Some of

phosphoenzyme intermediates may contain other protein-phosphate linkages, namely phosphoamidate and phosphoanhydride bonds. Since phosphoenzyme intermediates (cf. ref.16) are involved in catalytic mechanisms and do not regulate the functions of proteins, they will not be discussed here. The phosphoproteins referred to here are those with phosphoester linkages, unless otherwise stated.

A) Phosphoesters

Phosphoserine was first obtained by Lipmann and Levene from vitellinic acid (17). Subsequently it was isolated from an acid hydrolysate of casein in 1933 (18). Two decades later, from the same source the second phosphoamino acid, phosphothreonine, was isolated (19). The characteristic of the linkage is that the phosphate groups are readily hydrolyzed in dilute alkali solution at room temperature, e.g., in 0.25 N NaOH at 25°, but that they are stable in acid under the same condition (20). In contrast to the great lability of phosphoproteins in dilute alkali solution, the free phosphohydroxyamino acids are stable in this medium (21). This indicates that the chemical properties of the phosphoester bonds in free phosphoamino acid are different from those in phosphoproteins, in which the phosphoamino acids are sensitive to the adjacent molecular

configuration and to the conformation of the peptides. Neither the phosphotyrosine nor the phosphohydroxyproline linkage has been identified in phosphoproteins.

B) Phosphoamidates

Because of the relatively stable linkage of the phospho-ester bond, the conditions of phosphoprotein manipulation are frequently not mild enough to rule out the possible existence of a variety of labile phosphate bonds, such as phospho-amidate and phosphoanhydride bonds. This is why the phosphoamidate bonds escaped detection until the late 1960's. The phosphoamidate linkages are labile under acidic conditions but relatively stable in alkali (22,23). Phosphohistidine has been identified as the active site for several phospho-enzyme intermediates (16). The nitrogen in both positions in the imidazole ring can be phosphorylated. There are indications that other phosphoamidate bonds, namely phospholysine (24-26) and phosphoarginine (27), may be found in nature.

C) Phosphoanhydrides

Phosphoanhydride bonds, like phosphoamidate bonds, may be considered high energy chemical bonds. Acyl phosphates are labile in both high and low pH ranges but are relatively stable at neutral pH (28,29). They are,

however, labile in the presence of hydroxylamine or molybdate (28,29). Several phosphoenzyme intermediates are in the form of phosphoglutamates (16). Besides phosphoglutamate, the phosphate may also be linked to aspartate in a phosphoanhydride bond (28).

D) Other linkages and possible artifacts

There is no hard evidence for phosphoamidate or phosphoanhydride linkages at free amino or free carboxyl terminals of proteins. The protein-bound phosphate does not seem to cross-link, as far as it is known, despite the early suggestion that the phosphodiester bond may be a possible cross-linkage in casein (30). There appears to be no serious chemical attempt to look for cross-linking phosphate groups which may be cleaved under normal conditions of phosphoprotein study. Several pyrophosphorylated enzyme intermediates have been reported (31-33) suggesting that pyrophosphate may be covalently linked to proteins in the same way as orthophosphate.

Several possible artifacts which may occur during the study of phosphoproteins have been discussed in a recent review by Taborsky (34), such as the strong but noncovalent association of phosphate with protein and the indirect association between protein and phosphorus (e.g., binding

of lipid, carbohydrate, and nucleoside phosphate to protein). In studying protein kinase and phosphoprotein phosphatase, which catalyze the formation and hydrolysis of the phosphoester bond, one should also be aware of other covalent linkages besides phosphoester bonds (i.e., phosphoanhydride, phosphoamidate, and pyrophosphorylation) which may arise as artifacts. In addition, macromolecules containing phosphate, such as polyphosphate (35), may also be present, which also can be precipitated by trichloroacetic acid, a common method for separating phosphoprotein from small molecules (e.g., P_i and ATP). Polyphosphate kinases, which transfer γ -phosphate from ATP to polyphosphate, have been isolated from several species of bacteria (36,37) and the enzymic reaction is stimulated by histone or protamine. Thus, when these basic proteins are used as protein substrates for studying protein kinase in bacterial systems (38,39), the phosphorylated product of the reaction should be chemically characterized carefully.

II. Phosphoproteins

As components of biological systems, phosphoproteins maintain structures, provide nutrients, promote and regulate metabolic reactions, mediate neural and hormonal control mechanisms, and play an important role in several other cellular functions. In this part of the review, I will

discuss the enzymes and regulatory proteins which have been shown to be metabolically interconvertible through phosphorylation-dephosphorylation reactions, the structural and nutrient phosphoproteins, and their possible functions and potential regulatory role in biological systems.

A) Phosphorylation of enzymes

The interconversion of key enzymes between active and inactive forms through cycles of covalent modification is an important control mechanism involved in the regulation of many biological processes (1-5). Phosphorylation (6-10) and nucleotidylation (5,11) are the best known examples. In eukaryotes, phosphorylation has been found to be one of the most important metabolic regulatory mechanisms which is involved in mediating the function of a number of hormones (e.g., ACTH, glucagon, and epinephrine, etc.) (12).

Among many phosphorylated enzyme systems recognized (Table 1), the mammalian glycogen metabolic system (352-379, 469) is one of the most intensively studied. The system is summarized in Scheme 2.

First indication that interconversion of phosphorylase might involve phosphates was found by Cori and Green in the mid-forties (460,461). Ten years later, the interconversion was correlated with phosphorylation both in muscle (352,462, 463,467) and in liver (464-466). It was the study of the

SCHEME 2

Phosphorylation and Dephosphorylation of Mammalian Glycogen Metabolic System

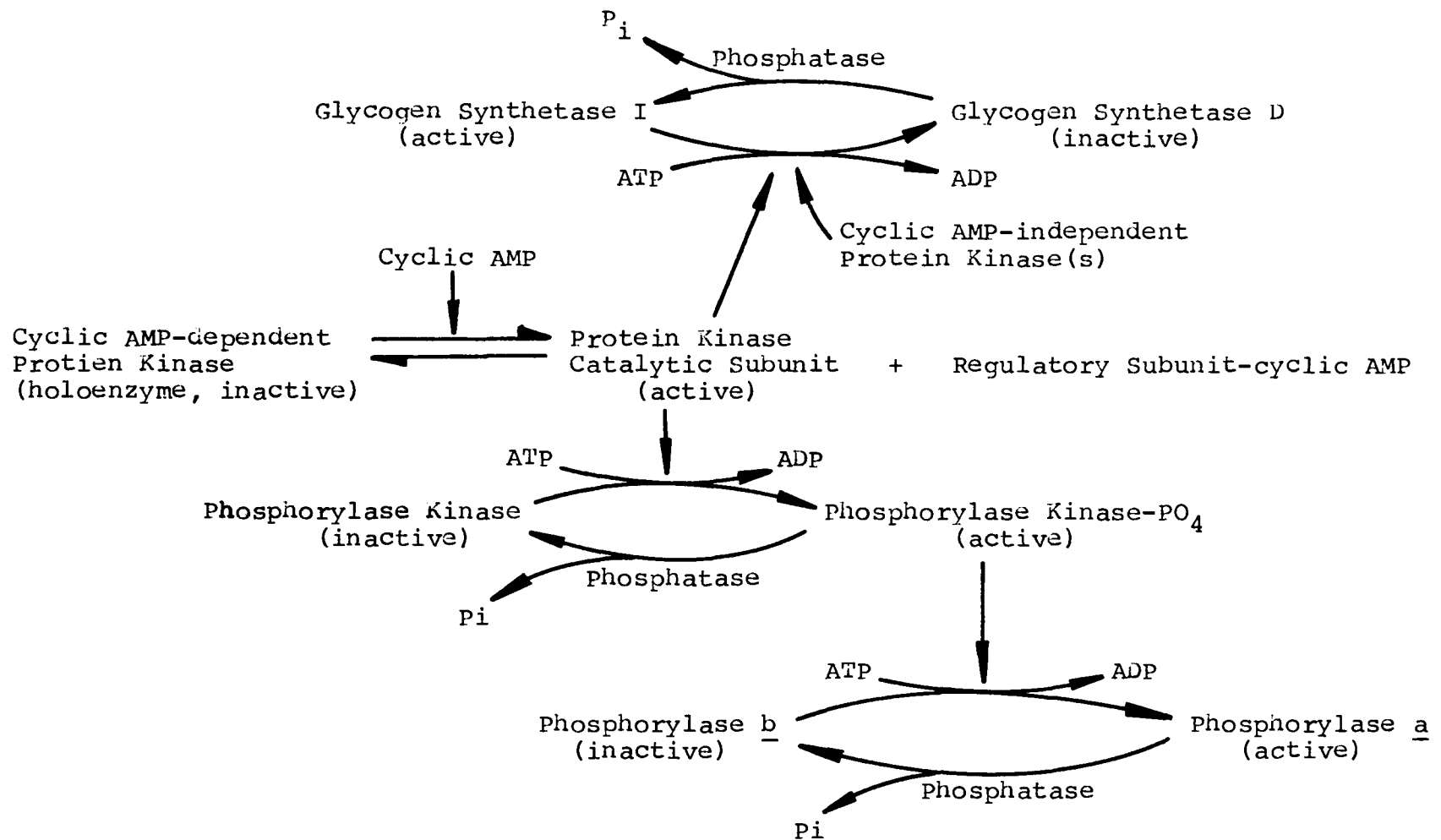


TABLE 1
Phosphorylation of Enzymes

Enzymes	Phosphorylation Sites	Activity Changed by Phosphorylation	References
<u>Glycogen Phosphorylase</u>			
(subunit: $M_r=100,000$)	-Lys-Gln-Ile-Ser(P)-Val-Arg- (muscle)	increasing	343-357 352-357
	-Arg-Gln-Ile-Ser(P)-Ile-Arg- (liver)	(AMP independent)	352-354
<u>Phosphorylase Kinase</u>			
Subunit α ($M_r=145,000$) (or Subunit A, $M_r=118,000$)	-Arg-Leu-Ser(P)-Ile-Ser-Thr- (cyclic AMP PK)	stimulating dephosphorylation of β subunit	358-365, 384 362, 379
Subunit β ($M_r=128,000$) (or Subunit B, $M_r=108,000$)	-Lys-Arg-Ser-Gly-Ser(P)-{ ^{Val} _{Ile} }- -Tyr-Glu-Pro- (cyclic AMP PK)	increasing (pH 6.8/8.2 ratio)	379
<u>Glycogen Synthetase</u>			
(subunit: $M_r=85,000-90,000$)	-{ ^{Lys} _{Arg} }-Ser-Asn-Ser(P)-Val-Asp- (cyclic AMP PK)	?	366-378, 469 371, 377

TABLE 1 contd.

	-{Lys} Arg}-Arg-Ala-Ser(P)- (cyclic AMP PK)	decreasing (glucose-6-phosphate dependent)	371
	-{Lys} Arg}-Glu-Ile-Ser(P)-Val-Arg-	?	366
<u>Lipase</u>			
Triglyceride, Diglyceride & Monoglyceride Hydrolases	Phosphoester (cyclic AMP PK)	increasing	380-386
<u>Acetyl-CoA Carboxylase</u>			
(dimer: $M_r = 215,000$)	Phosphoester	decreasing	387-391
<u>Fatty Acid Synthetase</u>	-	decreasing	423-425
<u>Palmityl-CoA Synthetase</u>	-	increasing	392-394
<u>Pyruvate Kinase</u>			
L-type (subunit: $M_r = 57,000-61,000$)	-Leu-Arg-Arg-Ala-Ser(P)-Leu- (cyclic AMP PK)	decreasing	395-402
<u>Pyruvate Dehydrogenase</u>			
(α -subunit: $M_r = 41,000$)	-Tyr-His-Gly-His-Ser(P)-Met-Ser-	decreasing	403-422
	-Pro-Gly-Val-Ser(P)-Tyr-Arg-	?	409-411
	-Gly-Met-Gly-Thr-Ser(P)-Val-Glu-Arg-	?	409
<u>Phosphofructokinase</u>			
(subunit: $M_r = 24,000$)	Ser-P	increasing	426-428

TABLE 1 contd.

Fructose 1,6—Diphosphatase

(M _r =130,000; subunit:M _r =65,000 ?)	-	decreasing	429,430
	(cyclic AMP PK)		

Cholesterol Side Chain Cleavage Enzyme System

-	increasing	431
(cyclic AMP PK)		

Cholesterol Esterase

-	increasing	383,432-437
(cyclic AMP PK)		

Hydroxymethylglutaryl-CoA Reductase

-	decreasing	481-483

Phenylalanine Hydroxylase

(subunit:M _r =50,000)	Phosphoester	increasing	438-440
	(cyclic AMP PK)		

Tyrosine Hydroxylase

indirectly	activator (?)	increasing	441-445
	(cyclic AMP PK)		

Tyrosine Aminotransferase

(subunit:M _r =50,000)	Ser-P	releasing from polysome ?	446,447
	(cyclic AMP PK ?)		

TABLE 1 contd.

Carbonic Anhydrase

(carbonic anhydrase I $M_r=30,000$)	- (cyclic AMP PK)	increasing	448

Reverse Transcriptase

(β subunit: $M_r=100,000$)			86
(ϕ subunit: $M_r=15,000$)	phosphoester	increasing	81
			83

DNA Dependent RNA Polymerase

<u>E. coli</u> RNA polymerase	Thr-P (major)	decreasing	70
(β and β' subunits)	Ser-P (bacteriophage induced protein kinases)		
(σ factor)	Ser-P (cyclic AMP PK)	increasing	75,449

RNA polymerase I

(subunits:SA ₁ ,SA ₃ ,SA ₅ ,SA ₆)	Ser-P & Thr-P (cyclic AMP PK)	increasing	449-452

RNA polymerase II

(subunits: $M_r=25,000$ & 180,000)	Ser-P & Thr-P (cyclic AMP PK)	increasing	452-454

Yeast RNA polymerase

Ser-P & Thr-P	?	455,456

TABLE 1 contd.

<u>Adenylate Cyclase</u>	?	decreasing	155-157
<u>Polynucleotide Phosphorylase</u>	?	increasing	76
<u>Pepsin</u>	-Glu-Ala-Thr-Ser(P)-Glu-Glu-Leu-	?	64,457

(cyclic AMP PK) indicates the phosphorylation sites which were phosphorylated by cyclic AMP-dependent protein kinase.

activation effect of glucagon and epinephrine on liver phosphorylase that led to the discovery of cyclic AMP by Sutherland in the late fifties (14,15). During its activation, one phosphate was found to be transferred from ATP to each subunit ($M_r=100,000$) of phosphorylase and the phosphorylation site was determined (352-355) (Table 1). Although the phosphorylation converted muscle phosphorylase from a dimer to a tetramer, the phosphorylation of liver phosphorylase did not induce molecular aggregation (355). (For review of phosphorylase, cf. 356,357.) Subsequently, the stimulation effect of cyclic AMP was demonstrated to be mediated by a cyclic AMP-dependent protein kinase which phosphorylated the phosphorylase b kinase. The phosphorylase b kinase was converted into an active form by the phosphorylation (358,359,468) which could then phosphorylate phosphorylase b. Two phosphorylation sites on phosphorylase b kinase have been identified, one on subunit α (or A) and the other on subunit β (or B) (360,362,379) (Table 1). The phosphorylation of subunit β was accompanied by activation of phosphorylase b kinase. The phosphorylation of subunit α did not activate the enzymatic activity, but stimulated the rate of dephosphorylation of subunit β by phosphoprotein phosphatase (364,365,378). Multisite phosphorylation of

enzyme proteins have also been found in glycogen synthetase (369-373,375,376), pyruvate dehydrogenase (407,409,411), and RNA polymerase (449-456), and multisite phosphorylation has been suggested to have a potential regulatory function (378).

Larner and colleagues established the existence of two forms of glycogen synthetase, namely D form (glucose-6-phosphate dependent) and I form (glucose-6-phosphate independent) (470, 471). Analogous to the phosphorylase system, the two forms of glycogen synthetase were interconvertible by phosphorylation and dephosphorylation, except that the phosphorylated glycogen synthetase was the less active form (D form) (for review, cf. 368). The phosphorylation and inactivation of glycogen synthetase was shown to be catalyzed by the cyclic AMP-dependent protein kinase, which also phosphorylated phosphorylase b kinase (469). The phosphorylated glycogen synthetase was reported to contain up to 6 moles of phosphate per enzyme subunit ($M_r=85,000-90,000$) (472). However, it seems only one or two phosphorylation sites are involved in determination of glucose-6-phosphate dependency (369-371, 375-377, 469). Amino acid sequences of some of the phosphorylation sites have been determined (Table 1). Recently, a number of reports have shown that the phosphorylation and inactivation of glycogen synthetase could be catalyzed by a

cyclic AMP-independent protein kinase(s) (369,370,373,375, 376), which is clearly distinguished from the cyclic AMP-dependent protein kinase (469). The potential regulatory significance of the cyclic AMP-independent protein kinase remains to be elucidated. The properties of protein kinase and phosphoprotein phosphatase are discussed in following sections.

A number of enzymes, besides the glycogen metabolic system, have been reported to be phosphorylated and/or regulated by phosphorylation (Table 1). However, some of these experiments need further study to substantiate this fact (e.g., palmityl-CoA synthetase, adenylate cyclase and polynucleotide phosphorylase). Phosphorylation of some enzymes, such as tyrosine aminotransferase (446,447), yeast RNA polymerase (455,456) and pepsin (64,457) has been demonstrated in vitro and in vivo, but no functional change has been correlated to this chemical modification.

The activities of lipase (380-386) and palmityl-CoA synthetase (392-394), the catabolic enzymes in fatty acid and lipid metabolism were increased by phosphorylation. On the other hand, the activities of fatty acid synthetase (424,425) and the rate limiting enzyme in fatty acid synthesis, acetyl-CoA carboxylase (387-391), were decreased

by phosphorylation. Moreover, recently, there has been some indication that the first enzyme in lipid synthesis, glycerol-3-phosphate acylate transferase, may also be inactivated by phosphorylation (473).

Phosphorylation of L-type pyruvate kinase has been suggested to mediate the stimulation effect of cyclic AMP on gluconeogenesis (399,401). One phosphate was shown to be incorporated into each subunit ($M_r=57,000-61,000$) and the site of phosphorylation was also determined (Table 1). The activity of pyruvate kinase was found to be decreased by phosphorylation (395-400,402).

The phosphorylation of pyruvate dehydrogenase (403-422) resulted in a decrease in enzymic activity. The phosphorylation was stimulated by acetyl-CoA and NADH (474), instead of cyclic AMP (406-417), and inhibited by CoA and NAD^+ (474). The phosphorylation sites were located in the α -subunit ($M_r=41,000$) (405,407,409,411), and only one of the phosphorylation sites was correlated with the decrease of the enzymic activity (409,411) (Table 1).

Another control point in glycolysis and gluconeogenesis, phosphofructokinase and fructose 1,6-diphosphatase, were reported to be phosphorylated. The activity of phosphofructokinase was increased by phosphorylation (426-428).

On the other hand, the activity of fructose 1,6-diphosphatase was decreased by phosphorylation (429,430). If this phosphorylation control is related to the regulation of gluconeogenesis by hormones (e.g., epinephrine or glucagon), these hormones must stimulate the dephosphorylation of phosphofructokinase and fructose 1,6-diphosphatase. The mechanism of this regulatory function remains to be solved.

Activation of cholesterol esterase by cyclic AMP-dependent phosphorylation may play a role in hormonal stimulated (e.g., ACTH and LH) steroidogenesis (432-437). The activated cholesterol esterase may liberate the cholesterol from the cholesterol ester-riched lipid droplets and maintain the supply of free cholesterol to the mitochondria, where the rate-limiting step in steroidogenesis takes place, i.e., the cholesterol side-chain cleavage reaction. The enzyme system which catalyzes this reaction was also reported to be activated by phosphorylation (431). A "rapidly turning-over protein" in adrenal cortex was proposed to be essential for ACTH stimulated steroidogenesis and may be phosphorylated (475). Further experiments are needed to identify the "rapidly turning-over protein," the phosphorylation site(s), and its functions.

Hydroxylmethylglutaryl-CoA reductase catalyzes the

rate-limiting reaction of hepatic cholesterologenesis under most physiological conditions. Interconversion of the active and inactive enzyme has been observed (481-483). The enzymic activity was inactivated by an inactivator protein ($M_r > 150,000$) in the presence of ATP and Mg^{++} (481,482), and was reactivated by an activator protein ($M_r = 20,000 - 30,000$) (482) or purified liver phosphorylase phosphatase ($M_r = 35,000$) (483). The interconversion of the reductase was suggested to be modulated by phosphorylation-dephosphorylation (481), and the inactivator and activator to be protein kinase and phosphoprotein phosphatase, respectively (483). However, the direct phosphorylation of the reductase has not been demonstrated yet. (The direct phosphorylation of the reductase by both Rodwell and Beg was cited recently as personal communication by Ingebritsen et al. (483).)

In amino acid metabolism, the regulation of phenylalanine hydroxylase and tyrosine hydroxylase were found to be associated with phosphorylation. These two enzymes are involved in synthesis of catecholamines (e.g., dopamine, epinephrine, and norepinephrine), which are involved in adrenal endocrine function and neurotransmission. One phosphate was found in each phenylalanine hydroxylase subunit ($M_r = 50,000$), and the enzymic activity was stimulated by phosphorylation

(438-440). The stimulation of tyrosine hydroxylase by phosphorylation may be indirect, since no phosphate has been found to be incorporated into the enzyme per se (441-445). The phosphorylation of tyrosine aminotransferase has also been demonstrated. The phosphorylation site was shown to be a serine residue, but no activity change was correlated with the phosphorylation (446). However, the phosphorylation of tyrosine aminotransferase may induce the release of the enzyme from polysomes (447).

Carbonic anhydrase was also activated by cyclic AMP-dependent phosphorylation (448). This implies that the mechanism of cyclic AMP stimulated gastric juice secretion may involve the activation of carbonic anhydrase by phosphorylation in gastric mucosa.

Viral RNA-dependent DNA polymerase (reverse transcriptase) was found to be phosphorylated (81,83,86). The phosphorylation form of the enzyme was more active than the dephosphorylated form (83,86). The β -subunit ($M_r=100,000$) of the enzyme was shown to be phosphorylated (81) and the phosphorylation sites located on a small, acidic protein ($M_r=15,000$), which may be a proteolytic product of the β -subunit (83) (cf. Bacterial and viral phosphoproteins section).

Subunit β and β' of E. coli RNA polymerase was phosphorylated, mainly at threonine residue, by bacteriophage T_7 -induced protein kinase. The enzymic activity was inhibited by this phosphorylation (70), which may be the shut-off mechanism of host RNA synthesis. In addition, the σ factor of the E. coli RNA polymerase was phosphorylated by the cyclic AMP-dependent protein kinase from rabbit skeletal muscle (75,449). This phosphorylation increased the enzymic activity. The phosphorylation of eukaryotic RNA polymerase I (449-452) and RNA polymerase II (452-454) was cyclic AMP-stimulated and the phosphorylation was correlated with increasing enzymic activities. Aside from histones, nuclear acidic proteins, and nucleoli proteins (cf. Nuclear phosphoproteins section), these RNA polymerases may be another possible phosphorylation site which regulates transcription.

As discussed above, phosphorylation seems to be involved in regulation of metabolism of carbohydrates, fatty acids, lipids, steroids, amino acids, replication, transcription, and translation. It is interesting to see if any key enzymes in the metabolism of purines, pyrimidines, nucleosides, and nucleotides are regulated by phosphorylation. These are the only important intermediary metabolism which

have not yet been correlated with phosphorylation.

B) Enzyme cascade systems

The metabolic enzymes described in the previous section may be regulated by the following mechanisms: (1) Biosynthesis and degradation of the enzyme proteins. Enzyme induction and gene expression have been extensively studied in the field of molecular biology. Very little, however, is known about the degradation of enzyme proteins at the present time. Future investigation about the enzyme degradation is essential to elucidate the regulation of enzyme protein level in the cell. This kind of control mechanism is relatively long term and energy consuming (comparing ATP required for protein synthesis and phosphorylation modification). (2) Non-covalent modification of the enzyme. The modification effectors, which may be micromolecules (e.g., metabolites) or macromolecules (e.g., inhibitor proteins), are bound to the enzyme protein by non-covalent bonds. These non-covalent interactions may alter the enzymic activity and kinetic properties even though the binding sites may not be at the reaction sites. The sites on an enzyme protein for these allosteric effectors are limited and the regulation effects are discontinued when these effectors have disappeared (e.g., feedback mechanism). (3) Covalent modification of

the enzyme. Enzymic phosphorylation, nucleotidylation, adenosine diphosphate ribosylation, oxidation, and reduction of some enzyme proteins have been found to change the activity of these interconvertible enzymes (1-5). These chemical modifications are generally reversible reactions, which are catalyzed by two separate "converter" enzymes. Irreversible chemical modification by selective proteolysis (458,562) (e.g., blood clotting process and activation of zymogens) may be viewed as a step in functional protein synthesis.

The discovery of covalent modification of interconvertible enzymes led to the development of a new concept of metabolic regulatory function—the enzyme cascade system (476-478). As shown in Scheme 2, glycogen synthetase represents a monocyclic enzyme cascade system, and phosphorylase and phosphorylase kinase represent a multicyclic enzyme cascade system.

A theoretical analysis of enzyme cascade systems (476, 477) shows that the systems display a remarkable flexibility for the amplification of regulatory inputs and an extensive capacity for the integration of different allosteric stimuli. Stadtman and Chock (476) suggested that, in order to maintain these abilities, the enzyme interconversion seems to be in a dynamic process rather than in an "all-or-

none" reciprocal control of the opposing converter enzymes. ATP decomposition, which is caused by cyclic cascades, supplies the energy needed to maintain concentrations of modified enzymes at steady-state levels, and, therefore, it is not a futile process.

A number of effectors are known to be able to modulate the converting reaction by interacting with the converter enzymes (e.g., cyclic AMP and heat stable protein inhibitor for cyclic AMP-dependent protein kinase, Ca^{2+} for phosphorylase kinase). It has been suggested that modulation at substrate level represents an important regulatory mechanism for the protein modification reactions (520,569,570,581,582), e.g., glucose for phosphorylase kinase (726) and AMP for phosphorylase (582). Although the allosteric effector-substrate enzyme interaction can greatly affect the protein modification reactions, it has not been considered in the theoretical treatment of the enzyme cascade system by Stadtman and Chock (476,477). Steady-state expression of the modulation at substrate level in a monocyclic system is similar to that of the modulation at the converter enzyme level (Similar equations can be derived for the effectors to modulate the fractional activity via interaction with

substrate enzymes).^{*} This suggests that modulation at substrate enzyme level may be a regulatory mechanism as important as modulation at the converter enzyme level for the covalent modification reactions.

Besides modulating the fractional activities of the target enzyme, the allosteric modification at substrate enzyme level can also modulate the effects of the primary signal (e.g., cyclic AMP). Both the maximum fractional activity ($F_{\max}$) of the target enzyme produced by the primary signal and the concentration ($[e_1]_{1/2}$) of the primary signal required to produce the half maximum fractional activity are functions of the concentration of the allosteric effectors.^{**}

^{*}For example: The inactive form (I_i) of substrate enzyme for E_a , the active converter enzyme, is generated from the interaction of I_i' with an effector (e_4). K_4 is the dissociation constant of the interaction. Therefore,

$$I_i \xrightleftharpoons{K_4} I_i' + e_4; [I_i] = \frac{[e_4]}{K_4} \times [I_i']$$
 The steady-state expression for fractional activity, F , can be derived under the assumption made by Chock and Stadtman (476,477).

$$F = \frac{[I_a]}{[I]} = \left[\frac{\alpha_r [R][e_2] (K_1 + [e_1])}{\alpha_f [E][e_1] (K_2 + [e_2])} \times \frac{K_4 + [e_4]}{K_4} + 1 \right]^{-1}$$

^{**}Effector e_4 interacts with the substrate enzyme as described above. If $B = \frac{\alpha_r [R][e_2]}{\alpha_f [E] (K_2 + [e_2])}$ is a constant.

From the equation described above, the following equations can be derived: $F_{\max} = f_1([e_4]) = \frac{K_4}{(B+1)K_4 + B[e_4]}$ and

$[e_1]_{1/2} = f_2([e_4]) = K_1 \left(1 - \frac{K_4}{(B+1)K_4 + B[e_4]} \right)$. Similar equations can be derived when the effector worked as either an activator or an inactivator for the converter enzymes, E or R , at the substrate level.

In the case of allosteric effector-substrate enzyme interaction results in an inactivation of the converting reaction, the equation indicates that the effect of the primary signal can be totally blocked at high concentration of the allosteric effector (when $e_4 \longrightarrow \infty$, therefore $F_{\max} \longrightarrow 0$)^{**} (see footnote , p. 27).

The above discussion clearly indicates that the allosteric effector-substrate enzyme interaction not only can increase the flexibility for amplification of the primary signal but also can increase its capacity for integration of different metabolic signals. The amplification properties of the enzyme cascade system enable the target enzyme to fully respond to the primary signal, which may have a concentration much lower than that of the target enzyme. Because of its flexibility, the enzyme cascade system can adjust its response to the primary signal according to different cellular metabolic conditions. The adjustment is achieved by integrating different metabolic signals into the cascade system. The multisite phosphorylation (378) of enzyme proteins makes this system even more sophisticated.

In order to elucidate the regulation of interconvertible reactions, the properties of converter enzymes must be well understood. The converter enzymes in phosphorylation

modification are protein kinases and phosphoprotein phosphatases, which will be discussed in later sections.

C) Membrane phosphoproteins

A large number of physiological functions of living organisms take place at biological membranes (e.g., water permeability, transduction of neural signals, transport of biological substances). Since membrane is a rich source of protein-bound phosphate, the study of the possible regulatory role of membrane protein phosphorylation certainly is an interesting field. Membrane phosphoproteins and their possible physiological functions are summarized in Table 2.

1) Nerve cells and synaptic membranes

Brain contains about 10 nmol of phosphoserine and 1 nmol of phosphothreonine per mg protein (185). Some of these phosphates turn over rapidly (186), and this dynamic metabolism may relate to brain function (187). Cyclic AMP has been proposed to modulate some types of synaptic transmission by protein phosphorylation in the central and peripheral nervous systems (115-120). Two synaptic membrane proteins were found to be regulated by cyclic AMP, namely, protein I ($M_r=86,000$) and protein II ($M_r=48,000$) (120,121). Protein I is composed of two types of polypeptides, I_a ($M_r=86,000$) and I_b ($M_r=80,000$), both of which have been

TABLE 2
Membrane Phosphoproteins

Phosphoproteins	Phosphorylation Sites	Possible Functions	References
<u>Synaptic Membranes</u>			
Protein I _a (M _r =86,000)	Ser-P	synaptic functions ?	120-124
Protein I _b (M _r =80,000)	Ser-P	synaptic functions ?	122
Protein II (M _r =48,000)	Ser-P	SCARP ^a (?)	120,121
<u>Myelin</u>			
Myelin Basic Protein (M _r =15,000-18,000)	-Arg-His-Gly-Ser(P)-Lys-Tyr- 12	?	126-132
	-His-Arg-Asp-Thr(P)-Gly-Ile- 35		
	-Lys-Arg-Gly-Ser(P)-Gly-Lys- 56		
	-Arg-Gly-Leu-Ser(P)-Leu-Ser- 110		
	CH ₃		
	phosphohistidine & phosphoarginine	?	27

TABLE 2 contd.

Disk Membrane of Rod Outer Segment

rhodopsin ($M_r=35,000$)	Ser-P & Thr-P	Regulation of light sensitivity of visual system.	134-139
minor components	-		136

Erythrocyte Membranes

band II ($M_r=215,000$)	phosphoester	fibrillar network formation	140-148
subunit of spectrin complex		(maintain cell shape)	
protein of $M_r=240,000$	- (cyclic AMP sensitive)	cation permeability	149
band III ($M_r=88,000$)	phosphoester	binding & regulating(?) glyceraldehyde-3-phosphate dehydrogenase	142,148,150, 151
band IV _C ($M_r=50,000$)	phosphoester	SCARP (?)	141,148,152,153

Steroid- and Cyclic AMP-Regulated Phosphoprotein (SCARP)

protein D (Toad Bladder)	-	sodium transport and water permeability	163-166
($M_r=49,000-50,000$)			
SCARP	-	biological effects of steroid hormones	165,166

TABLE 2 contd.

Secretory Membranes

adenohypophyseal plasma	Ser-P & Thr-P	secretory process	167,168
membrane & secretory granule			

islet cells ($M_r=40,000$)	-	secretory process	169

zymogen granules	-	secretory process	170

Sarcoplasmic Reticulum

phospholamban ($M_r=20,000-22,000$)	phosphoester	Ca^{2+} transport	171-180

protein of $M_r=95,000$	phosphoester	Ca^{2+} -ATPase (?)	180

Sarcolemma

proteins of $M_r<30,000$	phosphoester	Ca^{2+} transport	181-184

Smooth Muscle Membranes

proteins of $M_r=130,000$ & 100,000	-	cyclic GMP mediated cholinergic effect	162

Other Plasma Membranes

fat cell ($M_r=16,000$ & 22,000)	-	glucose transport	154

TABLE 2 contd.

polymorphonuclear granulocytes	-	regulating adenylate cyclase	155-157
platelet			
liver	-	permeability	158,159
kidney ($M_r=35,000$)	Ser-P	amino acid transport	160,161

^aSCARP: Steroid- and Cyclic AMP-Regulated Phosphoprotein (165).

purified (121,122). Protein I appears to be specific to neuronal membranes (121). Its appearance correlates with synaptogenesis in the brain of developing rat and is localized in the synaptic region (122). These facts indicate that protein I may play an important role in synaptic functions and is regulated by phosphorylation. Besides cyclic AMP, Ca^{2+} has also been shown to induce protein I phosphorylation in rat brain synaptosomes. Calcium-dependent phosphorylation of protein I may play a role in presynaptic functions, and cyclic AMP-dependent phosphorylation of the protein may play a role in postsynaptic functions (124,125).

In contrast to protein I, protein II appears to be a nonspecific membrane protein. It appears to be the Steroid- and Cyclic AMP-Regulated Phosphoprotein (SCARP) described by Liu and Greengard (165). The SCARP with a molecular weight of about 50,000 has been suggested to be the regulatory subunit of cyclic AMP-dependent kinase (122,166, 188).

In the sixties, before the discovery of cyclic AMP-dependent protein kinase and cyclic AMP mediated nervous function, cyclic AMP-independent protein kinase (186) and phosphoprotein phosphatase (211) in nervous systems received considerable attention. The regulatory properties and

physiological functions of this phosphatase and protein kinase, however, remain unclear. In nerve cells, besides synaptic membrane phosphorylation systems, there are phosphoproteins, phosphatases, and protein kinases in cytoplasm and subcellular organelles. Even within the synaptic system, there are a number of phosphoproteins which are not regulated by cyclic AMP (121). Their physiological functions, properties, and regulation of phosphorylation are yet to be elucidated.

Myelin of brain and spinal cord contains a basic protein ($M_r=15-18,000$) which constitutes about 30% of its total protein (126-132). The presence of both protein kinase (129) and phosphatase in myelin (130), which use myelin basic protein as an effective substrate, indicates that these enzymes may control the phosphorylation of the protein. The four phosphorylation sites have been reported (Table 3) (126,131,189). Besides phosphoserine and phosphothreonine, myelin basic protein may also contain phosphoarginine and phosphohistidine as its phosphate-protein linkage (27). The physiological significance of the phosphorylation of myelin basic protein remains to be solved.

2) Rod outer segment of the lens

The major protein component of rod outer segment

of lens is rhodopsin ($M_r=35,000$), which is localized in the disk membranes. Rhodopsin is the visual pigment which mediates the light absorption function known as the bleaching process (133). In the early seventies, the rhodopsin in rod outer segment was found to be phosphorylated in vitro by ATP and the phosphorylation was induced by light (134-136). Light-dependent phosphorylation and dephosphorylation of rhodopsin were demonstrated later in living frogs (139). Both phosphoserine and phosphothreonine were present in phosphorylated rhodopsin (134,137). Besides rhodopsin, some other minor components in rod outer segment may also be phosphorylated (136). A water soluble protein kinase has been isolated from rod outer segment (137,138). This cyclic AMP-independent protein kinase acts only on photo-bleached rhodopsin, but not the unbleached one (137,138). Dephosphorylation of rhodopsin in rod outer segment is very slow (136-138). The time course of rhodopsin phosphorylation is too slow to be involved in the direct response to light. It has been postulated, however, that the phosphorylation is involved in slow, long-term changes in the sensitivity of the visual system for the adaptation of the photoreceptors to light or dark conditions (137,139), i.e., the physiological significance of the phosphorylation

may be in the regulation of light sensitivity rather than in the mediation of the light signal. The phosphorylation seems to be independent of photopigment regeneration (137, 139). The possible regulatory site(s) has been suggested to be involved in decreasing sodium conductance (134) or closing transmitter channels (137).

3) Erythrocyte membranes

About a dozen membrane proteins have been identified in erythrocytes by SDS gel electrophoresis (140,143). Three ($M_r=215,000$; 88,000; 50,000) of them may serve as substrates for endogenous protein kinase (141,142,148). Cyclic AMP increased the rate of phosphorylation of proteins of $M_r=88,000$ and 50,000, but has little effect on the phosphorylation of the protein of $M_r=215,000$ (141,142,148). Both phosphoserine and phosphothreonine are present in these phosphoproteins (141,142). Protein of $M_r=215,000$ (Band II, ref. 143) may be a subunit of the spectrin complex (140), and appears to have a single major phosphorylation site which is located in a CNBr cleaved fragment of $M_r=19,000$ (145). The phosphorylation of spectrin seems to lead to polymerization or fibrillar network formation of the spectrin complex on the membrane, which in turn induces the disk (or cup) shape change in erythrocytes (144,145).

This indicates that the phosphorylation of spectrin plays a role in maintaining cell shape (144,145,147). Cyclic AMP-dependent phosphorylation of a membrane protein ($M_r=240,000$) in the Band II region has been suggested to be involved in mediation of the β -adrenergic effects on increasing the cation permeability of the avian erythrocyte membrane (148).

Protein of $M_r=88,000$ (Band III, ref. 143) spans the erythrocyte membrane and is a glycoprotein (190). There are binding sites on the protein at the inner surface of the membrane for glyceraldehyde-3-phosphate dehydrogenase (150,191) and for a band 4.2 protein ($M_r=72,000$) (191). The physiological role of the phosphorylation, which has yet to be proved, may be involved in regulating glyceraldehyde-3-phosphate dehydrogenase.

Protein of $M_r=50,000$ (Band IV_C, ref. 143) may be similar to the protein found in synaptic membrane (protein II), toad bladder membrane (protein D; cf. following section), and other tissues (121,166) and has been suggested to be the SCARP or the regulatory subunit of the cyclic AMP-dependent protein kinase (122,166,188,192). Its existence, however, in rabbit erythrocytes (152), which do not contain cyclic AMP-dependent protein kinase or cyclic AMP-binding activity, and experiments which separated the cyclic

AMP-binding activity from [^{32}P] phosphoprotein IV_C (153) indicate that there may be several phosphoproteins in the Band IV_C region (on SDS gel electrophoresis).

4) Steroid- and Cyclic AMP-Regulated Phosphoprotein (SCARP)

The antidiuretic hormone (vasopressin) and mineral corticoid (aldosterone) regulate the flow of water and sodium across renal epithelial cells. The hormonal actions seem to be associated with the phosphorylation states of a membrane phosphoprotein of $M_r=50,000$ (163-165,188). The protein was named protein D (163), which appears in several other tissues (166). The name subsequently was changed to SCARP, for Steroid- and Cyclic AMP-Regulated Phosphoprotein, by Liu and Greengard (165). The SCARP has been found in cytosol fractions as well as in membrane fractions of a variety of tissues, including kidney and toad bladder (165, 166). By using membrane fractions from renal epithelial cells purified by free flow electrophoresis, Dr. I. L. Schwartz and his colleagues (194) have localized the adenylate cyclase in the serosal membrane (marker: Ca^{2+} -ATPase) and the cyclic AMP-dependent protein kinase and its endogenous substrates in the mucosal membrane fraction (marker: HCO_3^- -ATPase). This indicates that vasopressin action is mediated by generating cyclic AMP at the serosal membrane where the

vasopressin receptor is located, and the cyclic AMP, in turn, regulates the phosphorylation of the mucosal membrane and changes the permeability of the membrane. Dr. Greengard and his colleagues (163) have demonstrated that cyclic AMP mediated the vasopressin action by decreasing the phosphorylation of the protein of $M_r = 50,000$ (protein D). The protein D (SCARP) was suggested to be the regulatory subunit of cyclic AMP-dependent protein kinase (166). Later, the dephosphorylation of the regulatory subunit of cyclic AMP-dependent protein kinase by highly purified phosphoprotein phosphatase was shown to be stimulated by cyclic AMP, which increases the accessibility of the regulatory subunits to the phosphatase by interacting with the substrate protein (520). Although the studies carried out in Greengard's laboratory have been criticized for using unfractionated "microsomal membranes", its existence and the cyclic AMP-stimulated dephosphorylation of SCARP in plasma membrane from dog renal inner medulla have been recently reported by Huang et al. (188). The aldosterone action, which stimulates sodium transport without altering cyclic AMP levels in the toad bladder, may be mediated by stimulating the activity of SCARP phosphatase (164). Effects of several steroids on its target tissue may in part resemble that of aldosterone on the toad bladder via reducing the rate of phosphorylation of SCARP (10, 165), e.g., testosterone on

prostate, estradiol on uterus, and cortisol on liver. The proposed mechanism of SCARP in regulating sodium transport and membrane permeability is yet to be elucidated.

5) Secretory membranes

A model for the regulation of the secretory process by calcium and protein phosphorylation has been proposed by Rasmussen (195). The regulation of protein phosphorylation might involve cyclic AMP, which mediates some of the actions of physiological agents for activating secretory processes in several secretory tissues, such as anterior pituitary (167), islets of Langerhans (196), salivary gland (197), and thyroid (198). Interaction of storage granules with cell plasma membrane is essential for the exocytosis accompanying the secretory process. Phosphorylation of the adeno-hypophyseal plasma membrane (167) and secretory granule (168) at serine and threonine residues have been reported to be cyclic AMP stimulated. Several phosphopolypeptides from the phosphorylated adeno-hypophyseal plasma membrane have been identified by SDS gel electrophoresis (167), and may play an important role in cyclic AMP stimulates secretion of adeno-hypophyseal hormones.

Specific protein(s) with molecular weight about 40,000 have been found to be phosphorylated by endogenous protein

kinase in the plasma membranes of fish islet cells. The phosphorylation was enhanced by cyclic AMP (169). This implicates that the insulin and glucagon secretion from the islet cells may be regulated by this membrane phosphoprotein(s). On the other hand, phosphorylation of membrane proteins of zymogen granules have been shown to be stimulated by pancreozymin and caerulein (a pancreozymin related decapeptide) in pancreatic acinar cells (170). The function-phosphorylation relationship of those membrane phosphoproteins mentioned above and the mechanism in mediation of secretory process are still very far from established. Besides the granule and plasma membrane phosphoproteins, components of the microtubular-microfilamentous system and calcium related proteins are the other possible phosphorylation sites involving the secretory process (196).

6) Sarcoplasmic reticulum and sarcolemma

It is generally accepted that cyclic AMP mediates the positive inotropic effect of β -adrenergic stimulation on heart by altering the availability of intracellular calcium to contractile proteins (171). The membrane phosphorylation catalyzed by cyclic AMP-dependent protein kinase has been suggested to regulate the active transport of calcium ion across membranes. This hypothesis provides

a possible mechanism of cyclic AMP action in muscles (172-176). A specific small membrane protein of $M_r=20-22,000$ was identified as the phosphate receptor in cardiac sarcoplasmic reticulum (SR), by Katz and colleagues (175) and also by LaRaia and Morkin (172). The protein was named phospholamban (phosphate receiver) (175). The phosphoester linkage and molecular weight of the phospholamban are clearly distinct from the SR Ca^{2+} -ATPase of $M_r=100,000$ which has an acyl-phosphate enzyme intermediate. Similar results were obtained by using SR from slow contracting skeletal muscles but not from the fast contracting ones (176,180). The phosphorylation of the phospholamban has been correlated with stimulation of calcium uptake by SR as well as of the Ca^{2+} -ATPase activity in SR (172-176). The dephosphorylation of phospholamban was correlated with a decreased calcium uptake by SR (178). In contrast, Schwartz et al. (180) have reported that calcium uptake by SR from fast skeletal muscle were also stimulated by protein kinase and cyclic AMP. However, the phosphorylation site in the SR of fast skeletal muscle was in the protein fraction of $M_r=95,000$, which was different from phospholamban. In addition, phosphorylase b kinase was shown to stimulate calcium uptake and to phosphorylate the protein of $M_r=95,000$ in SR from a number of muscle

tissues (180). They have suggested that the $M_r=95,000$ component may be the Ca^{2+} -ATPase. But it should be noticed that there were large amounts of phosphorylase b in their SR preparation (180), and phosphorylase b with subunit molecular weight about 100,000 is a very good substrate for phosphorylase b kinase. The physiological role of phosphorylation of phospholamban may be associated with mediation of β -adrenergic effects on contractivity. The increased rate of calcium uptake into SR by phosphorylating phospholamban could increase the rate of relaxation directly as well as enhance contractivity by increasing available calcium to be released from SR. The increasing rate of calcium uptake into SR may be a result of the stimulation of the calcium pump (Ca^{2+} -ATPase) by phospholamban. Sarcolemma (plasma membrane of muscle) isolated from skeletal (181,184) and cardiac (182,183) muscle contain cyclic AMP-dependent protein kinase and phosphoprotein phosphatase. The protein kinase-catalyzed phosphorylation of sarcolemma were correlated to an increase in the Ca^{2+} -ATPase activity (181,183) and sarcolemmal calcium accumulation (181-183). Three sarcolemmal proteins with molecular weight less than 30,000 were phosphorylated with phosphoester bonds (184). The reversibility of the system catalyzed by phosphoprotein

phosphatase has also been demonstrated (181-183). It seems like there is a phosphorylation regulated calcium active transport system present in sarcolemma in addition to SR. Whether the phosphoprotein in sarcolemma is phospholamban as in SR remains to be elucidated.

7) Smooth muscle membranes

The phosphorylation of two proteins ($M_r=130,000$ and $100,000$) by endogenous cyclic GMP-dependent protein kinase in the membrane preparations from several different smooth muscles may mediate the actions of cyclic GMP in these tissues (162), and may be associated with cholinergic effects. The protein of $M_r=50,000$ (SCARP), which is regulated by cyclic AMP, was also present in these membrane preparations (162). Since the study was carried out with the "microsomal membranes," the intracellular location of these membrane phosphoproteins remains to be determined.

8) Other plasma membranes

Cyclic AMP has been found to stimulate the phosphorylation of two membrane proteins ($M_r=16,000$ and $22,000$) in fat cells. The phosphorylation was associated with inhibition of insulin-stimulated glucose transport, and these proteins have been suggested to have possible carrier function (154). The reported cyclic AMP-stimulated

phosphorylation of liver plasma membrane has been suggested to regulate the structure and function of the membrane, in particular the permeability of the membrane (158,159).

Membranes of polymorphonuclear granulocytes and platelets can be phosphorylated by ATP (155), and this phosphorylation may activate the activity of adenylate cyclase (156,157).

A small acidic protein ($M_r=35,000$) has been shown to be involved in binding amino acids (161) and can be phosphorylated and dephosphorylated (160). This indicates that the phosphorylation of the acidic protein of $M_r=35,000$ may be connected with the stimulatory effect of the parathyroid hormone and cyclic AMP on the active uptake of amino acids in kidney cortex (193).

D) Contractile and microtubular phosphoproteins

Although myosin was reported to be a phosphoprotein more than ten years ago (199), serious investigation into the phosphorylation of contractile proteins did not begin until Bailey and Villar-Palasi showed in 1971 (223) a cyclic AMP-dependent phosphorylation of troponin. A component of the light chain of skeletal muscle myosin was first reported by Perry and his colleagues (200) to be phosphorylated by protein kinase. The pair of myosin light chains of $M_r=18,000 \sim 20,000$ were termed P light chains (203), which are

TABLE 3

Contractile and Microtubular Phosphoproteins

Phosphoproteins	Phosphorylation Sites	Possible Functions	References
<u>Myosin</u>			
P light chain ($M_r=18,000-20,000$)	-Glu-Gly-Gly-(Ser,Ser(P))-Asn-Val-	regulating actin- activated ATPase	200-215,223
heavy chain ($M_r=180,000-220,000$)	-	regulating actin- activated ATPase	216-219
<u>Troponin</u>			
inhibitory subunit (TNI) ($M_r=28,000$)	Cardiac: Ser-20, Ser-37, Ser-146 skeletal muscle: Thr-11, Ser-118	mediated β -adrenergic effects ?	224-238
tropomyosin-binding subunit (TNT) ($M_r=37,000$)	(Asx,Ser(P),Glx)-Glu-Val- -Ala-Leu(Ser,Ser(P))-Met-Gly- -Ala-Asn-Tyr-(Ser,Ser(P))-Tyr-	 ?	228,240-242
<u>Other Contractile Proteins</u>			
actin($M_r=45,000$)	-	-	242

TABLE 3 contd.

tropomyosin ($M_r=33,000$)	Ser-P	-	243,244
filamin (subunit: $M_r=240,000-250,000$)	-	-	251,252
<u>Microtubule</u>			
tubulin			
(β subunit: $M_r=53,000$)	Ser-P	microtubule assembly and/or functions	256-259,264
microtubule-associated proteins ($M_r=70,000-300,000$)	-	microtubule assembly and/ or functions	259-262

identical to the 5,5'-dithiobis-(2-nitro benzoic acid) (DTNB) light chain (245). The presence of one phosphoserine residue per light chain and the phosphorylation site have been determined (Table 3) (201). Subsequently, the P light chain was demonstrated to be present in cardiac muscle (202,204,216), smooth muscle (203,205-207,213), and non-muscle myosins, such as platelet (209,210), myoblast (212), astrocyte (211), and fibroblast (217). The specific myosin light chain kinase (cyclic AMP-independent) was purified from striated muscle (220) and platelet (221), and a specific myosin light chain phosphatase was identified by Morgan et al. (222). Phosphorylation of the myosin heavy chain ($M_r=180,000 \sim 220,000$) has also been demonstrated in cardiac muscle (216), fibroblast (217), and Acanthamoeba castellanii (219). Muhlrads and Oplatka (217) suggested that two different myosin kinases are present in fibroblast; a myosin light chain kinase and a myosin heavy chain kinase. The A. castellanii cofactor protein ($M_r=97,000$) required for actin activation of Mg^{2+} -ATPase activity of Acanthamoeba myosin I (218) was shown to be a myosin heavy chain kinase (219).

The phosphorylation of smooth muscle (205-207,213) and non-muscle myosin (210,212) was shown to be a prerequisite for actin-activated ATPase activity (208). The regulation

of actin-activated ATPase activity of myosin by Ca^{2+} -dependent phosphorylation (activation) and Ca^{2+} -independent dephosphorylation (inactivation) provides a possible mechanism for Ca^{2+} mediated smooth muscle contraction and relaxation (205-208,213). In contrast, however, the myosin kinases from non-muscle tissue do not require Ca^{2+} (219,221). Although there is no functional change in the skeletal muscle myosin due to phosphorylation of the P light chain (222), the myosin of skeletal muscle (201,215,223) and cardiac muscle (204,216) was shown to be phosphorylated in vivo and related to contractile activity.

Troponin has been found to contain three functional components (246), namely inhibitory subunit (TNI, $M_r=24 \sim 28,000$), tropomyosin-binding subunit (TNT, $M_r=35 \sim 37,000$), and Ca^{2+} -binding subunit (TNC, $M_r=19 \sim 21,000$). TNI inhibits actomyosin ATPase activity and TNC blocks the inhibition by TNI in the absence of Ca^{2+} . The TNI was shown to be phosphorylated by phosphorylase kinase (225,228,230,238), cyclic AMP-dependent protein kinase (223,228,230,231,233, 235), and cyclic GMP-dependent protein kinase (236,237). The phosphorylation sites, two in skeletal muscle TNI and three in cardiac muscle TNI, have been determined (Table 3) (227-229,231). Besides TNI, TNT was shown to be the other

subunit of troponin that could be phosphorylated (228,238, 240-242). The phosphorylation of TNT could be catalyzed by phosphorylase kinase (228,240,241), but not significantly by cyclic AMP-dependent protein kinase (223,228,235,241). Three phosphorylation sites in skeletal muscle TNT were identified (Table 3) (241). There is no evidence to date, however, that indicates TNC may be phosphorylated.

No physiological significance for the phosphorylation of the skeletal muscle troponin has been proven. The phosphorylation in vitro of skeletal muscle TNI and TNT in the troponin complex was blocked by TNC (238,241). In addition, very little, if any, TNI was phosphorylated under in vivo conditions where TNI, TNT, and TNC exist as a complex (239). However, the skeletal muscle TNT does contain covalently bound phosphate in vivo (238,240). Although the skeletal muscle TNT could be phosphorylated by phosphorylase kinase, it seems very unlikely that the phosphorylation is important in the response of tissue to epinephrine (215), since the I-strain mice, deficient in phosphorylase kinase, did respond normally to epinephrine (8). In addition, phosphorylation has no significant effect on the ability of troponin to regulate the actomyosin ATPase activity (238).

On the other hand, the β -adrenergic effect on cardiac muscle was correlated with phosphorylation of cardiac TNI (204,232-235). The cardiac TNI could be phosphorylated by either phosphorylase kinase or cyclic AMP-dependent protein kinase. The TNC blocked the phosphorylation of cardiac TNI by phosphorylase kinase, but not by cyclic AMP-dependent protein kinase (230). The additional phosphorylation site (Ser #20) of cardiac TNI may be responsible for the β - adrenergic function (204,231-233). However, the functional changes of cardiac TNI due to phosphorylation have yet to be elucidated.

Besides myosin and troponin, actin (242) and tropomyosin (243,244) also have been reported to be phosphorylated. But no functional change has been correlated to their phosphorylation. The phosphorylation of erythrocyte myofilament protein, spectrin, has been described in a previous section (cf. Membrane phosphoproteins). Another actin-binding high-molecular-weight protein (M_r =240,000-250,000) was purified from smooth muscle (247-250,252) and non-muscle tissues (247,251), and was named filamin (247). The filamin was shown to be a substrate for cyclic AMP-dependent protein kinase (251,252). The physiological function of filamin and the significance of its phosphorylation remain to be established.

As discussed in the Membrane phosphoproteins section, the phosphorylation of microtubular-microfilamentous system may play an important role in secretory process, such as synaptic transmission and endocrine secretion. The phosphorylation of non-muscle contractile protein of the microfilamentous system has been discussed above. The cyclic AMP-stimulated phosphorylation of neurotubular subunits was first demonstrated by Goodman et al. in 1970 (253). Cyclic AMP-dependent protein kinase was found to be very closely associated with tubulin, and may be an integral component of the microtubules (254,255,259,261). The β subunits ($M_r=53,000$) of tubulin have been shown to be phosphorylated in vitro and in vivo (257,258,264). Several other minor proteins exist in "polymerization-purified" tubulin, and it seems likely that one or more of these high-molecular-weight proteins are also substrates for endogenous protein kinase (259-262). The possible physiological significance of the phosphorylation of microtubules and the role on regulation of secretory process are still not well understood. Besides secretory processes, other microtubules mediated cellular functions, such as mitosis, may also be regulated by phosphorylation. Using HeLa cells, tubulin was shown to be phosphorylated to different degrees

during varying stages of the cell cycle (263). Cyclic GMP was reported to oppose the cyclic AMP-dependent phosphorylation of a tubulin-related brain protein, and a cyclic GMP-stimulated phosphoprotein phosphatase was postulated (265) to cause this phenomenon.

E) Nuclear phosphoproteins

Among various histone fractions, the lysine-rich histone (H1*) was the first one reported to be phosphorylated in 1966 (279,280). Since that time, phosphorylation of histones has been widely studied (266-284,290). All types of histones, including the nucleated avian erythrocyte H5 (275) and trout H6 (268), were reported to be phosphorylated both in vitro and in vivo. The phosphorylated sites of different histones are summarized in Table 4. The sites of phosphorylation by cyclic AMP-dependent protein kinase and cyclic AMP-independent protein kinase are different (Table 4). A characteristic of the phosphorylation of H2A and H4 is that the N-terminal serine is both phosphorylated on hydroxyl side chain and acetylated on its free amino group (268,270,277). These modifications of H2A and H4 may be linked to processing of newly synthesized histone molecules for entry into the

*The histone nomenclature used is according to that adopted at Ciba Foundation Symposium 28 in 1974 (Bradbury, E. M. (1975) in *The Structure and Function of Chromatin*, Ciba Foundation Symp. 28, 1-4, Elsevier, Amsterdam).

TABLE 4
Nuclear Phosphoproteins

Phosphoproteins	Phosphorylation Sites	References
<u>Histones</u> ^a		
H1 (lysine-rich) (M _r =22,000)	-Arg-Lys-Ala-Ser(P)-Gly-Pro-Pro- 38 (cyclic AMP PK) ^b -Ala-Ser-Gly-Ser(P)-Phe-Lys-Leu- 105 -Thr(P)-Pro-Val-Lys- 20 -Gly-Ala-Ala-Thr(P)-Pro-Lys- 136 -Lys-Thr(P)-Pro-Lys- 153 -Lys-Ser(P)-Pro-Lys- 156 & 180	266,269 266 267 267 267 267,268 277
H2A (slightly lysine rich) (M _r =13,000- 15,000)	N-Acetyl-Ser(P)-Gly-Arg-Gly-Lys- 1 Acetyl -Thr-Arg-Ser-Ser(P)-Arg-Ala- 19 (cyclic AMP PK)	268,270,277 269
H2B (slightly lysine rich) (M _r =13,800)	-Arg-Lys-Arg-Ser(P)-Arg-Lys-Glu- 32 -Ser(P)-Tyr-Ser-Val- 36 (cyclic AMP PK) -Lys-Lys-Gly-Ser(P)-Lys-Ale- 14 (cyclic AMP PK) -Pro-Ale-Lys-Ser(P)-Ala-Pro-Lys- 6 Acetyl	269,271, 272 269 268,270
H3 (arginine rich) (M _r =15,300)	-Arg-Lys-Ser(P)-Thr-Gly- 10 Methyl -Arg-Lys-Ser(P)-Ala-Pro- 28 Methyl	268,270, 274

TABLE 4 contd.

H4 (arginine rich) ($M_r=11,300$)	N-Acetyl-Ser(P)-Gly-Arg-Gly-Lys- 1 Acetyl	268,270, 273,277
H5 (lysine rich) ($M_r=21,500$) (nucleated avian erythrocytes)	H ₂ N-Thr-Glu-Ser(P)-Leu-Val-Leu- 3 -Ser(P)-Pro-Ala- 7 C-terminal phosphorylation sites similar to H1	479 275
H6 (lysine rich) ($M_r=14,500$) (trout tissues)	-Arg-Lys-Ser(P)-Ala-Thr- 5	268
<u>Protamine</u>		
($M_r \approx 5,000$)	-Arg-Arg-{Ser(P)} ₃ -Arg-Pro- -Arg-Val-Ser(P)-Arg- -Ala-Ser(P)-Arg- -Arg-{Ser(P)} ₂ -Arg-	268,276
<u>Nucleoli Proteins</u>		
heterogenous (~40 phosphoproteins)	Ser-P	296-299,306
<u>Nuclear Acidic Proteins(non-histone proteins)</u>		
$M_r=10,000-170,000$	Ser-P & Thr-P	279,290- 295,305
<u>Nuclear Matrix Proteins</u>		
$M_r=60,000-70,000$ (3 main proteins)	phosphoester	310

^a The histone nomenclature used is according to that adopted at Ciba Foundation Symposium in 1974; Bradbury, E.M. (1975) in the Structure and Function of Chromatin, Ciba Foundation Symp. 28, 1-4, Elsevier, Amsterdam.

^b (cyclic AMP PK) indicates the phosphorylation sites which were phosphorylated by the cyclic AMP-dependent protein kinase.

nucleus and their subsequent association with chromatin (268,270,273). In vivo investigation has shown that H1 is phosphorylated during S phase (274,281) and prior to or during mitosis (274), and H3 is significantly phosphorylated during mitosis (274). Thus, the phosphorylation of histones is implicated as an important control of chromosome replication and condensation.

In rat liver the amount of H1 phosphorylated in response to hormones (e.g., glucagon, insulin) or cyclic AMP was quite small, comprising only approximately 1% of the total lysine-rich histone (266). However, in the regenerating rat liver, H1 was shown to be phosphorylated more extensively (281). The differences in histone phosphorylation occurring in dividing and non-dividing cell led to the following proposed phosphohistone function suggested by Langan (282,283): In dividing cells the entire chromosome is manipulated in a non-gene specific manner to provide for replication. Under these conditions, histone phosphorylation is massive, involving histone associated with the entire genome. In non-dividing cells, the extent of cyclic AMP regulated histone phosphorylation is limited. The selective manipulation of the genome are required for the activation of specific gene transcription.

Phosphorylation of nucleosomal histones (H2A, H2B, H3, and H4) may play an important role in regulating the formation of chromatin subunit structures. Recently, Sung (275) has shown that H5 was highly phosphorylated in immature avian erythrocytes, and was dephosphorylated during maturation. A maturation pathway of H5 was suggested by Sung (275): the phosphorylation of H5 orients the binding of H5 to DNA (479). During this maturation process, the properly oriented H5 is sequentially dephosphorylated and tightly associated with DNA. Therefore, the mature erythrocyte chromatin is condensed under well-controlled conditions. During spermatogenesis in trout testis, the metabolism of another basic protein, protamine, appears to be similar to H5 (268,276,277). Protamine may also play a similar role in the condensation of chromatin, although in spermatogenesis the entire complement of histone is replaced by protamine during condensation (277). The repeating phosphoserine sequences in phosvitin and casein (Table 7) also occur in protamine (Table 4).

Because of their abundance and defined structure, histones and protamine are often used as model substrates with basic properties for studying protein kinases and phosphoprotein phosphatases.

Besides phosphoester linkages, phosphohistones may also contain phosphoamidate linkages (25,26) (e.g., phosphohistidine

in H4 and phospholysine in H1). The acid-labile nature of phosphoamidate linkage prevents their detection under usual conditions employed for the extraction and electrophoretic analysis of histones (cf. Protein-Phosphate Linkage section). Since the acid-labile phosphorylation of histone was well correlated with the onset of DNA synthesis during liver regeneration (25), Chan et al. (26) have suggested that phosphorylation on histidine in H4 (may also be lysine in H1) loosens the interaction among the histones of nucleosome in such a way that DNA replication may proceed. Post-synthetic covalent modifications of histones other than phosphorylation have also been reported, such as methylation (284-286), acetylation (268,270,284,285,287), and adenosine diphosphoribosylation (288,289), which will not be discussed here.

In general, evidence indicates that histones express negative control in gene replication and transcription. Histones inhibited gene replication and transcription by complexing with DNA; phosphorylation of histone released the inhibition. On the other hand, phosphorylation of nuclear acidic proteins were implicated in positive control of gene expression at the level of transcription (290-295). The phosphorylated heterogeneous nuclear acidic proteins

with molecular weight ranging from 10,000 to 170,000 (291, 294) contain more than 90 percent of the nuclear protein-bound phosphorus (279,292). The phosphorylation sites were identified as phosphoserine and phosphothreonine residues (279,293). The nonhistone chromosomal proteins include (a) enzymes, (b) proteins that are involved in determining the structure of chromatin, and (c) proteins that serve as recognition sites for binding of regulatory agents (300). Phosphorylation may regulate their functions. RNA polymerase activity was shown to be enhanced by phosphorylation (cf. Phosphorylation of enzymes section). In vivo, the rate of phosphorylation of the nuclear acidic proteins was most active at periods when RNA synthesis was high (phase G₁ and S) and was minimal in the phase of G₂ and M when RNA synthesis was suppressed (291). The ability of nuclear acidic proteins to bind homologous DNA and to stimulate transcription in vitro increases with increasing phosphorylation and decreases upon dephosphorylation (293-295). The phosphorylation of different nuclear acidic proteins are modified at times of gene activation by steroid hormones (295,301-303) and are stimulated by cyclic AMP (295,304,305). While it is clear that phosphorylation of nuclear acidic protein plays an important role in the

regulation of gene expression, the mechanism by which positive control of RNA synthesis is specifically facilitated is not known. It is speculated, however, that the specificity involves binding of the phosphoprotein to specific nucleotide sequences in the DNA (293-295,300). The reversibility of phosphorylation provided by protein kinase and phosphoprotein phosphatase fits a cyclical mechanism that genes are turned-on and turned-off by interaction with control proteins whose conformation and DNA-binding properties are regulated by the phosphorylation. Identification and separation of individual phosphoproteins from heterogeneous nuclear acidic proteins will greatly facilitate the elucidation of the physiological function and regulation of those proteins.

About forty of the one hundred proteins isolated from nucleoli were found to be phosphorylated via a phosphoserine linkage (296). A protein kinase was identified to associate with nucleoli (298). These proteins were suggested to be involved in regulation of ribosomal RNA transcription or in ribosomal RNA processing and preribosomal particle formation (306), which take place in nucleolus. A nucleolus-specific phosphoprotein ($M_r=120,000$) was found to be essential for preserving the stereo-specific structure of the nucleoli.

Phosphorylation of the protein was catalyzed by a cyclic GMP-dependent protein kinase from the nucleoli (299).

Recently, Coffey and colleagues (307-310) have isolated a nuclear protein matrix from rat liver nuclei. The nuclear protein matrix provided a framework structure, which maintains the spherical shape of the nucleus. In the regenerating rat liver, the protein matrix was associated with newly synthesized DNA (308). The matrix is composed primarily of three acidic polypeptides in the molecular weight ranging from 60,000 to 70,000 (307). Phosphorylation of nuclear matrix proteins in vitro was subsequently reported and suggested to be important in the regulation of DNA synthesis in the nucleus (310).

F) Ribosomal phosphoproteins

Eukaryotic ribosomal proteins were reported to be phosphorylated in vivo and in vitro by cyclic AMP-dependent and -independent protein kinases (311-321,325). The reported ribosomal phosphoproteins are summarized in Table 5. In general, the phosphorylation of the 60S subunit is more complexed than that of the 40S subunit (7,8,312,313,325). Several proteins associated with the 60S subunit were shown to accept phosphates (312-315). In the 40S subunit, only a single major protein ($M_r=28,000$)

TABLE 5
Ribosomal Phosphoproteins

Phosphoproteins	Phosphorylation Sites	References
<u>60S Subunit</u>		
5~8 proteins	Ser-P & Thr-P (cyclic AMP PK)	312-315
protein of $M_r=21,000$	Ser-P & Thr-P	311
<u>40S Subunit</u>		
protein of $M_r=28,000\sim35,000$	Ser-P & Thr-P (cyclic AMP PK)	311-317
protein of $M_r=50,000$	Ser-P & Thr-P	315
protein of $M_r=70,000$	Ser-P & Thr-P	311
<u>Eukaryotic Initiation Factor 2</u>		
protein of $M_r=38,000$	-	480 317-319
protein of $M_r=49,000\sim52,000$	Ser-P	319, 320
<u>Eukaryotic Initiation Factor 3</u>		
protein of $M_r=65,000$ 70,000	Ser-P	319, 320
protein of $M_r=120,000$ 125,000	Ser-P	319-320
protein of $M_r=140,000$	Ser-P (cyclic AMP PK)	320
protein of $M_r=160,000$	-	319
<u>Prokaryotic Initiation Factor 3</u>		
(phosphorylated by bacteriophage T_7 protein kinase)	Thr-9, Ser-162, Thr-165	74
<u>Other Ribosomal Proteins</u>		
protein of $M_r=67,000$	-	319
acidic protein of $M_r=14,000\sim16,000$	-	321

(cyclic AMP PK) indicates the phosphorylation sites which were phosphorylated by cyclic AMP-dependent protein kinase.

35,000) was phosphorylated in vitro and in vivo (311-317). The phosphorylation was stimulated by cyclic AMP and it was suggested that this protein may play an important role in mediating several hormonal functions at the level of translation (6,7,312-314). The mechanism of phosphorylation in regulating eukaryotic ribosomal functions remains to be solved (320,324).

Recently, London and his coworkers (317-319) demonstrated that the heme-regulated translational inhibitor was closely associated with a cyclic AMP-independent protein kinase. The initiation of protein synthesis in reticulocyte lysates could be inhibited by the translational inhibitor activated in heme deficiency. The phosphorylation of eukaryotic initiation factors 2 and 3 by protein kinases (317-320, 480) supports this hypothesis, but the mechanism by which phosphorylation regulates the function of the initiation factors has yet to be elucidated.

Although ribosomal proteins (77) and initiation factor 2 (323) of E. coli could be phosphorylated in vitro by protein kinase from skeletal muscle, it seems that the prokaryotic ribosomes do not contain phosphoproteins in vivo (322). However, the host ribosomal proteins were shown to be phosphorylated by a cyclic nucleotide-independent protein

kinase which appears in E. coli following infection with bacteriophage (69-73) (cf. Bacterial and viral phosphoproteins section). The bacteriophage-induced protein kinase was also found to phosphorylate initiation factor 3 from E. coli (74). Although the phosphorylation was suggested to be the mechanism by which the bacteriophage modifies host metabolism, the functional change of the phosphorylated ribosomal proteins has not yet been demonstrated (69,74).

G) Bacterial and viral phosphoproteins

There are very little, if any, phosphoproteins which can be found in E. coli. Most protein-bound phosphate in E. coli exists primarily in the form of phosphoenzyme intermediates (cf. Protein-Phosphate Linkage section). A cyclic nucleotide-independent protein kinase, however, appears in E. coli following infection with bacteriophage (e.g., T₇, T₃) (69-72). Some bacterial proteins have been found to be endogenous substrates for the phage-induced protein kinase, such as ribosomal proteins (73), DNA-dependent RNA polymerase (70), and initiation factor IF-3 (74). Early reports of protein kinases in prokaryotic organisms (38,39) may be explained by the existence of a polyphosphate kinase that is stimulated by histone (36,37). Phosphorylation of E. coli DNA-dependent RNA polymerase

by a phage-induced protein kinase may be the "early transcription control" of the production of early phage RNAs and shut-off host RNA synthesis. The phosphorylation occurs on the threonine residues of the β' subunit of RNA polymerase (70). Several other *E. coli* proteins have been found to be phosphorylated by protein kinases of mammalian tissues (75-77). The functional significance of such heterologous systems, however, needs further study to elucidate.

Since the discovery of protein kinase and phosphoproteins in Rauscher murine leukemia virus by Strand and August in 1971 (84), many RNA- and DNA-animal viruses have been found to contain protein kinase and/or phosphoproteins (Table 6) (78-114). The major phosphoprotein in mammalian type C oncornaviruses is the structural component p12 (protein with $M_r=12,000$) (87,88). On the other hand, the structural protein p19 was demonstrated to be the major phosphoprotein in avian sarcoma virus (78,79). The phosphoprotein patterns in rhabdoviruses, however, varies from species to species (89-92). In addition, some or all of the structural proteins of various other viruses can be modified by phosphorylation (Table 6). Most of the phosphate-protein linkages have been demonstrated to be phosphoester bonds.

TABLE 6

Viral Protein Kinases and Phosphoproteins

Viruses	Protein Kinase	Phosphoproteins	References
<u>RNA VIRUSES</u>			
<u>A. RETRAVIRIDAE</u>			
Oncornavirus C:			
Rous sarcoma virus (RSV)	+	p19, p12, p23	78-80, 86
Avian sarcoma virus B 77		β subunit of reverse transcriptase	81
Avian myeloblastosis virus (AMV)	+	ϕ factor from reverse transcriptase (?)	82-85
Murine sarcoma virus (Kirsten) (K-MSV)		p10, p12	87, 88
Murine leukemia virus (Rauscher) (R-MLV)	+	p12	80, 84, 85, 87
Murine Erythroblastosis virus		p12, gp74	87
Feline leukemia virus	+	p12	80, 87
Feline endogenous virus (RD 114)		p12, p16	87, 88
Primate sarcoma virus		p12	88
Primate endogenous virus (baboon)		p12, p16	88
Hamster leukemia virus	+	-	80
Viper virus	+	?	80, 113
Oncornavirus B:			
Mouse mammary tumor virus	+		80
<u>B. RIABDOVIRIDAE</u>			
Vesicular stomatitis virus (VSV)	+	NS, M(?), L(?)	84, 89-92, 113
Rabies virus	+	N	89, 90, 113

TABLE 6 contd.

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Kern Canyon virus	+	N, G	89,90
Cocal virus		NS	89
several other rhabdoviruses		structural proteins	89
<u>C. PARAMYXOVIRIDAE</u>			
Sendai virus (parainfluenza virus type 1)	+	structural proteins	93,94,113
Parainfluenza virus type 3	+		80
<u>D. ORTHOMYXOVIRIDAE</u>			
Influenza virus type A	+		80
Influenza virus type B	+		80
<u>E. REOVIRIDAE</u>			
Mammalian reovirus type 3	-	λ , μ_2	85,95
<u>F. TOGAVIRIDAE</u>			
Semliki Forest virus	+	structural proteins	96,113
Sindbis virus	+	structural proteins	96,97,113
<u>DNA VIRUSES</u>			
<u>A. POXVIRIDAE</u>			
Vaccinia virus	+	proteins of $M_r=38,500$ & 11,700	85,98-101, 113
	($M_r=62,000$)		
<u>B. IRIDOVIRIDAE</u>			
Frog virus 3	+	p111, p16, p14 & others	85,102-104, 113
	($M_r=65,000$)		
<u>C. PAPOVAVIRIDAE</u>			
Simian virus 40 (SV 40)	-	structural proteins	105,106
<u>D. ADENOVIRIDAE</u>			
Adenovirus	-	DNA binding proteins & structural proteins	84,107-110
<u>E. HERPESVIRIDAE</u>			
Herpes simplex virus (type 1)	+	structural proteins	85,112,113

TABLE 6 contd.

Equine herpesvirus (type 1, equine abortion virus)	+	all viral proteins	111
Pseudorabies virus	+		113

Virion-associated protein kinases have been found in enveloped viruses, both membrane-maturing and intracytoplasmic-maturing species (Table 6), but not in nonenveloped viruses, such as simian virus 40 (105), adenovirus, poliovirus (84), and reovirus (85). Viral protein kinases from frog virus 3 (103), avian myeloblastosis virus (82), and vaccinia virus (100,101) have been purified and characterized. In general, these viral enzymes are cyclic nucleotide independent, utilize GTP as well as ATP as a phosphate donor, have a molecular weight of 50,000 to 62,000, are not inhibited by the heat-stable protein inhibitor of cyclic AMP-dependent protein kinase (103), and probably related to the cyclic nucleotide-independent protein kinase (9,113). The origin of the virion-associated protein kinase could be: 1) a host cellular enzyme that is incorporated into virion during virus maturation, 2) host coded and virus induced, 3) virus coded. Since the virion-associated protein kinases can only be found in enveloped viruses, it is very possible that the protein kinase is cellular in origin and is captured during virus maturation (91,92). The observations that different viruses grown in the same host cell have protein kinases with different specific activities (113) indicated that the protein kinase is not captured randomly by the

virus during maturation, whether it is of cellular origin or not. Frog virus 3 protein kinase has been demonstrated to be a virus-specified enzyme, i.e., a viral gene product (104). More studies have to be done in order to elucidate the problem of the origin of the virion-associated protein kinase. The presence of virion-associated protein kinase is not necessarily associated with the presence of viral phosphoproteins, and vice versa. Simian virus 40 (105,106) and adenovirus (110) do not carry protein kinases and yet contain phosphoproteins. On the other hand, although the viper C-type virus and Moloney murine leukemia virus exhibit protein kinase activity, they do not contain phosphoproteins (90,105). Besides protein kinase, phosphoprotein phosphatase activities have also been detected in the Alphavirus (96), frog virus 3 (85), and many other enveloped viruses (85). Some of the phosphatases required Mn^{2+} for activity (85).

The biological roles of the viral protein kinases and phosphoproteins may be involved in regulation of viral metabolism and/or virus-mediated modification of host cell metabolism. Some of the viral phosphoproteins bind to viral genome (e.g., p12) (114), and might be involved in the regulation of the transcription of viral genome. The

RNA-dependent DNA polymerase can be phosphorylated (81,83,86) and activated (83,86). The phosphorylation sites are located on the β subunit (81) and may be removed as the small acidic protein ϕ (83). A phosphorylation model for enzymic modification of the reverse transcriptase has been proposed by Tsiapalis (83) (cf. Phosphorylation of enzymes section). Some other viral phosphoproteins with unknown function may play other regulatory roles in viral metabolism, such as maturation.

H) Structural and nutrient phosphoproteins

About 80% of milk protein is casein, which is defined as the phosphoprotein component of milk (34). The electrophoretically distinct casein components α , β , γ (59) and κ (60) are heterogeneous. Heterogeneity may be the consequence of a partial dephosphorylation of these components. The complete primary structure of α_{s1} -casein (40) and β -casein (41) has been determined. γ -casein is a fragment of β -casein and represents residues 29 to 209, which contains only one phosphoserine (position 35). The phosphate groups in all caseins appear to be linked only on serine or threonine side chains. The repeating phosphorylated serine sequence is characteristic of casein (Table 7) and also appears in the egg protein phosvitin. The principal phosphoprotein

TABLE 7
Structural and Nutrient Phosphoproteins

Phosphoproteins	Phosphorylation Sites	References
α_s -casein (M_r =23,600-25,500)	-Ile-Gly-Ser(P)-Glu-Ser(P)-Thr-Glu- 46 48 -Ala-Glu-Ser(P)-Ile-{Ser(P)} ₃ -Glu-Glu- 65 70 -Pro-Asn-Ser(P)-Val-Gln- 75 -Pro-Asn-Ser(P)-Ala-Glu-Glu- 115 -Asp-Gln-Thr(P)-Met-Glu- (variant D) 53	40 40
β -casein (M_r =24,000-25,000)	-Val-Glu-Ser(P)-Leu-{Ser(P)} ₃ -Glu-Glu- 15 20 -Phe-Gln-Ser(P)-Glu-Glu- 35	41
γ -casein	same as Ser-35 in β -casein	41
κ -casein (M_r =20,000-26,000)	-Glu-Ala-Ser(P)-Pro-Glu- -Pro-Thr-Ser(P)-Pro-Thr- carbohydrate	42 43
Ovalbumin (M_r =44,000)	-Gly-Asp-Ser(P)-{Glu,Ile}-Ala- -Val-Gly-Ser(P)-Ala-Glu-	44
Phosvitin (M_r =35,000)	-Asp-{Ser(P)} ₆ -Arg-Asp-({Ser(P)} ₆ , Lys)-({Ser(P)} ₆ ,Lys)-Arg-({Ser(P)} ₆ , His)-{Ser(P)} ₆ -His- -Gly-Val-({Ser(P)} ₃ ,Glu)-Lys-({Ser(P)} ₃ , Leu)-His-His-Arg-{Ser(P)} ₆ -Arg-His- -Ser-Asn-Ser-Gly-{Ser(P)} ₈ -Arg-Ser- carbohydrate	45,46
Lipovitellin (Subunit: M_r =30,000)	Ser-P	47

TABLE 7 contd.

Egg white flavin-binding Protein ($M_r=32,000-36,000$)	?	54
Eggshell protein ($M_r=57,500$)	Ser-P	55,56
Dentinal phosphoproteins		
(1) collagen α -chain	Ser-P	48
(2) protein of $M_r=39,000$	Ser-P	49,50,67
Phosphoproteins in dental enamel		
E_3 ($M_r=5,000-6,000$)	-Glu-Ser(P)-Leu-	51
E_4 ($M_r=5,000-6,000$)	-Glu-Ser(P)-Tyr- (position:5,24 & 34)	52
Phosphoproteins in bone	Ser-P	53
Collagens and gelatins	Ser-P(?)	57,58

obtained in a highly purified form from hen egg yolk, named "Phosvitin" in 1949 (61), contains exceptionally high levels of both phosphate and serine. More than 50% of the amino acids of phosvitin are serine, almost all of which are phosphorylated. An unusual characteristic of phosvitin is the repeating oligophosphoserine sequences up to six residues in length accompanied by basic amino acid residues (45,46). The octaphosphoserine peptide sequence, which runs nearby the carbohydrate side chain, is the longest oligophosphoserine found in any phosphoprotein up to date (Table 7). Because of their abundance, casein and phosvitin are often used as a model substrate with acidic chemical properties for protein kinase and phosphoprotein phosphatase.

Lipovitellin (62) refers to the major lipoprotein fraction of the yolk granule in which phosvitin and other minor components have been removed. Ten percent of the amino acids of lipovitellin is serine, and about one-half of it is phosphorylated (47). Together with phosvitin, lipovitellin forms an ordered structure in the yolk platelet. Ovalbumin is the major protein component of egg white and is a phosphorus-containing protein which was first demonstrated in 1900 (63). The phosphates are linked at two different serine sites by ester bond (64)

(Table 7) and the partial amino acid sequence at the phosphorylated sites has been reported (44). Phosphoproteins of egg and milk that occur in bulk are considered to be a store for amino acids, phosphates, and metals, and their biological significance may include providing these important components to the young animal or developing embryo. Since the high energy nature of the phosphate bond in the repeating phosphoserine sequence, phosvitin has also been considered as an energy source (65,66).

Besides the major phosphoproteins described above, a major component ($M_r=32,000-36,000$) of the egg white protein was reported to contain phosphorus and flavin (54). The nature of the phosphate-protein bond and the function of the phosphate remain unclear. There are structural proteins in the eggshell with a striking high phosphate content (7.6%) which have been shown to exist in the phosphoserine linkage (55,56).

Because of their possible role in the mineralization process, much attention has been recently focused on the phosphoproteins in teeth and bones. The dentin phosphoprotein has a molecular weight of about 39,000 and three-quarters of the total amino acids are serine and aspartic acid residues. Both the carboxylate group of aspartate and

the phosphate group of phosphoserine appear to be involved in providing high-affinity binding sites for calcium ions (50). Some of the dentin phosphoproteins may be covalently bound to dentinal collagen (49,67). It was suggested that the soluble phosphoprotein may be the immediate precursor of the covalently bound phosphoprotein in the mature dentin matrix, where it might serve as nucleation sites in the matrix mineralization process (49). About 20% of the proteins of embryonic dental enamel are phosphorylated polypeptides (68), E₃ and E₄ being the two major ones (Table 7). All three serine residues in phosphopeptide E₄ are phosphorylated and exist in a Glu-Ser(P)-Tyr sequence (52). All three serine residues in phosphopeptide E₃ are also phosphorylated; however, they are all in the Glu-Ser(P)-Leu sequence (51). Similar to the phosphoprotein in teeth, phosphoserine has been identified in both bound and soluble phosphoprotein isolated from bone (140). The bone phosphoproteins also have very high levels of serine and acidic amino acids and may play a similar role in bone mineralization process as does the dentin phosphoprotein in teeth. Collagens in dentin (48) and skin (57, 58) have also been reported to contain organic phosphates.

The structural and nutrient phosphoproteins and their

phosphorylation sites are summarized in Table 7.

I) Miscellaneous phosphoproteins

In the blood, fibrinogen was shown to contain phosphate (326,327). Fibrinopeptide A, which was the peptide removed from the N-terminal ends of fibrinogen by thrombin, was found to be a phosphorylated protein isolated from dog (328) and human (329). And the phosphorylation sites were determined as:

-Thr-Asp-Ser(P)-Glu-Gly-Lys- (dog)

-Ala-Asp-Ser(P)-Gly-Glu-Asp- (man)

Although early studies suggested that dephosphorylation of fibrinogen resulted in increased clotting time (330), recent studies with purified fibrinogens raised doubt about this hypothesis (331). Further studies are needed for elucidating the significance of the phosphorylation.

An acidic, calcium binding protein has been purified from brain (332,333), adrenal medulla (334), and adult testis (335). The protein binds one mole of calcium per mole of protein ($M_r=11,900$). Treatment with phosphatase results in loss of calcium binding activity (332). The phosphoprotein was suggested to be associated with adrenergic neurons and to play a role in calcium-dependent secretion of a neurotransmitter (334) (for calcium and secretory processes, cf. ref. 195-198 and Membrane phosphoproteins

section). Recently, Wolff and Brostrom (336) have shown that the acidic, calcium-binding protein is identical to the calcium-dependent regulator of cyclic nucleotide phosphodiesterase from brain. Calcium stimulates the phosphodiesterase activity by binding to the phosphoprotein. This implicates that the phosphorylation may play a feedback role in regulating cyclic nucleotide metabolism; i.e., the cyclic AMP-stimulated phosphorylation may inactivate adenylate cyclase (155-157) and activate the phosphodiesterase through calcium-dependent regulatory phosphoprotein, thereby decreasing the cyclic AMP concentration and terminating the cyclic AMP-stimulated phosphorylation.

Recently, two highly purified phosphoproteins, protein A ($M_r=9,900 \sim 11,000$) and protein C ($M_r=16,300$), from human parotid saliva were reported to bind calcium (337-340). Those proteins contain two mole of phosphate per mole of protein (337,339), probably as phosphoserine. The phosphate seems to be important for the calcium-binding ability (340). Although Bennick has suggested that these phosphoproteins may associate with the secretory granule in the human salivary glands and interact with hydroxyapatite at the tooth surface (337-339), no physiological activity has been demonstrated for these proteins yet.

A phosphoprotein from rat liver cytosol containing both phosphoserine and phosphothreonine was suggested to be an iron carrier, which transports iron from cytosol to mitochondria (341). This protein is analogous to phosvitin which acts as iron carrier during the development of chick embryo (342) (cf. Structural and nutrient phosphoproteins section). The iron binds to the phosphorylated protein and is released by phosphoprotein phosphatase.

III. Protein Kinases

Protein kinases are the enzymes which catalyze the transfer of the γ -phosphate of nucleotide triphosphates to the side chain of amino acid residues in protein substrates. Although the receptor amino acids may be histidine and lysine (and arginine) (25-27,484), generally protein kinases are referred to the enzymes which catalyze the formation of phosphoester bonds with serine and threonine residues (for reviews, cf. 7-9,283,485-489).

Since the recognition that the activity of phosphorylase kinase was stimulated by cyclic AMP in 1968 (494), protein kinases have been categorized into cyclic nucleotide-independent protein kinases and cyclic nucleotide-dependent protein kinases. The criteria for the classification have been suggested by Traugh et al. (495), namely, that the cyclic AMP-dependent protein kinase can bind cyclic

AMP, and be stimulated by cyclic AMP and be inhibited by inhibitor protein (496). Although the catalytic subunit of the cyclic AMP-dependent protein kinase does not bind cyclic AMP and is not stimulated by cyclic AMP, the activity of the catalytic subunit is inhibited by the regulatory subunit of cyclic AMP-dependent protein kinase and by the heat-stable inhibitor protein. None of the effects mentioned above is applicable to cyclic nucleotide-independent protein kinases (Type III, cf. 495).

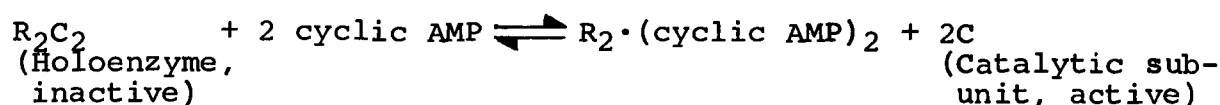
A) Cyclic nucleotide-dependent protein kinases

The cyclic nucleotide-regulated protein kinases are stimulated by low concentrations of cyclic 3',5'-purine nucleotides* and prefer to utilize ATP as phosphate donor. Cyclic AMP-dependent protein kinases are ubiquitously distributed in eukaryotes (13,503) and have been purified to homogeneity from rabbit skeletal muscle (505) and from bovine heart (506). Cyclic GMP-dependent protein kinases, which have greater affinity for cyclic GMP than for cyclic

*Recently, Bloch (497-499) has demonstrated the occurrence of cyclic 3',5'-pyrimidine nucleotides in leukemia L-1210 cells and liver cells, and cyclic CMP was suggested to be associated with cell division. Subsequently, the cytidylate cyclase (500) and cyclic CMP specific phosphodiesterase (501,502) were reported. It is interesting to see if there is any cyclic CMP regulated protein phosphorylation and/or dephosphorylation.

AMP, have also been isolated from a variety of tissues (504,507,508,528,531).

The holoenzymes of cyclic AMP-dependent protein kinase are composed of a regulatory subunit dimer (R_2 ; monomer R , $M_r=49,000 \sim 55,000$), which binds cyclic AMP, and two catalytic subunits (C , $M_r=38,000 \sim 42,000$), which catalyze the phosphotransfer reaction (488). In the presence of saturating levels of cyclic AMP, the inactive holoenzyme binds two moles of cyclic AMP per mole of enzyme and dissociates into a regulatory subunit dimer and two active catalytic subunit monomers (486-488):



Whether the dissociation proceeds through an intermediate

R_2C requires further investigation. The cyclic GMP-dependent protein kinase ($M_r=140,000$) from lobster muscle was reported to be dissociated by cyclic GMP into a cyclic GMP-binding protein and a catalytic subunit ($M_r=40,000$) (507). However, the enzymes isolated from the silkworm pupae ($M_r=140,000$) (508) and from bovine lung ($M_r=165,000$, two subunits of $M_r=81,000$) (528) could not be dissociated by cyclic GMP. The mechanism of cyclic GMP stimulation has yet to be established.

The activity of cyclic nucleotide-dependent protein

kinase may also be regulated by a heat- and acid-stable protein (496,504,509-513). The cyclic AMP-dependent protein kinase inhibiting protein ($M_r=26,000$) was shown to interact with the catalytic subunit (509,510). A similar protein ($M_r=34,000$) was isolated from lobster tail muscle (511,512). The ability of the "modulator" to either stimulate or inhibit protein kinase activity depended on assay conditions and the protein substrate used (511,512). Although it appears that the same modulator inhibited cyclic AMP-dependent protein kinase and stimulated cyclic GMP-dependent protein kinase in arthropod tissue (511), there are separate modulators for these two regulatory activities in mammalian tissues (513). The physiological significance of these heat- and acid-stable regulatory proteins has yet to be elucidated.

The soluble cyclic AMP-dependent protein kinases can be further classified as Type I and Type II isozymes according to their ability to be dissociated by histone or 0.5M NaCl and to their affinity for DEAE-cellulose (514). Type I protein kinases, eluted with 0.05-0.10M NaCl from DEAE-cellulose, were rapidly dissociated by histone or 0.5M NaCl. In contrast, the type II protein kinases, eluted with 0.15-0.25M NaCl from DEAE-cellulose, were slowly dissociated by histone or salt. The Type I

and II isozymes correlate to the peak I and II protein kinase activities, respectively, named in the purification of protein kinases from rabbit skeletal muscles (505,515). Both types of isozymes exist in a variety of tissues, and their distributions depend on the tissues and species (8, 514,529). The Type I isozyme was purified from rabbit skeletal muscle by Krebs and colleagues (505), and the Type II isozyme was purified from bovine heart by Rosen and colleagues (506). The two types of isozymes may have similar or identical catalytic subunits ($M_r=40,000$) but differ in their regulatory subunits (516). The regulatory subunits of Types I and II isozyme have molecular weights of 49,000 and 55,000, respectively. In contrast to that of Type II isozyme, the regulatory subunit of Type I isozyme is easily dissociated from the catalytic subunit, has high-affinity binding sites for Mg^{2+} -ATP, and cannot be autophosphorylated (516,529,530). The Type II isozyme can catalyze the phosphorylation of its own regulatory proteins (2 moles of phosphates per mole of holoenzyme) (517,518,530,545) and phosphorylation site has been identified as phosphoserine (489). The self-phosphorylation reaction was rapid both in the presence or absence of cyclic AMP. When the reaction rate was slowed by lowering the concentration of ATP to 0.06 μM , a two-fold stimulation of the self-phosphorylation reaction by cyclic AMP (5 μM) was

apparent (487). The phosphorylation of Type II isozyme reduced its dissociation constant for cyclic AMP from 2.8 μM to 0.4 μM and reduced the rate of reassociation of the dissociated regulatory and catalytic subunits (519). The phosphorylated Type II isozyme can be dephosphorylated by a phosphoprotein phosphatase ($M_r=30,000$) purified from bovine heart (520). The dephosphorylation reaction requires the addition of cyclic AMP, which appears to interact with the substrate (520). Whether these isozymes have different physiological roles remains to be solved.

The cyclic AMP-dependent protein kinases have a broad substrate specificity (7-9,283,458,494) (e.g., histone, casein, phosphorylase kinase, glycogen synthetase, lipase, etc.). However, the basic residues on the NH_2 -terminal side of the phosphorylated serine residue may be important substrate specificity determinants for the cyclic AMP-dependent protein kinases (379,521-525) (cf. Tables 1-7). The substrate specificity of cyclic GMP-dependent protein kinase is very similar to that of the cyclic AMP-dependent enzyme (527). Both of them catalyzed the phosphorylation of pyruvate kinase (527), glycogen synthetase (527), histone (527), fructose 1,6-diphosphatase (527), phosphorylase b kinase (384,527), lipase (384), troponin inhibitor subunit (236,237), and several synthetic peptides (527). Besides substrate specificity, the two cyclic nucleotide-dependent protein kinases have some similarities in physical

properties and amino acid compositions (527). Lincoln and Corbin (527) have proposed that the two classes of enzyme are homologous proteins and evolved from a common ancestral protein.

B) Cyclic nucleotide-independent protein kinases

Early studies of protein kinase in the 1950's were concentrated on classical nutrient phosphoproteins, such as casein and phosvitin (490,491). Since the enzyme was not restricted to organs which synthesize the food protein (9,491), the functional significance and the entities of these protein kinases remain unclear. It was postulated that the repeating serine sequences in casein, phosvitin (cf. Structural and nutrient phosphoprotein section) and some tissues phosphoproteins were responsible for the specificity of such protein kinases (9,485,492). Recently, Pinna et al. (493) reported that the repeating serine blocks were not required for the phosphorylation reaction, but, instead, a lysine residue may play a critical role.

Compared to cyclic nucleotide-dependent protein kinase, the cyclic nucleotide-independent protein kinases are more substrate specific and are named in terms of their preferred substrates (e.g., phosphorylase kinase, histone kinase, casein kinase, phosvitin kinase). Most of them can utilize

both ATP and GTP as phosphate donors. Some of the cyclic nucleotide-independent protein kinases have been purified and studied (100-104,266,532-544).

Several cyclic nucleotide-independent protein kinases, which utilize acidic protein as substrate (e.g., casein and phosvitin), have been isolated from brain (532), liver (533-536,542,543), reticulocytes (537,538), erythrocytes (539), mammary gland (540), heart (541), and other tissues (7,9). The casein kinase found in the Golgi apparatus of lactating mammary gland (540) and the phosvitin kinase found in the cytosol of avian liver (533) may be directly associated with the post-translational modification of the respective nutrient proteins. Besides casein and phosvitin, the enzymes found in rat liver also phosphorylate nuclear acidic proteins but not histone or protamine (536) (cf. Nuclear phosphoprotein section). Glycogen synthetase was shown to be phosphorylated and inactivated by this type of protein kinase (369,370,373,375) (cf. Phosphorylation of enzymes section).

Basic proteins, such as histones and protamine, may serve as substrates for another type of cyclic nucleotide-independent protein kinase. An histone kinase has been identified by Langan (266,283), and a protamine kinase has been purified from dogfish skeletal muscle (544). This

type of protein kinase may be responsible for the cyclic AMP-independent phosphorylation of nuclear basic proteins, histone, and protamine (cf. Nuclear phosphoproteins section).

The protein kinase induced by bacteriophage in E. coli was found to be cyclic nucleotide independent (69-72). The virion-associated protein kinases have also been demonstrated to be cyclic nucleotide-independent (82,100-104,113) (Table 6) (cf. Bacterial and viral phosphoproteins section). Aside from eukaryotes, no evidence of cyclic nucleotide-regulation of protein phosphorylation has been found up to date.

Phosphorylase kinase has been the most intensively studied cyclic nucleotide-independent protein kinase since its discovery in 1956 (466,546). The enzyme catalyzes the phosphorylation of phosphorylase (356-,357) and troponin inhibitory subunit (225). Hayakawa et al. (360) and Cohen (361) have purified the enzyme to homogeneity and identified the subunits (Table 1). The phosphorylation of the β subunit (or subunit B, ref. 360) by cyclic AMP-dependent protein kinase was correlated with the stimulation of enzymic activity (cf. Phosphorylation of enzymes section). Besides phosphorylase, which is regulated by phosphorylase kinase, the activities of pyruvate dehydrogenase (406,417) and acetyl-CoA carboxylase (387-390) have also been demonstrated

to be regulated by cyclic AMP-independent protein kinase(s). Recently, a myosin light chain kinase was reported to be independent of cyclic nucleotides (220,221,548).

Calcium may play an important role in the regulation of cyclic nucleotide-independent protein kinase. The enzymic activities of phosphorylase kinase (547) and myosin light chain kinase (220,221) was dependent on μM levels of calcium. Recently, the stimulating effect of Ca^{++} on the myosin light chain kinase was shown to be mediated by the Ca^{++} -dependent modulator protein of cyclic nucleotide phosphodiesterase (549,550). Wang and colleagues (550) suggested that the calcium effects in many cellular processes may be mediated by this "modulator-dependent" protein kinase. Limited proteolysis does activate phosphorylase kinase (551) and other protein kinase (552). The physiological significance of this regulation remains to be established. The interaction between substrate proteins and effectors (e.g., metabolites) is another possible way to regulate the activity and specificity of cyclic nucleotide-independent protein kinases (cf. Enzyme cascade systems section). For example, the pyruvate dehydrogenase kinase was shown to be inhibited by thiamine pyrophosphate which bound to the substrate, pyruvate dehydrogenase (405,410).

IV. Phosphoprotein Phosphatases

Although the enzyme converting phosphorylase a to b was described by Cori and Green (460) as PR (prosthetic-removing) enzyme in 1943, the term of phosphoprotein phosphatase, which catalyzes the hydrolysis of the phosphate from phosphoproteins, was first described in the frog egg by Harris in 1946 (692). Subsequently, phosphatases were demonstrated in spleen (681-690) and other mammalian tissues (682,685,686) by several different investigators using casein, phosvitin, and/or vitellin as a substrate. In addition, it was demonstrated that the PR enzyme was a phosphorylase phosphatase (463). In comparison to protein kinases, much less is known about phosphoprotein phosphatases, despite their earlier discovery. This section will discuss the current knowledge of phosphoprotein phosphatases including their molecular forms, substrate specificities, and the regulation of their activities. The phosphoprotein phosphatases from various tissues and the effectors, which were shown to alter the enzymic activity, are summarized in Table 8.

A) Molecular forms of phosphoprotein phosphatases

The early work done by Meisler and Langan (622) demonstrated that there are at least two different phosphohistone phosphatases in the cytosol of rat liver (Table 8).

TABLE 8
Phosphoprotein Phosphatases

Sources & References	Physical Properties (Molecular Weights)	Substrates	Activators	Inhibitors
<u>HEART</u>				
<u>Bovine heart</u>				
Thomas (563,564)	65,000-70,000	GS, bK, C, H, PL	Mg ²⁺ , Mn ²⁺ (inhibit PL phosphatase)	ATP, UTP
Chou & Rosen (487,520)	31,000	H, C, PL, cyclic AMP-dependent protein kinase	cyclic AMP	
Lee (384,386,614)	35,000	PL, lipases		
<u>Rat heart</u>				
Thomas (565)	50,000-100,000	GS	Mg ²⁺ , Mn ²⁺ , G-6-P,	glycogen
Nuttall (559)	-	GS	insulin <u>in vivo</u>	
Lee (613)	35,000	PL		
<u>Procine heart</u>				
Wieland (566)	92,000-95,000	pyruvate dehydrogenase	Mg ²⁺ , Mn ²⁺ , (Ca ²⁺ ?)	NaF
Takeda (567)	250,000	H, PL	Mg ²⁺ , Mn ²⁺ (H as substrate)	Zn ²⁺
	30,000 (SDS:34,000)	H, PL		

TABLE 8 contd.

Reed(411)	100,000	pyruvate dehydrogenase	Mg^{2+}, Ca^{2+}	
<u>Canine heart</u>				
Li & Hsiao (568,569)	161,000; 7.1S	H,C,PL	KCl,ATP,GTP, etc. (H as substrate)	PPi,Pi
	34,800; 3.45S	H,C,PL	KCl (H as substrate)	ATP,GTP,PPi, etc.
Li & Hsiao (570)	61,000; 3.8S	H,C,PL	Mn^{2+}, Co^{2+} (dependent)	$Zn^{2+}, Fe^{2+}, Cu^{2+}$ ATP,PPi,Pi, etc.
Sutherland(463)	-	PL		
Kirchberger & Li (177,178)	sarcoplasmic reticulum	H, phospholamban	Mn^{2+}, Mg^{2+}	Pi,NaF
<u>SKELETAL MUSCLE</u>				
<u>Rabbit skeletal muscle</u>				
Kato & Bishop(571)	-	GS,H	Mn^{2+}, Mg^{2+}	NaF,Pi,PPi
Kato(572)	(I) 300,000; 6.7S	H,GS	Mn^{2+}, Co^{2+}	
	(II) 150,000; 3.8S	H,GS	Mn^{2+}, Co^{2+} (dependent)	
Kato(573)	I & II	PL	G-6-P,glucose	$M^{2+}, AMP, ATP, Pi,$ NaF,glycogen
Kato(574)	7.3S, 8S, 6.7S convert to 4S	H,PL		
Kato(575)	3.8S	H,PL	$Mn^{2+}, Mg^{2+}, Co^{2+}$	ATP
Glinsmann(576,577)	-	GS,bK,P		spermine
Glinsmann(578-580)	70,000 convert to 52,000	PL		phosphorylated inhibitor-1
		phosphorylated inhibitor-1	Mn^{2+} (dependent)	
Graves(581-584)	50,000(592)	PL, PL peptide	glucose,G-6-P, glycogen	G-1-P,AMP,Pi, M^{2+} ,fluorophos- phate

TABLE 8 contd.

Hers(585)	50,000(592)	PL	glucose	AMP
Cori(460,461,706)	PR enzyme	PL	cysteine, Mn^{2+} (?)	Mg^{2+} , salt, NaF, G-1-P
Cohen (364,586-589)	(I) 300,000 (II) 170,000 (IIIA) 75,000 (IIIB) 46,000	H(some bK, PL, GS) H, α -bK(some GS, PL) (same as IIIB ?) H, β -bK, GS, PL	Mn^{2+} Mn^{2+} Mn^{2+} Mn^{2+}	 inhibitors 1 & 2 inhibitors 1 & 2
Krebs(590)	-	bK	Mg^{2+} , Ca^{2+} , Mn^{2+}	NaF, glycogen
Fischer(591-593)	50,000 (glycogen particle)	PL	G-6-P	AMP, ATP
England & Krebs (266)	50,000(593)	PL, TNI		NaF
Fischer(594-596)	33,000-120,000 2,7S & 4.6S (glycogen particle)	PL, TNI	glucose, G-6-P	Pi, salt, nucleotides
Bot(597-599)	-	PL	G-6-P, glucose, caffeine	non-activated bK AMP, G-1-P, etc.
Villar-Palasi(600)	-	GS, PL		glycogen, oligosaccharides
Perry(222)	70,000	myosine light chain	Mg^{2+} , Mn^{2+} , Co^{2+} (dependent)	
Lee(608)	35,000	PL		
<u>Rat skeletal muscle</u>				
Nuttall(601)	-	GS	G-6-P	ATP, ADP, UDP, glycogen
England(602)	(I) >250,000 (II) 50,000-250,000 (III) <50,000	TNI TNI, H, PL TNI, PL	Mn^{2+} Mn^{2+}	

TABLE 8 contd.

Bargoni <u>et al</u> (603)	-	C,phosvitin, phosphoserine	Mn ²⁺ ,Mg ²⁺	Ag ⁺ ,Cu ²⁺ ,Ba ²⁺
Lee (613)	35,000	PL		
<u>Pigeon muscle</u>				
Torres (604-606)	-	PL	theophylline, caffeine,ATP-Mg ²⁺	nucleotides,PPi
<u>Bovine muscle</u>				
Wasner (607)	-	H	cyclic AMP antagonist	cyclic AMP
<u>LIVER</u>				
<u>Rabbit liver</u>				
Lee (608-611)	34,000; 2.9S	PL GS bK,cyclic AMP-dependent protein kinase	theophylline Mg ²⁺ & G-6-P	Mg ²⁺ , Mn ²⁺ ,Ca ²⁺
Kato & Sato (615)	(I) 139,000; 7.0S	H,PL	Mn ²⁺	
	(II) 164,000; 7.8S	H,PL	Mn ²⁺	
	(III) 105,000; 5.8S	H,PL	Mn ²⁺	
	47,000; 3.4S	H,PL	-	
Khandeival <u>et al</u> (616-618)	(I) 34,500; 3.4S (SDS:30,500)	PL,GS,bK,H,C,TNI		M ²⁺ ,NaF,PPi,Pi, PNPP,nucleotides, AMP (PL phosphatase)
	(II) 34,500; 3.4S (SDS:34,000)	same as (I)		same as (I)
Najjar (159)	membrane bound	H,PL		ATP,GTP,Pi
<u>Rat liver</u>				
Lee (609,613,614)	(H) 130,000; 5.77S	PL		
	(C) 35,000	PL		

TABLE 8 contd.

Steinberg(385)	35,000(?)	lipases		
Tsuiki(620,621)	115,000-500,000	GS	Mg ²⁺ (dependent), G-6-P	
	-	PL		
Langan(622)	185,000 & 190,000	H, protamine	salt	PPi, NaF
Whelan(623)	-	PL	glucose	AMP
Moret(624-626)	cytosol multiple forms	C(Thr-P)		
Moret(624-627)	mitochondria:32,000	C(Ser-P), phosvitin, PNPP, ATP, ADP, PPi		Mg ²⁺
Nuttall(628-630)	glycogen particle	GS	G-6-P, glucose	ATP, EDTA, salt, NaF, PPi
	glycogen particle	H	salt	NaF, PPi
Nuttall(631,632)	multiple forms	GS, PL, H		
Titanji(397,633, 634)	32,000	pyruvate kinase & its phosphopeptides, protamine, H	Mn ²⁺ , Mg ²⁺ , Ca ²⁺ F-1,6,-P ₂	ATP, ADP, Pi
<u>Canine liver</u>				
Sutherland(456)	-	PL, phosvitin, C		salt, NaF
Merlevede(635-640)	215,000; 77,000; 8.3S convert to 45,000	PL		ATP, nucleotides, NaF
	138,000; 4.35S	PL	ATP-Mg ²⁺ (dependent)	
Merlevede(640)	51,000 (glycogen particle)	PL		ATP, Mg ²⁺
<u>Mouse liver</u>				
Hers(642,644,645)	-	GS	glucose, caffeine	glycogen, AMP, NaF
Hers(643-645)	-	PL	glucose	AMP
Paigen(646,647)	-	C(pH<6)	thioglycolate, cycteine, M ²⁺	1,10-phenanthro- line, molybdate

TABLE 8 contd.

ADRENAL CORTEXBovine adrenal cortex

Perlman (648)	75,000 (sucrose gradient)	H, protamine PL	salt, Mn^{2+} caffeine, glucose	ATP, ADP, NaF, Zn^{2+} salt, G-1-P, G-6-P, Zn^{2+} , NaF, Pi, ATP, ADP, AMP
		bK, PNPP		
Merlevede (637, 638, 649, 650)	195,000; 7.7S, 4.15S	PL		Mg^{2+} , ATP, nucleotides & analogs
	88,000; 4.15S	PL	ATP- Mg^{2+} (dependent)	
Rosemont (651)	25,000-60,000	PL	Ni^{2+} , Co^{2+} , Mn^{2+} (not Mg^{2+})	
	30,000 (Ni^{2+} activated)	PL	lost ATP- Mg^{2+} dependency	

BRAINRat brain

Greengard (652)	multiple forms	H, protamine	Mn^{2+}	Zn^{2+} , Cu^{2+} , Fe^{3+} , NaF, Pi
Miyamoto (653)	myelin fraction	myelin basic protein H, protamine		

Bovine brain

Miyamoto (653)	multiple forms	myelin basic protein H, protamine		
Rodnight (654)	membrane bound	endogenous membrane protein	Mn^{2+} , Mg^{2+}	EDTA, Zn^{2+} , Cu^{2+} , salt, ATP- Mg^{2+} , molybdate
Rose (655-657)	-	C	Mn^{2+} , Mg^{2+}	EDTA, Zn^{2+} , Co^{2+} , Fe^{2+} , NaF

Bovine pineal gland

Yang et al (658)	-	H	GTP, ATP, Mn^{2+} (activator ?)	Zn^{2+} , NaF

TABLE 8 contd.

SMOOTH MUSCLERat uterus

Kram(561)	45,000	H,protamine	Zn ²⁺
Davis(661)	-	C	inhibitor(?)

Procine uterus

Roberts et al(659, 660)	32,000	phosvitin(pH 4.9) PNPP,PPi,ATP	mercaptoethanol Zn ²⁺ ,NaF, molybdate

Bovine Tracheal smooth muscle

Sands(662)	50,000-80,000 (multiple forms)	H	Zn ²⁺ ,ATP,Pi,NaF, spermidine, molybdate
		muscle specific substrate protein (M _r =19,000)	Mn ²⁺ (dependent) same as above

BLOOD CELLHuman erythrocytes

Avruch(663)	-	spectrin	salt	ATP,GTP,PPi,Pi, 2,3-diphospho- glycerate

Human lymphocytes

Kemp(664)	-	H	salt	ATP(prevent by Mg ²⁺),phospho- enol pyruvate

Rabbit reticulocytes

Traugh(665-667)	(I)	C	hemin	
	(II)ribosomal	H, ribosomal 40S subunit	Mn ²⁺ ,Ca ²⁺ ,Mg ²⁺ (H as substrate)	
	(III)	C,H	same as (II)	

TABLE 8 contd.

Canine platelet

Najjar(668) - phosphoglucomutase NaF, prostaglandin E_1

Polymorphonuclear leukocytes

Najjar(668) membrane bound phosphoglucomutase NaF, prostaglandin E_1
 endogenous substrate

Weissmann(669) 45,000 & 18,700 H Mn^{2+}, Mg^{2+} $Zn^{2+}, Fe^{2+}, Cu^{2+}$

KIDNEY

Villar-Palasi(670) -

PL

Huang & Schwartz (I) 100,000
 (188,699)

H, protein p49 cyclic AMP
 (p49 as substrate) ATP, ADP, AMP,
 Pi, Zn^{2+}, Cu^{2+}

(II) 60,000

H, C, protein p49 same as (I) same as (I)

NUCLEIHeLa cell nuclei

Jeanteur(671) ribonucleoprotein
 particle

H, endogenous substrate M^{2+} , ATP & analogs

Novikoff hepatoma nucleolei

Olson(672) -

nucleolar protein EDTA, M^{2+}

Rat liver nuclei

Yang & Li(673) multiple forms

H ATP, Mn^{2+}, Mg^{2+} , salt

Calf liver nuclei

Yang(674) 32,000

H, protamine, phosphovitin ATP

RAT DIAPHRAGM

Mansour(619) -

PL G-6-P, glycogen $Pi, F-1,6, -P_2$
 glucose

TABLE 8 contd.

BOVINE EPIDIDYMAL SPERMATOTZOA

Hoskins(675)	-	H		Zn ²⁺ ,NaF

BOVINE CORPUS-LUTEUM

Menon(676)	6.7S & 4.8S (plasma membrane)	H,C,protamine	dithiothreitol, mercaptoethanol	M ²⁺ ,PPi,Pi,NaF, nucleotides

CALF THYROID

Burrow(677)	(I)	H,proteamine	Mn ²⁺ ,Mg ²⁺	Co ²⁺ ,NaF
	(II)	H,protamine	Mn ²⁺ ,Mg ²⁺	Co ²⁺ ,NaF
	(III)	H,protamine	Mn ²⁺	Co ²⁺ ,NaF

ADIPOSE TISSUESProcine adipose tissue

Mayer(678)	55,000; 4.1S (glycogen particle)	PL	glucose,G-6-P, theophylline	M ²⁺ ,AMP,ATP, NaF,G-1-P
		TNI		
Steinberg(385)	-	lipase		

N. CRASSA (ASCOMYCETE FUNGUS)

Torres(679)	-	PL	ATP-Mg ²⁺	NaF,ATP, nucleotides

CENTRAL NERVE SYSTEM OF M. SEXTA (TOBACCO HORNWORM)

Newburgh(680)	-	protamine	salt,Mn ²⁺	ATP,GTP,NaF

BONE

Vaes(691)	lysosomal(?)	C		

SPLEENRat spleen

Sundararajan & Sarma (681-683)	-	C,phosvitin,PPi,ATP, PNPP(pH<6)	thioglycolate	

TABLE 8 contd.

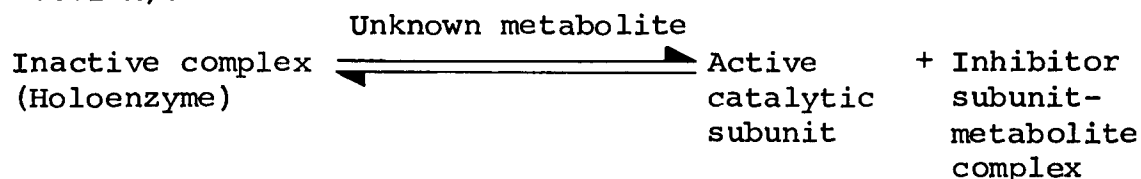
Feinstein(685)	-	C	Mn ²⁺ ,Mg ²⁺ ,Ba ²⁺ ,Ca ²⁺	Cu ²⁺
Norberg(686)	-	C		
<u>Bovine spleen</u>				
Sundararajan & Sarma (682-684)	-	C,phosvitin,PPi, vitellin,ATP, PNPP (pH<6)	thioglycolate	Cu ²⁺ ,Pb ²⁺ ,Zn ²⁺
Hofman(687,688)	-	C,phenylphosphate, PNPP,PPi (pH 5.0-5.5)	thioglycolate	
Revel(689,690)	-	C,phosvitin,PNPP, ATP,PPi,acyl phosphate, aromatic phosphoesters	thioglycolate	M ²⁺ ,molybdate, 8-hydroxyquinoline, 1,10-phenanthro- line
<u>EGG AND EMBRYO</u>				
<u>Frog egg</u>				
Harris(692,693)	-	egg yolk protein,C, vitellin,PNPP,ATP, acyl phosphate (pH<5)		Pi,molybdate
Nass(694)	-	yolk platelet (pH~5)		
<u>Fish oocyte</u>				
Tsusaka(695)	-	C		
<u>Sea urchin embryo</u>				
Vittorelli(696)	-	C,phosvitin (pH 5.5-6.0)	salt	Ca ²⁺ ,Mg ²⁺
<u>Chick embryo</u>				
Kind(697,698)	-	C,phosvitin		

TABLE 8 contd.

The abbreviations used in table 8 are: H, histone; C, casein; PL, phosphorylase; bK, phosphorylase b kinase (α & β indicate its subunits); GS, glycogen synthetase; G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; TNI, inhibitory subunit of troponin; F-1,6-P₂, fructose-1,6-diphosphate; PNPP, p-nitrophenyl phosphate; M²⁺, divalent cations.

The glycogen synthetase phosphatase isolated by the method of Kato and Bishop (57) and also used by several other groups (e.g., Glinsmann, 576; Thomas, 563,564), has a very broad elution profile from a DEAE-cellulose column. Multiple forms of phosphoprotein phosphatases have subsequently be demonstrated with different substrates in various tissues, such as liver (615,620,621,624-626,631,632,635-640), skeletal muscle (572-574,586-589,602), heart (568), adrenal cortex (638), brain (652,653), smooth muscle (662), reticulocytes (665-667), kidney (188), and thyroid (677) (see Table 8). Recently, several laboratories have found that the multiple forms of phosphoprotein phosphatases could be dissociated by treatment with ethanol (609,610,613) or 2-mercaptoethanol (572-574,615) to yield a catalytic subunit, with concomitant activation of the enzyme. Therefore, Lee and colleagues (610,613,700) have proposed a hypothesis which states that the multiple forms of phosphorylase phosphatase consists of an enzyme-inhibitory protein complex which contains a single, common catalytic subunit of molecular weight 32,000, and that the inactive complex might be activated by interaction of the complex with a metabolite—"second messenger." This model is analogous to the model for the activation of cyclic AMP-dependent protein kinase (cf. Protein kinases

section).



The multiple forms of phosphorylase phosphatase from liver were further suggested to be the products of partial proteolysis of the inhibitor protein (610,614,700,701). The purification of the catalytic subunit to homogeneity from rabbit liver (608) and the isolation of a heat-stable inhibitory protein(s) (609) have been subsequently reported.

Since then, the phosphoprotein phosphatases with a molecular weight of 30-35,000 have been highly purified by several other laboratories from rabbit liver (616), bovine heart (520), and rabbit skeletal muscle (595) (Table 8). The larger, less-active holoenzyme has been purified from rat liver with a calculated molecular weight of 130,000 ($S_{20,w}$ of 5.77S & Stokes radius of $51.5\overset{\circ}{\text{A}}$) by Lee et al. (614).

These workers suggested that the holoenzyme consisted of two catalytic subunits ($M_r=35,000$ each) and one regulatory subunit ($M_r=65,000$) (614). However, the simplified model proposed by Lee and colleagues (613,700) may not be applicable to all phosphatases in the liver or other tissues.

Evidence from the same laboratory (702) has indicated that a protamine phosphatase in rat liver could be distinct

from phosphorylase phosphatase(s) described above.

Besides the soluble phosphoprotein phosphatases, the enzyme has been found to be associated with different sub-cellular organelles, such as membranes (159,188,652-654, 663,668,676,699), ribosomes (665), nuclei (671-674), sarcoplasmic reticulum (177,178), glycogen particles (591-596, 628-630), and mitochondria (624-627). Although there is some evidence suggesting that the particle-bound phosphatases (e.g., phosphatases from glycogen particles (595), renal medullary membrane (188) and sarcoplasmic reticulum*) are very similar to the soluble enzymes, the relationship between the particle-bound and those established cytosol phosphoprotein phosphatases remains to be elucidated.

The study of a partial purified phosphoprotein phosphatase from canine heart in this laboratory (569) has indicated that dissociation of holophosphatase to its catalytic subunit ($M_r=35,000$) is accompanied by increased enzymic activity and a pronounced change in the catalytic properties of the enzyme. Thus, the holoenzyme and its catalytic subunit exhibit different pH profiles, substrate saturation kinetics as well as different responses to nucleoside phosphates, salts, and other ionic compounds. In general, the holophosphatase activity is much more sensitive

*H.-C. Li unpublished observation.

to the modification of the substrate (histone) by ATP and salts than is its catalytic subunit. These observations suggest that, following the dissociation, the regulatory properties of the enzyme are lost. This hypothesis was substantiated by the study of the holoenzyme complex ($M_r=240,000$) isolated from glycogen particles (705), which appears to be more responsive to modulation in the complex form than in the $M_r=33,000$ species. The catalytic subunit of the phosphorylase phosphatase isolated from glycogen particles has a minimum molecular weight of 33,000, and displays a strong tendency to aggregate with decreasing enzymic activity (594,595). Therefore, whether the conversion of holophosphatase to its catalytic subunits and the loss of regulation is due to the removal of a regulatory subunit(s) or to the dissociation of an oligomer of catalytic subunits remains unclear.

B) Protein inhibitors of phosphoprotein phosphatases

Heat-stable protein inhibitors of phosphoprotein phosphatases from various tissues have been demonstrated, and some of them have been purified to homogeneity (Table 9). Two types of protein inhibitors have been found, one being cyclic AMP-dependent and the other cyclic AMP-independent.

Huang and Glinzmann (578) have reported that a partially

TABLE 9

Protein Inhibitors of Phosphoprotein Phosphatases

Sources & References	Molecular Weights	Phosphoprotein Phosphatase	Notes
<u>RABBIT SKELETAL MUSCLE</u>			
<u>Inhibitor-1</u>			
Huang & Glinsmann (578,580)	26,000	phosphorylase phosphatase ($M_r=52,000$) (578)	activated and phosphorylated by cyclic AMP-dependent protein kinase; inactivated and dephosphorylated by Mn^{2+} -dependent phosphatase
Cohen et al (588,589)	25,000	phosphatase III (587)	phosphorylation site: -Arg-Arg-Pro-Thr(P)-Pro-Ala-
<u>Inhibitor-2</u>			
Huang & Glinsmann (579,580)	33,000	phosphorylase phosphatase ($M_r=52,000$) (578)	not phosphorylated by cyclic AMP-dependent protein kinase
Cohen et al (588)	65,000	phosphatase III (587)	not phosphorylated by cyclic AMP-dependent protein kinase
<u>RABBIT LIVER</u>			
Brandt et al (609)	50,000-250,000	phosphorylase phosphatase (608)	
Khandelwal & Zinman (618)	15,500 ^o (1.45S & 25.5A)	phosphorylase phosphatase (616)	not phosphorylated

DOG LIVER

Goris <u>et al</u> (673)	-	phosphorylase phosphatase(641)	cyclic AMP-dependent and -independent inhibitors

BOVINE ADRENAL CORTEX

Merlevede <u>et al</u> (638)	-	phosphorylase phosphatase	cyclic AMP-dependent and -independent inhibitors

MOUSE EHRLICH ASCITES CARCINOMA CELL

Baba & Tsuiki (704)	$M_r < 50,000$	glycogen synthetase phosphatase	

purified phosphorylase phosphatase from rabbit skeletal muscle can be inactivated by a reaction catalyzed by cyclic AMP-dependent protein kinase. The inactivation is accompanied by a decrease in molecular weight of the enzyme from 70,000 to 52,000 and by the formation of a phosphorylated inhibitory protein with a molecular weight of 26,000. Dephosphorylation of this phosphoprotein inhibitor by a Mn^{2+} -dependent phosphatase(s) results in the loss of its inhibitory activity on phosphorylase phosphatase. Furthermore, the phosphoprotein inhibitor does not affect the phosphorylase phosphatase activity toward phosphohistone or muscle glycogen synthetase D (578,580). This cyclic AMP-dependent protein inhibitor was named inhibitor-1 (580) and was purified to homogeneity (589). One mole of phosphate was found to be incorporated into one mole of inhibitor-1 at a threonine site (Table 9) by cyclic AMP-dependent protein kinase (589).

In addition to the phosphorylatable protein inhibitor-1 ($M_r=26,000$), Huang and Glinzmann (579,580) have also reported the isolation of a nonphosphorylatable protein inhibitor ($M_r=32,000$) from skeletal muscle, which was named inhibitor-2 (580). Cohen et al. (588) have reported that the inhibitor-2 ($M_r=65,000$, judged by gel filtration), isolated from skeletal muscle, inhibited the phosphatase

III ($M_r=46-75,000$) activity toward phosphorylase, β -phosphorylase kinase, and glycogen synthetase. Recently, Khandelwal and Zinman (618) have purified the inhibitor-2 ($M_r=15,500$) to homogeneity from rabbit liver.

These heat-stable protein inhibitors may play a very important role in regulation of phosphoprotein phosphatase activities. The phosphorylatable inhibitor-1 may mediate the regulatory effects of cyclic AMP on phosphoprotein phosphatase activities, which has been suggested to be regulated by cyclic AMP-mediated phosphorylation-dephosphorylation interconversion (604-606,638,649). The possibility that these heat-stable inhibitors are the regulatory subunit(s) of the holophosphatase(s), as suggested by Lee and colleagues (613,700) has yet to be established.

C) Substrate specificities of phosphoprotein phosphatases

The early studies on the phosphorylase phosphatase of rabbit skeletal muscle (592) indicated that the phosphoprotein phosphatases might have high substrate specificity. Subsequently, the existence of phosphatase specific for histone and protamine were demonstrated in liver by Meisler and Langan (622). Kato and Bishop (571) isolated a partial purified glycogen synthetase phosphatase which was active

toward histone but could not convert phosphorylase a to b. However, by using an enzyme preparation similar to that of Kato and Bishop (571), Zieve and Glinsmann (576) and Nakai and Thomas (563,564) were able to show the same enzyme preparation could convert glycogen synthetase from D to I, phosphorylase from a to b, inactivate phosphorylase kinase and dephosphorylate glycogen synthetase, phosphorylase, phosphorylase kinase, histone, and casein. Therefore, they postulated that the enzyme preparation contained a general phosphoprotein phosphatase with a broad substrate specificity. The concept of a general phosphatase with a broad substrate specificity was proven by the homogeneous preparation of the phosphatase catalytic subunits of $M_r=30-35,000$ (520,611,616), which was shown to be active toward histone, casein, phosphorylase, glycogen synthetase, phosphorylase kinase, regulatory subunit of cyclic AMP-dependent protein kinase, lipase, and inhibitory component of troponin (Table 8).

Although the general phosphatase with a broad substrate specificity has been well established, phosphoprotein phosphatases with narrow substrate specificity toward α and β subunit of phosphorylase kinase (587), inhibitory subunit of troponin (602), myosin light chain (222), glycogen

synthetase (621,632), phosphorylase (621,632), and casein (626) have been only recently reported. The substrate specificity of these phosphatases may be due to the association of the general phosphatase catalytic subunit with different regulatory subunits, aggregation of the catalytic subunits, or it may be due to enzyme entities completely different from the general phosphatase.

Early studies of phosphatases from frog egg (692,693), chick embryo (697,698), and various mammalian tissues (655-657,681-690) indicated that there were two types of phosphatases, one being specific for phosphoproteins, and the other nonspecific, which is active toward phosphoproteins as well as towards the lower-molecular-weight phosphomonoesters at acidic pH (e.g., *p*-nitrophenyl phosphate, *PPi*, *ATP*) (Table 8). Most phosphoprotein phosphatases studied in recent years are specific for phosphoproteins. The homogeneous catalytic subunits of $M_r=30-35,000$ with broad specificity toward phosphoproteins were shown to be inactive toward the low-molecular-weight phosphomonoesters (520,608, 616). However, the partially purified preparations of phosphoprotein phosphatase from rat liver mitochondria ($M_r=32,000$) (627) and from bovine adrenal cortex ($M_r=75,000$) (648) did hydrolyze *p*-nitrophenyl phosphate. The *E. coli*

alkaline phosphatase (707) and the human placental alkaline phosphatase (708) active toward phosphoproteins were also reported recently.

D) Effectors

Effectors, which activate or inhibit phosphoprotein phosphatase activity, are summarized in Table 8. In general, the phosphorylase phosphatases were activated by glucose, glucose-6-P, theophylline, caffeine and inhibited by glycogen, glucose-1-P, AMP, and other nucleotides. The glycogen synthetase phosphatases were activated by glucose-6-P, glucose, and inhibited by glycogen, nucleoside di- and tri-phosphates. Most of these effectors have been suggested to exert their effects by binding to the substrate protein, phosphorylase, or glycogen synthetase (565,581,582,585,597,598,623,678). NaF, Pi, and PPi were more general phosphoprotein phosphatase inhibitors than those metabolites described above (Table 8). Ionic strength was reported to alternate the phosphatase activities, especially when a highly charged protein (e.g., histone) was used as a substrate (569). In most cases, nucleoside triphosphates were inhibitory (Table 8). However, several nucleoside triphosphates which stimulate histone phosphatases (568, 569,630,658) have been reported. The stimulatory effect of nucleoside phosphates and salts were interpreted as being

a consequence of anion—histone interaction. Presumably, the anions neutralize the positively charged histone and make it a better substrate for the enzyme (569). In addition, the regulatory effects of pyruvate (404), thiamine pyrophosphate (405,410), the acetyl CoA/CoA and NADH/NAD⁺ ratios (474) on the phosphorylation-dephosphorylation of pyruvate dehydrogenase (409) and the stimulatory effects of alanine and phenylalanine on the phosphorylation of pyruvate kinase (398) were shown to act at the substrate level. The cyclic AMP stimulated dephosphorylation of the regulatory subunit of cyclic AMP-dependent protein kinase was similarly interpreted (520). One may conclude, therefore, that the interaction of metabolites with substrate proteins plays an important role in the regulation of the phosphorylation-dephosphorylation reactions and can modulate the output of the entire enzyme cascade systems (cf. Enzyme cascade systems section).

The effects of divalent cations on phosphoprotein phosphatase activity seem to be diverse (Table 8). Generally, Zn²⁺, Fe²⁺, and Cu²⁺ were inhibitory. The effects of Mg²⁺, Mn²⁺, Co²⁺, and Ca²⁺ depended on the enzyme preparation and substrate protein used in the study. The PR enzyme described by Cori and Cori (461) in 1945 was stimulated by

Mn^{2+} , but they could not later reproduce the result with a partially purified enzyme preparation (706). Several Mn^{2+} stimulated phosphoprotein phosphatases were isolated from various tissues (178,222,563-575,578,587,590,602,603,615,633,658) (Table 8). Some of them are highly dependent on Mn^{2+} for the enzymic activities (222,566,570,572,590,662,669). Mg^{2+} stimulated phosphoprotein phosphatases were also reported. In addition to those summarized in Table 8, Mg^{2+} has been shown to activate phosphatase activity toward lipase (385), acetyl-CoA carboxylase (387,388), pyruvate dehydrogenase (403,414), phosphofructokinase (426), fatty acid synthetase (425), and cholesterol esterase (432) in some crude preparation. On the other hand, the divalent cations, including Mn^{2+} , Mg^{2+} , and Co^{2+} , exhibited either no effect or inhibitory effect toward the homogeneous catalytic subunit of $M_r=30-35,000$ (520,608,617). The discrepancy of these divalent cation effects probably arises from using different enzyme preparations and different substrates. Some of the metal ion inhibitory effects may be explained by the interaction with substrates (582). Although the mechanism by which they exert their effects remains unclear, the divalent cations certainly play an important role in modulating the phosphoprotein phosphatase activities.

The controversial results obtained from the study of the molecular forms and the substrate specificity of phosphoprotein phosphatases in the past twenty years may be mainly due to different assay conditions employed by different laboratories. As discussed above, the ionic strength (e.g., salt), the metabolite(s) (e.g., AMP, theophylline) and divalent cations (e.g., Mg^{2+} , Mn^{2+}) present in the assay mixture could alter the phosphatase activity dramatically. These effects should be seriously considered when experimental results are interpreted or compared to prevent misleading conclusions.

E) Phosphoprotein phosphatases from canine heart

In 1975, H.-C. Li (568) reported that phosphohistone phosphatase activity in the 0-55% ammonium sulfate fraction of canine heart extracts can be resolved by DEAE-cellulose chromatography into four major fractions which were arbitrarily designated as phosphatases A, B, C, and U. The enzymic activity of each fraction showed different responses to nucleoside triphosphates and metal ions. Phosphatase A was inhibited by ATP, phosphatase B was stimulated by ATP, phosphatase C required Mg^{2+} for maximum activity and phosphatase U was totally dependent on Mn^{2+} or Co^{2+} for activity. The phosphatase U was further fractionated in this laboratory

by gel filtration on a Sephadex G-200 column, and the Mn^{2+} -activated enzyme was eluted with a Stokes radius of $39\overset{O}{\text{\AA}}$ (570). The enzyme was active toward phosphohistone, phosphorylase a, and phosphocasein. However, the elution profile of phosphocasein phosphatase was not exactly superimposable with that of phosphohistone phosphatase but shifted slightly to the range of a smaller molecular size on gel filtration chromatography. In addition, $MgCl_2$ can markedly activate the phosphatase U activity toward phosphocasein but not phosphohistone nor phosphorylase a (570). The results indicated that the phosphatase U preparation may contain two different enzymic activities: an Mg^{2+} and Mn^{2+} -activated phosphocasein phosphatase and an Mn^{2+} -activated enzyme which dephosphorylates both phosphohistone and phosphorylase a, but not phosphocasein.

The purpose of this study was to examine those multiple forms of phosphoprotein phosphatase in canine heart and to study the mechanism which regulates the enzymic activities.

V. Summary

A) Phosphoproteins are important components of biological systems. They regulate metabolic processes, control specific cellular functions, mediate neural hormonal signals, maintain structures, and provide nutrient for embryonic development and newborns.

B) Phosphoproteins discussed in this review are referred to those covalently linked to the phosphates through a phosphoester bond at serine or threonine residues. Phosphoproteins found in nature include enzymes, membrane proteins, contractile proteins, microtubular proteins, nuclear proteins, ribosomal proteins, bacterial and viral proteins, structural and nutrient proteins and some other unclassified proteins.

C) Many proteins phosphorylated under hormonal control are mediated by cyclic nucleotides. In addition to cyclic nucleotides, several other regulatory agents also affect the phosphorylation-dephosphorylation of specific proteins in their target cell, such as steroid hormones, insulin, hemin, and calcium.

D) Protein phosphorylation reactions are catalyzed by protein kinases which transfer γ -phosphate of nucleoside triphosphates to an acceptor protein. There are cyclic nucleotide-dependent and -independent protein kinases. Cyclic nucleotides and calcium play important roles in regulating protein kinase activities.

E) Phosphoprotein phosphatases, which catalyze the dephosphorylation reactions, may exist in multiple forms. Some of those multiple form can be dissociated into a single general catalytic subunit of $M_r=30-35,000$, with

concomitant activation of the enzyme. The multiple forms may be complexes of catalytic subunit and regulatory subunit or oligomers of catalytic subunits.

F) Both phosphoprotein phosphatases with broad and narrow substrate specificities have been reported.

G) Two classes of heat stable protein inhibitor of phosphoprotein phosphatases have been identified. Inhibitor-1 can be phosphorylated by cyclic AMP-dependent protein kinase and requires phosphorylation for its inhibitory activity. On the other hand, inhibitor-2 does not require phosphorylation for its inhibitory activity.

H) Control at substrate level is an important regulatory mechanism on the phosphoprotein phosphatases.

I) The reported diversified effects of metabolites (e.g., ATP) and divalent cations on phosphoprotein phosphatase activities may be due to the varied enzyme preparations and protein substrates used from study to study.

J) Phosphoprotein phosphatases from canine heart can be separated into four fractions by DEAE-cellulose chromatography, namely, phosphatases A, B, C, and U. The enzymic activity of each fraction showed different response to nucleoside triphosphates and metal ions. Phosphatase U is totally dependent on metal ions for activity. Thus,

the various regulatory properties of the phosphatase isozymes may be of physiological importance.

EXPERIMENTAL PROCEDURES

I. Materials

[γ -³²P]ATP and [8-¹⁴C]ATP were purchased from New England Nuclear. Rabbit skeletal muscle phosphorylase b was purified from frozen tissue by a modified (547) method of Fischer and Krebs (709) or purchased from Sigma Chemical Co. (2x crystalline). Calf thymus histone mixture (type II), phosphorylase a, glucose-1-phosphate, phosphorylase kinase, polyphosphates, glycogen, and other nucleotides were obtained from Sigma. (β , γ -methylene) adenosine 5'-triphosphate was from Miles Laboratory. ATP and dithiothreitol were from Calbiochem. Vitamin-free casein was purchased from Nutritional Biochemicals and was dephosphorylated as described by Reimann et al. (515) prior to use. Rabbit skeletal muscle myosin was a gift from Dr. G. Bailin, prepared according to Bárány and Oppenheimer (710). The myosin was dephosphorylated by Escherichia coli alkaline phosphatase (P-L Biochemicals) according to Perrie et al. (201), prior to use. The catalytic subunit of cyclic AMP-dependent protein kinase was purified from beef heart according to Erlichman et al. (711). All chemicals used were of reagent grade.

II. Preparation of ^{32}P -labeled Protein

Phosphorylase b was converted to ^{32}P -labeled phosphorylase a by a modified method of Torres and Chelala (604). Phosphorylation of 200-300 mg phosphorylase b was catalyzed by phosphorylase b kinase (300 μMolar units according to Sigma) at 30° for 20-30 min in a reaction mixture containing 50 mM Tris·HCl, pH 8.6, 20 mM magnesium acetate, 10 μM CaCl_2 , 1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and 20 mM 2-mercaptoethanol. The reaction was stopped by addition of equal volume of a buffer containing 100 mM NaF, 80 mM β -glycerophosphate, pH 6.8, 40 mM 2-mercaptoethanol, and 20 mM EDTA. The labeled phosphorylase a was crystalized by cooling in ice and was collected by centrifugation at $20,000\times g$ for 10 min. The precipitate was resuspended in 10 ml solution containing 50 mM NaF, 40 mM β -glycerophosphate, pH 6.8, 20 mM 2-mercaptoethanol, and 10 mM EDTA plus 2 mM ATP and 5 mM KPi (pH 7.0). The mixture was stirred at 37° until the enzyme dissolved. The ATP treatment inactivated phosphorylase phosphatase activity present in the $[\text{}^{32}\text{P}]$ phosphorylase a preparation. After 5-8 recrystallization, the phosphorylase a was dissolved in 10-15 ml buffer containing 5 mM β -glycerophosphate, pH 6.8, 10 mM 2-mercaptoethanol, and 1 mM EDTA, and dialyzed against the same buffer at 4° overnight. The

enzyme solution was then dialyzed against a buffer containing 20 mM Tris·HCl, pH 7.4, and 10 mM 2-mercaptoethanol with several changes. Finally, the enzyme was stirred with 300 mg Norite A at 30° and the active carbon was removed by repeating centrifugation (20,000 x g, 10 min) at 15°. The [³²P]phosphorylase a so prepared was adjusted to 50 μM concentration (in terms of ³²Pi) and stored at 4°.

Histone and casein were phosphorylated with [γ-³²P]ATP and the catalytic subunit of cyclic AMP-dependent protein kinase by a method similar to that of Meisler and Langan (622). The incubation mixtures contained 50 mM 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino] ethanesulfonic acid, pH 7.0, 10 mM magnesium acetate, 0.02% (w/v) sodium azide, 0.6 mM EGTA, 0.1-0.2 mM [γ-³²P]ATP, catalytic subunit of cyclic AMP-dependent protein kinase (40 μg/ml for casein; 20 μg/ml for histone) and 10 mg/ml casein or 2.5 mg/ml histone. Incubation was carried out at 30° for 4 hours (histone) or 24 hours (casein). At the end of incubation, phosphohistone and phosphocasein were precipitated by adding 1/3 volume and 1/8 volume of 1 g/ml trichloroacetic acid, respectively. The phosphoproteins were dispersed in 2 ml of ice cold 0.25 M NaOH and precipitated again with 25 ml trichloroacetic acid (25 g/100 ml for histone; 10 g/100 ml for casein). The washing processes were repeated four more times as

described above, except 2 ml of water instead of 0.25N NaOH was used for dispersing. The washed phosphoproteins were dissolved in 2 ml of 0.25N NaOH and pH of the solution was adjusted to 7.0 by adding more 0.25N NaOH. The solubilized phosphoproteins were dialyzed against 2l of 0.1 M KCl and 0.1 mM EDTA with three changes, followed by changing two times of distilled water. The [^{32}P] proteins were adjusted to 50 μM concentration (in terms of ^{32}Pi) and stored at -20° .

Rabbit skeletal myosin was phosphorylated in a reaction mixture containing 50 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 0.25 M KCl, 10 mM MgCl_2 , 0.1 mM [γ - ^{32}P]ATP, 3.6 mg/ml myosin, and 0.11 mg/ml of protein kinase catalytic subunit. After incubating at 30° for 30 min, ^{32}P —labeled myosin was precipitated by adding 4 volumes of cold water and redissolved in a buffer containing 20 mM Tris·HCl, pH 7.4, 1 mM 2-mercaptoethanol, and 0.3 M KCl. The washing processes were repeated four more times. Polyacrylamide gel electrophoresis of the ^{32}P —labeled myosin preparation in the presence of sodium dodecyl sulfate (SDS) showed that the radioactivity was incorporated into the myosin light chain.

^{32}P -labeled cyclic AMP-dependent protein kinase prepared according to Chou et al. (520) was a gift from Dr. O. M. Rosen.

The $^{32}\text{P}_i$ content of phosphorylated proteins was 30-40 nmol per mg of histone, 2.5-5 nmol per mg of casein, 8-10 nmol per mg of phosphorylase a, 0.38 nmol per mg of myosin, and 5.4 nmol per mg of cyclic AMP-dependent protein kinase. The specific radioactivity of the [^{32}P]proteins used in the assay varied from 50-650 counts min^{-1} (pmol of [^{32}P]phosphate).⁻¹

III. Enzyme Assay

Phosphoprotein phosphatase activity was measured by the release of $^{32}\text{P}_i$ from [^{32}P]phosphoprotein at 30°. The standard assay mixture contained 50 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 0.1 M KCl, 10 μM [^{32}P]protein (calculated by the $^{32}\text{P}_i$ concentration; any phosphate present before phosphorylation was not considered) in a total volume of 50 μl . The reaction was initiated by the addition of the enzyme to the reaction mixture and terminated with 20 μl of 8% (w/v) trichloroacetic acid. A 50 μl aliquot was then withdrawn from the reaction mixture and applied on the sample spot of a 2 x 22-cm Whatman 31 ET chromatographic paper strip, which was marked with pencil as described by Li and Felmlly (714). The paper strip loaded with sample was transferred to a trough containing 5 g/100 ml of trichloroacetic acid for the development of a descending chromatogram at room temperature. The enzymic product, [^{32}P]phosphate, traveled along the solvent front and was

completely separated from the unreacted ^{32}P -labeled proteins, which remained at the origin. After the solvent front had traveled about 12 cm from the origin, which took less than 20 min, the part of the wetted paper strip 4 cm from the solvent front was separated from the rest. This 2 x 4-cm strip containing [^{32}P]phosphate was rinsed in ether and dried in an oven. The strip was placed into a vial containing 10 ml of toluene scintillation fluid (0.1 g of p-bis[2-(5-phenyloxazolyl)] benzene and 6 g of 2,5-diphenyloxazole per liter of toluene). The radioactivity was determined by counting in a liquid scintillation counter. The present paper chromatographic assay procedure has been compared with the assay procedure described by Meisler and Langan (622). The results were identical. The amount of phosphatase added was such that less than 10% of the substrate was dephosphorylated in a 10 min period of incubation. Reaction rates were linear with time and were proportional to the amount of enzyme. Blank values, i.e., ^{32}Pi obtained in the absence of enzyme, were subtracted from all assays. One unit of phosphoprotein

phosphatase activity was defined as that amount of enzyme which catalyzed the formation of 1 nmol of ^{32}Pi per min. Specific activity was defined as the number of units per mg protein. When cyclic AMP-dependent protein kinase was used as a substrate, phosphatase activity was measured in the presence of 0.1 mM cyclic AMP as described by Chou et al. (520). The phosphorylase phosphatase activity of converting phosphorylase a to b was determined in the assay mixture contained 50 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 0.1 M KCl, 0.5 mM EDTA, 5 mM theophylline, and 0.2 mg/ml phosphorylase a. After incubated at 30° , the reactions were stopped by addition of equal volume of NaF buffer (0.2 M NaF, 1 mM dithiothreitol, 10 mM EDTA, 2 mg/ml bovine serum albumin, and 0.1 M TES, pH 6.8) (608). These phosphorylase solutions (50 μl) were then assay for phosphorylase activity by mixed with 50 μl assay solution (0.15 M glucose-1-phosphate; 2 g/100 ml glycogen; 50 mM TES, pH 6.8; and incubated at 30° for 10 min and with or without 1 mM ATP as indicated). The reactions were stopped by the addition of 2 ml of 0.072 M H_2SO_4 , and the inorganic phosphate released was determined by the method of Fiske and Subbarow (712). To the mixture 0.5 ml 1.25% (w/v) ammonium molybdate (in 2.5 N H_2SO_4) solution

was added. At timed intervals, 0.1 ml of Fiske and Subbarow reagent(Sigma) was added and the absorbance at 660 nm was measured exactly 10 min later.

For assaying the phosphatase activity toward various nonprotein phosphoesters, 20 mM of a phosphoryl compound was substituted for 10 μ M [^{32} P]protein as a substrate and Pi released as determined colorimetrically by the method of Martin and Dorty (713) as described by Meisler and Langan (622). After incubation, protein in the 0.5 ml assay mixture was precipitated by addition of 1 ml of 5 mM silicotungstic acid in 0.005 N H₂SO₄ and removed by centrifugation at 5000 x g for 5 min. To 1 ml of the deproteinized sample, 0.5 ml of 5% (w/v) ammonium molybdate in 4 N H₂SO₄ was added. The phosphomolybdate complex was extracted with 1.5 ml of isobutyl alcohol-benzene (1:1). A ml of the isobutyl alcohol-benzene extract was mixed with 0.5 ml of ethanol-H₂SO₄ (490:10) and 0.05 ml of stannous chloride reagent (713) was added. Absorbance at 660 nm of the mixture was measured spectrophotometrically by using a control without enzyme as a blank. Cyclic AMP-dependent protein kinase activity was measured at 30^o in an incubation volume of 0.1 ml containing 50 mM TES buffer, pH 7.0, 10 mM MgCl₂, 0.2 mg of histone mixture, or

0.5 mg of casein, 50 μM [γ - ^{32}P]ATP (10-100 cpm/pmol), and the enzyme in the presence or absence of 2 μM cyclic AMP. Incubation was carried out at 30° for 5 min. The ^{32}P -labeled protein formed was isolated and the radioactivity was determined by the method of Li and Felmlly (714). One unit of protein kinase activity was defined as the amount of enzyme which catalyzed the transfer of 1 nmol of [^{32}P]phosphate from [γ - ^{32}P]ATP to histone per minute.

IV. Determination of ATP Hydrolysis

Hydrolysis of ATP by phosphatase S was determined by measuring $^{32}\text{P}_i$ released from [γ - ^{32}P]ATP by a modified method of Shamoo et al. (715). The 100 μl assay mixture containing 50 mM Tris·HCl, pH 7.4; 1 mM dithiothreitol; 5 mM [γ - ^{32}P]ATP (specific activity 1000 cpm per pmol) and different concentration of metal ions and salts as indicated (e.g., 20 mM MgCl_2 ; 10 mM MgCl_2 and 5 mM CaCl_2 ; or 5 mM MgCl_2 , 0.1 M NaCl, and 15 mM KCl) was incubated at 30° for 20 to 40 min. The reactions were terminated by addition of 25 μl trichloroacetic acid (25 g/100 ml). Phosphomolybdate complex was formed by addition of 25 μl 5% (w/v) ammonium molybdate in 0.1 N H_2SO_4 and was extracted with 0.2 ml isobutanol by vibratory buzzing for 25 sec. After separation of the organic phase by centrifugation, 0.15 ml of isobutanol layer was added to 5 ml of Scinti Verse

(Fisher Scientific Co.) for measurement of $^{32}\text{P}_i$ in a Parkard liquid scintillation counter.

When nonradioactive ATP was used, P_i released was determined chemically according to Martin and Doty (713). The phosphomolybdate complex was extracted with 0.2 ml of isobutanol-benzene (1:1). An 0.1 ml of the isobutanol-benzene extract was mixed with 1 ml of ethanol- H_2SO_4 (490:10) and 25 μl of stannous chloride reagent (713) was added. The developed color was read at 660 nm.

V. Buffer Solutions

The following buffer solutions were used in the study.

Buffer A: 20 mM Tris·HCl, pH 7.4, 10 mM 2-mercaptoethanol, 2 mM MgCl_2 , 1 mM EDTA, and 50 mM KCl.

Buffer AM: 5 mM MgCl_2 in Buffer A.

Buffer G: 20 mM Tris·HCl, pH 7.4, 0.1 M KCl, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol.

Buffer S: 20 mM Tris·HCl, pH 7.4, 10 mM 2-mercaptoethanol, and 50% (v/v) glycerol.

VI. Purification of Phosphoprotein Phosphatases from Canine Heart

A) Crude extract

The supernatant fraction of canine heart was prepared essentially according to Harigaya and Schwartz (716). Hearts were quickly removed from dogs anesthetized with pentobarbital

and washed with ice-cold distilled water. After removal of fatty and connective tissues, approximately 70-140 g of ventricular muscle was cut into small pieces. The heart muscle was mixed with 5 volumes of 10 mM NaHCO_3 and homogenized at high speed for 30 seconds in a Waring blender. The homogenate was centrifuged at $8,700 \times g$ for 20 min, and the supernatant was recentrifuged under the same condition to yield a second supernatant fraction. This second supernatant fraction was then centrifuged at $37,000 \times g$ for 30 min. The supernatant obtained was the crude extract (Fraction I) used as an enzyme source for further purification.

B) Ammonium sulfate fractionation

Solid ammonium sulfate was added slowly with stirring to the crude extract to obtain 55% saturation (0.35 g added to each ml). The precipitate was collected by centrifugation, resuspended in one tenth volume of Buffer A and dialyzed overnight against the same buffer (Fraction II). Several individual preparations were purified through $(\text{NH}_4)_2\text{SO}_4$ fractionation, stored at -20° , and pooled prior to next step.

The ammonium sulfate fraction was either directly chromatographed on a DEAE-cellulose column for separation of phosphatases A, B, C, and U or used to purify phosphatases

S, U-1, and U-2. This latter procedure involved fractionation and concentration with DEAE Sephadex A-50 and ethanol precipitation prior to DEAE-cellulose chromatography.

C) Preparation of phosphatases A, B, C, and U

Ten ml of ammonium sulfate fraction were applied to a DEAE-cellulose (Whatman DE-52) column (1.5 x 60 cm) previously equilibrated with Buffer A. After sample application, the column was rinsed with Buffer A. Proteins that adsorbed to DEAE-cellulose were eluted by an increasing salt gradient as described by Li (568). Three equal size chambers were connected in series at same level and contained 330 ml Buffer A with different concentration of KCl (first one, 0.1 M; second one, 0.2 M; third one, 0.4 M). The gradient was withdrawn from the first chamber to the column. Fractions of 5.7 ml were collected. The DEAE-cellulose column resolved phosphohistone phosphatase into three peaks of activity, namely, peaks A, B, and C (Fig. 1). When the fractions were assayed in the presence of 5 mM MnCl_2 , an additional peak of activity (peak U) appeared between peaks A and B (data not shown, cf. Li and Hsiao (570)). The active fractions of peaks A (fractions 53-66), U (fractions 68-77), B (fractions 84-100), and C (fractions 120-147) were pooled and concentrated by membrane ultrafiltration with a Diaflo membrane (PM 10, Amicon Corporation)

to approximately 5 ml. The concentrated fractions were dialyzed overnight against 1 liter of Buffer G and stored at 4°.

D) Purification of phosphatases S, U-1, and U-2

The ammonium sulfate fraction was used to purify the basic catalytic entities in canine heart. The ethanol treatment technique (608,613) was employed as a step in the purification scheme for dissociating the larger molecular weight phosphoprotein phosphatases.

1) DEAE-Sephadex A-50 fractionation

The pooled ammonium sulfate fraction was mixed with 3 bed volume of DEAE-Sephadex A-50 (Pharmacia) presaturated with Buffer AM. The mixture was stirred for 1 hour and then filtered through a Whatman 54 filter paper. The resin was washed with two volumes of Buffer AM twice and two volumes of Buffer AM plus 0.05 M KCl twice. The enzyme absorbed on DEAE-Sephadex was extracted by mixing with two volumes of Buffer AM plus 0.45 M KCl, stirring for 1 hour and filtering through the Whatman filter paper. The extraction process was repeated with one volume of the same buffer twice. The enzyme in the filtrates was concentrated by precipitation with addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation and dialyzed overnight against Buffer AM.

2) Ethanol precipitation

The DEAE-Sephadex A-50 fraction was adjusted with 1 M MnCl_2 to obtain a final concentration of 5 mM MnCl_2 . One volume of the enzyme was then mixed with 5 volumes of 95% ethanol at room temperature, and the mixture was centrifuged immediately at 10,000 x g for 10 min. The enzyme in the precipitate was extracted four times with 1 volume of Buffer AM. The combined extracts were concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation and dialyzed overnight against Buffer AM.

3) DEAE-cellulose chromatography

The enzyme solution was applied onto a DEAE-cellulose (Whatman DE-52) column (2.5 x 50 cm) previously equilibrated with Buffer AM. After sample application, the column was rinsed with Buffer AM followed by Buffer AM plus 0.05 M KCl. The enzyme was then eluted with an increasing salt gradient from 0.1 to 0.4 M KCl in Buffer AM as described in the section of Preparation of phosphatases A, B, C, and U. The active fractions of Peak I (fractions 93-131), Peak II (fractions 132-148), and Peak III (fractions 149-172) (Fig. 2) were separately pooled and concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation. The precipitate was resuspended in a small volume of Buffer AM and dialyzed against the same Buffer overnight.

4) DEAE-Sephadex A-50 chromatography

Peaks I, II, and III obtained from previous step were separately chromatographed on a DEAE-Sephadex A-50 column (1.6 x 28 cm) pre-equilibrated with Buffer AM plus 0.05 M KCl. The chromatographic procedures for each fraction of the enzyme were described as follows:

a) Peak III: After sample application, the column was rinsed with about 300 ml of Buffer AM plus 0.05 M KCl and the enzyme was eluted with one liter of linear salt gradient from 0.1 to 0.3 M KCl in Buffer AM. The major peak (fractions 120-140) and the minor peak (fractions 145-160) of activities, as indicated in Fig. 3, were separately pooled, concentrated by membrane ultrafiltration (PM 10, Amicon) to 5-10 ml and dialyzed against Buffer G overnight.

b) Peak II: After sample application, the column was rinsed and the enzyme was eluted as described above. Fractions containing Mn^{2+} -activated phosphohistone phosphatase activity (phosphatase U-1) were pooled, concentrated, and dialyzed against Buffer G as described above.

c) Peak I: After sample application, the column was rinsed as described above and the enzyme was eluted with one liter of linear salt gradient from 0.1 to 0.25 M KCl in Buffer AM. Fractions containing the Mg^{2+} - and

Mn²⁺-activated phosphocasein phosphatase (Phosphatase U-2) activity were pooled, concentrated by membrane ultrafiltration (PM 10), and dialyzed against Buffer G plus 10 mM MgCl₂. The presence of MgCl₂ was essential for stabilizing this Mg²⁺-activated enzyme.

5) Sephadex G-100 chromatography

The concentrated solutions of Peaks II and III enzymes obtained from the previous step were separately filtered through a Sephadex G-100 (Pharmacia) column (2 x 98 cm) pre-equilibrated with Buffer G. The column was eluted with the same Buffer. The concentrated Peak I enzyme was subjected to the same gel filtration procedure except that 10 mM MgCl₂ was included in the buffer system. The active fractions of each column chromatography were pooled and dialyzed against Buffer S and stored at -20°. In the case of the Peak I enzyme, 10 mM MgCl₂ was added to Buffer S for dialysis and storage of the enzyme.

All operations were carried out at 4°, except where otherwise indicated.

VII. Polyacrylamide Gel Electrophoresis

Disc electrophoresis on 7% polyacrylamide gel was carried out by the procedure of Davis (717). After electrophoresis, gels were either stained with Coomassie Brilliant Blue, or transversely sliced into 1 mm sections and assayed

for phosphatase activities. Each slice was placed into a test tube containing 50 μ l of the assay mixture and incubated at 30° for 20 min. The reaction was stopped by the addition of 30 μ l of 8% (w/v) trichloroacetic acid and then 50 μ l aliquot was withdrawn for paper chromatographic separation of ^{32}Pi from the unreacted substrate as described under "Enzyme Assay."

Sodium dodecyl sulfate-gel electrophoresis was performed according to the procedure of Laemmli (718), employing 10% polyacrylamide gel. Bovine serum albumin, ovalbumin, carbonic anhydrase, and myoglobin were used as molecular weight markers.

VIII. Determination of Molecular Weights and Frictional Ratios

Molecular weights of phosphoprotein phosphatases were determined either by sodium dodecyl sulfate-gel electrophoresis described above or by the following method of Sigel and Monty (719). Molecular weights and frictional ratios (f/f_0) were calculated using the sedimentation coefficient ($s_{20,w}$) values derived from sucrose gradient centrifugation and Stokes radii obtained from gel filtration according to the following equations:

$$M = \frac{6\pi\eta N}{(1 - v_p)} a S$$

$$f/f_0 = a / \left(\frac{3vM}{4\pi N} \right)^{1/3}$$

where a = Stokes radius, S = sedimentation coefficient, N = Avogadro's number, η = viscosity of water, ρ = density of water, and v = partial specific volume of the protein (assuming 0.725 for phosphoprotein phosphatases). Axial ratio was estimated according to Schachman (720). $S_{20,w}$ values were determined by sucrose density gradient centrifugation as described by Martin and Ames (721). Centrifugation was carried out with an SW 50.1 swinging bucket rotor run at 39,000 rpm for 16 hours at 4°. Five ml of linear sucrose density gradient from 5-20% sucrose in 20 mM Tris·HCl, pH 7.4, was employed. Approximately 4 μ g of the phosphatase, together with marker proteins, bovine serum albumin (2 mg), pig heart malic dehydrogenase (20 μ g) and horse heart cytochrome c (0.4 mg) were applied to each gradient in a total volume of 100 μ l. After the run, each gradient tube was fractionated and assayed for the phosphatase activity and for the marker proteins. Stokes radii were determined by gel filtration on a Sephadex G-100 column (1.6 x 90 cm). The following proteins (Stokes radius) were used as internal standards: lactate dehydrogenase ($a = 42\overset{\circ}{\text{\AA}}$), liver alcohol dehydrogenase ($a = 36\overset{\circ}{\text{\AA}}$), bovine serum albumin ($a = 35\overset{\circ}{\text{\AA}}$), ovalbumin ($a = 28\overset{\circ}{\text{\AA}}$), carbonic anhydrase ($a = 24\overset{\circ}{\text{\AA}}$), and myoglobin ($a = 20\overset{\circ}{\text{\AA}}$).

IX. Determination of Protein Concentration

Protein concentration was determined by the method of Lowry et al. (722) following trichloroacetic acid precipitation. Bovine serum albumin was used as a standard.

RESULTS

I. Separation of Phosphoprotein Phosphatases by DEAE-Cellulose Column Chromatography

DEAE-cellulose column chromatography resolved phosphoprotein phosphatase present in the 55% ammonium sulfate fraction of canine heart extract into three major peaks (A, B, and C; Fig. 1A). When less enzyme from each fraction was assayed in the presence and absence of 2 mM ATP, the activity profile shown in Fig. 1B was obtained. At this concentration, ATP markedly stimulated the activity of peak B, inhibited the activity of peak A, and had no significant effect on the activity of peak C when phosphohistone was used as a substrate. When each fraction from the DEAE column was assayed in the presence of 5 mM MnCl_2 , the activity of peaks A, B, and C were all slightly stimulated. Furthermore, an additional peak of phosphoprotein phosphatase activity (fraction U) appeared between peaks A and B (570). The results of a typical separation are summarized in Table 10. The data indicate that either in the presence or absence of effectors fraction B contained the highest activity recovered from DEAE-cellulose chromatography.

II. Purification of Phosphoprotein Phosphatases S, U-1, and U-2

In order to purify and identify the basic catalytic

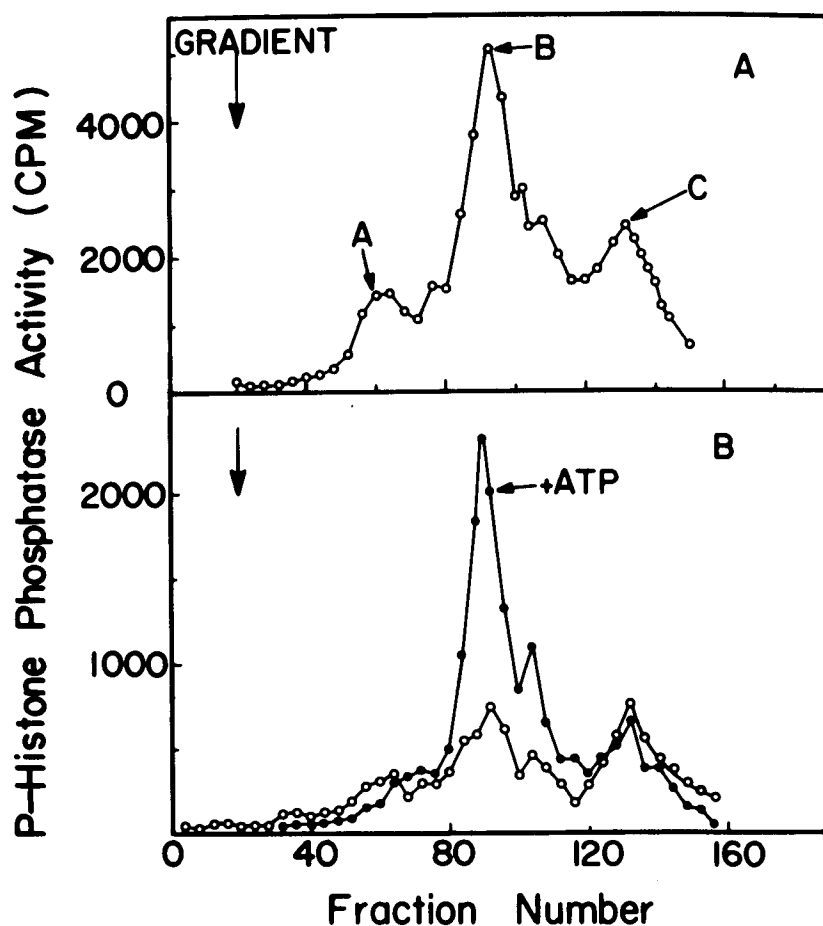


Fig. 1. Column chromatography of the $(\text{NH}_4)_2\text{SO}_4$ fraction on DEAE-cellulose. Ten ml of the $(\text{NH}_4)_2\text{SO}_4$ fraction (25 mg/ml) were applied on a DEAE-cellulose column (1.5 x 60 cm). The enzyme was eluted with a KCl gradient as described under "Experimental Procedures." Fig. 1A shows the elution profile of phosphoprotein phosphatase activity measured in the absence of ATP with 10 μl aliquots from the indicated fractions. Fig. 1B shows the elution profile of the enzymic activity measured in the absence of KCl and in the presence (●) and absence (○) of 2 mM ATP with 2 μl aliquots from the indicated fraction. Incubation was carried out at 30° for 10 min.

TABLE 10

Separation of Various Forms of PhosphoproteinPhosphatases from Canine Heart

Fraction	Total Protein (mg)	Specific Activity (unit/mg)			Total Activity (unit)		
		Control	ATP	Mn ²⁺	Control	ATP	Mn ²⁺
37,000xg supernatant	2985	0.47	0.62	0.6	1403	1851	1791
(NH ₄) ₂ SO ₄ Fraction	875	1.5	3.0	3.1	1312	2623	2711
DEAE-CELLULOSE							
Fraction A	37	1.3	0.6	2.1	48	22	77
Fraction B	92	5.3	14	12	485	1281	1098
Fraction C	29	3.3	4.6	6.4	95	133	185
Fraction U	42	1.0	1.5	8.4	42	63	353
Sephadex G-200							
Fraction B	27	13.7	28	19	370	756	513

Various forms of phosphoprotein phosphatase were isolated from 90 g of canine heart and the enzymic activity was measured in the absence and presence of 2 mM ATP or 5 mM MnCl₂ as described under "Experimental Procedures".

entities in canine cardiac muscle, an ethanol treatment was employed before DEAE-cellulose chromatography. A summary of the purification procedure is presented in Table 11 and Scheme 3.

As shown in Fig. 2, DEAE-cellulose chromatography resolved phosphoprotein phosphatase activities in the ethanol precipitation fraction (Fraction 4) into four active peaks. It appeared that Peak I was an Mg^{2+} - and Mn^{2+} -activated enzyme specific for phosphocasein. Peaks II and IV were Mn^{2+} -activated enzymes specific for phosphohistone and phosphocasein, respectively, and Peak III was a divalent cation-independent enzyme active toward either phosphoprotein substrate. The active fractions of Peaks I, II, and III were pooled separately and each of them was further purified by DEAE-Sephadex A-50 and Sephadex G-100 column chromatographies. Peak IV enzyme was not studied further.

A) Phosphatases S and SR

Chromatography of Peak III on a DEAE-Sephadex column yielded a major and a minor active peak which were eluted at 0.19 and 0.22 M KCl, respectively (Fig. 3). Both peaks were divalent cation-independent and were active toward either phosphohistone or phosphocasein. The major peak (fractions 120-140) and the minor peak (fractions 145-160)

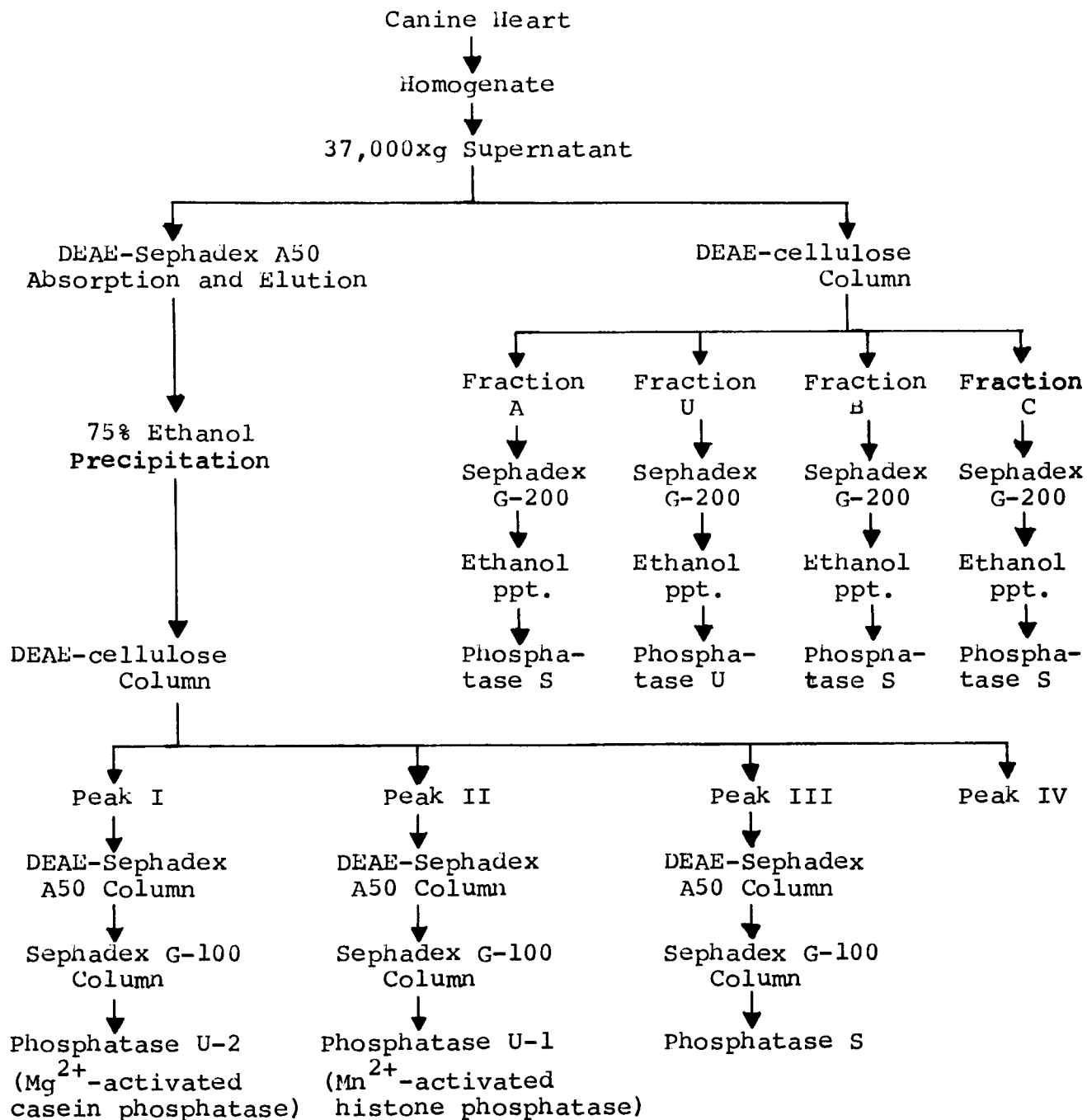
SCHEME 3Purification of Phosphoprotein Phosphatases from Canine Heart

TABLE 11

Purification of Phosphoprotein Phosphatases from Canine Heart

Phosphoprotein phosphatases were purified from 900 g of canine heart tissue as described in the text. At each stage of purification, enzymic activities were determined in the presence of 5 mM EDTA, 10 mM MgCl_2 or 5 mM MnCl_2 as described under Experimental Procedures

Fraction	Total protein	Specific activity on					
		Phosphocasein with			Phosphohistone with		
		EDTA	MgCl_2	MnCl_2	EDTA	MgCl_2	MnCl_2
	mg	units/mg					
1. 37000 $\times$ g supernatant	44600	0.22	0.47	0.45	0.22	0.27	0.36
2. 55% ammonium sulfate precipitation	17900	0.48	1.00	1.46	0.36	0.42	0.79
3. DEAE-Sephadex (A-50)	5790	0.75	1.84	3.24	0.86	1.09	2.11
4. Ethanol precipitation	1220	7.76	9.80	11.3	8.26	9.54	15.0
5-I. DEAE-Cellulose chromatography, peak I	95	0.52	20.2	22.2	1.33	4.49	5.38
6-I. DEAE-Sephadex chromatography	20.7	2.78	72.0	56.1	10.4	10.7	14.3
7-I. Sephadex G-100 chromatography	2.7	2.56	263.6	254.5	6.02	19.6	49.7
5-II. DEAE-Cellulose chromatography, peak II	85	1.50	4.06	6.09	5.11	13.1	25.7
6-II. DEAE-Sephadex chromatography	8.1	0.78	2.80	8.29	6.22	14.0	71.5
7-II. Sephadex G-100 chromatography	0.86	1.05	2.50	36.5	3.0	30.5	251.5
5-III. DEAE-Cellulose chromatography, peak III	149	50	50	61	55	58	63
6-III. DEAE-Sephadex chromatography	12.4	582	409	480	412	388	372
7-III. Sephadex G-100 chromatography	1.06	3454	1822	2375	2730	2842	2922

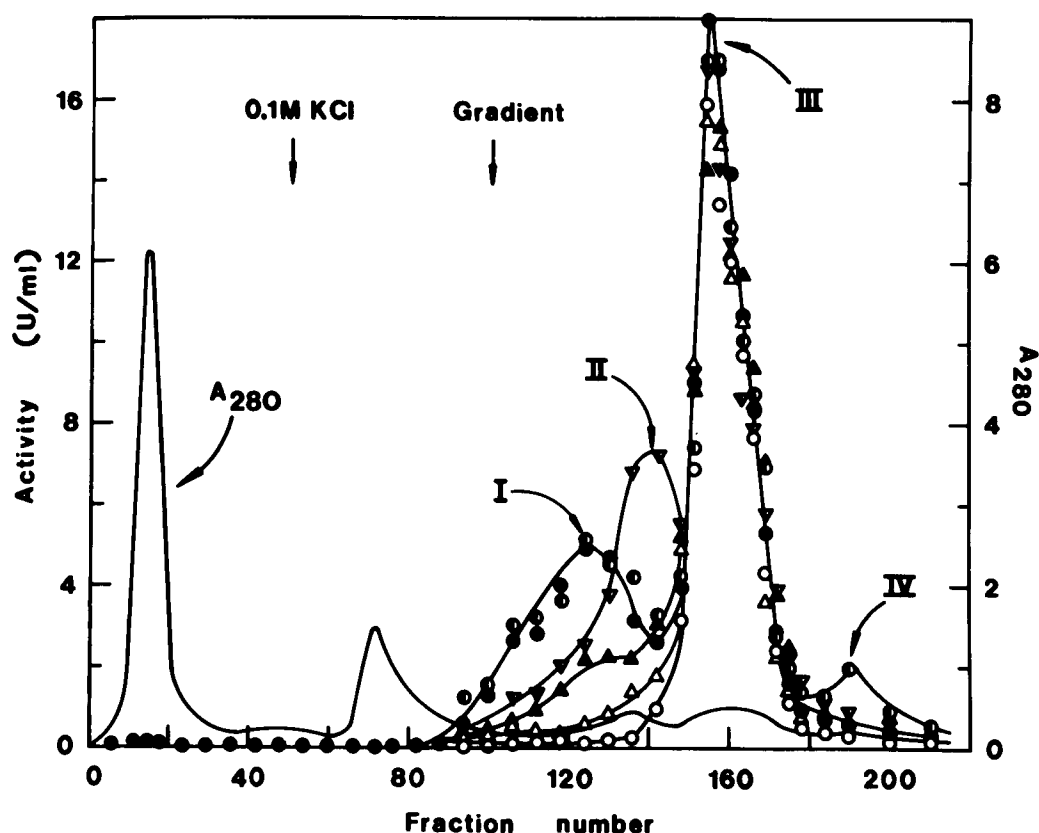


Fig. 2. Column chromatography of the ethanol precipitation fraction on DEAE-cellulose. The ethanol precipitation fraction (62 ml, 20 mg/ml) was applied on a DEAE-cellulose column (2.5 x 50 cm). After rinsing with Buffer AM followed by Buffer AM plus 0.05 M KCl (the final KCl concentration of the buffer solution was 0.1 M) as indicated, the enzyme was eluted with an increasing KCl gradient from 0.1 to 0.4 M in Buffer AM as described in the text. Fractions of 12.7 ml were collected. The enzymic activity toward phosphocasein in the presence of 5 mM EDTA (○), 5 mM MnCl_2 (◐) or 10 mM MgCl_2 (●) and the enzymic activity toward phosphohistone in the presence of 5 mM EDTA (△), 5 mM MnCl_2 (▼) or 10 mM MgCl_2 (▲) were determined with aliquots from the indicated fractions as described in the text. Absorbance at 280 nm was indicated by (—).

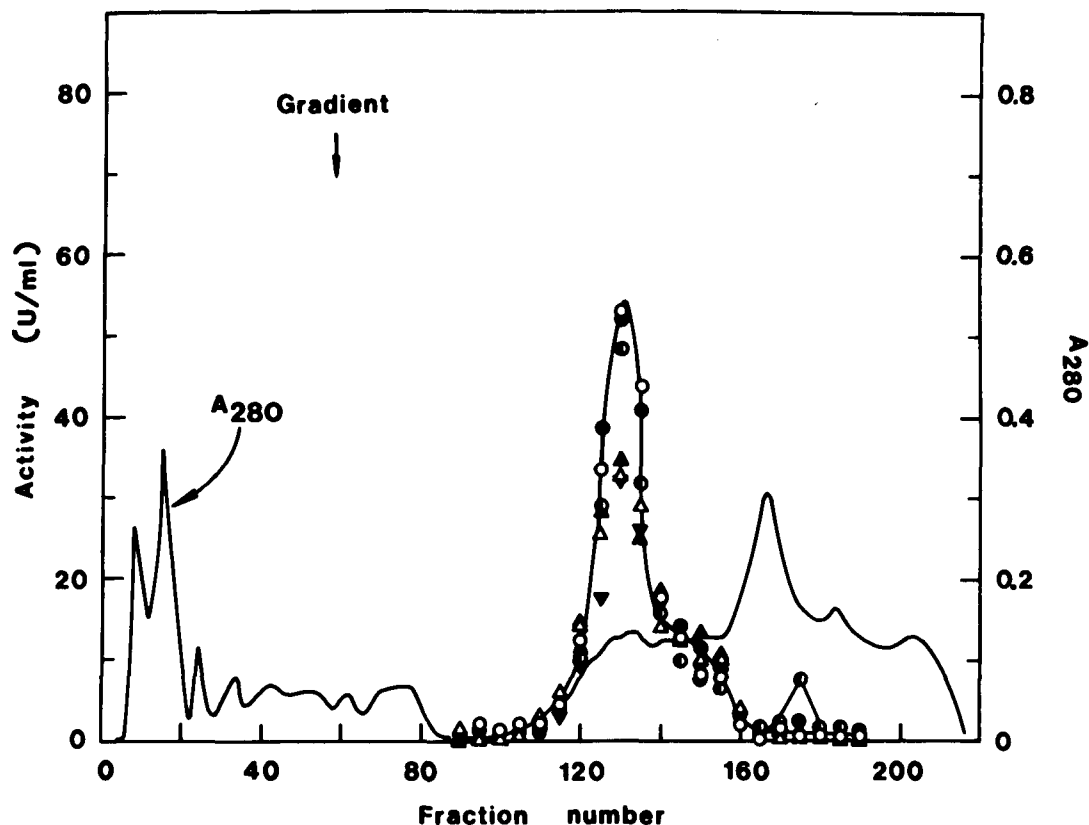


Fig. 3. Column chromatography of Peak III on DEAE-Sephadex A-50. The concentrated Peak III (7.2 ml, 21 mg/ml) obtained from DEAE-cellulose chromatography (Fig. 2) was chromatographed on a DEAE-Sephadex A-50 column (1.6 x 28 cm). After rinsing the column with Buffer AM plus 0.05 M KCl, the enzyme was eluted with a linear gradient of KCl from 0.1 to 0.3 M in Buffer AM. Fractions of 6.3 ml were collected. The enzymic activity toward phosphocasein in the presence of 5 mM EDTA (○), 5 mM MnCl_2 (●) or 10 mM MgCl_2 (●) and the enzymic activity toward phosphohistone in the presence of 5 mM EDTA (△), 5 mM MnCl_2 (▼) or 10 mM MgCl_2 (▲) were determined with aliquots from the indicated fractions as described in the text. Absorbance at 280 nm was indicated by (—).

were separately pooled, concentrated, and filtered through a Sephadex G-100 column. Fig. 4 shows the gel filtration pattern of the major active peak obtained from the DEAE-Sephadex chromatography. The data indicate that a single, symmetrical peak of divalent cation-independent, nonspecific phosphoprotein phosphatase activity coincided with one of the protein peaks. This purified enzyme was arbitrarily designated as phosphatase S. The Stokes radius of phosphatase S was estimated at $24.5\overset{\text{O}}{\text{\AA}}$. Chromatography of the minor peak shown in Fig. 3 on the Sephadex G-100 column also yielded a single peak of divalent cation-independent, nonspecific activity with a Stokes radius of $24.5\overset{\text{O}}{\text{\AA}}$ as that of a major peak, phosphatase S. This purified enzyme derived from the minor peak as indicated in Fig. 3 was arbitrarily designated as phosphatase SR in the following presentation.

In contrast to the Peak I and II enzymes, the final purified Peak III enzyme, phosphatase S, was fully active in the absence of a divalent cation and was about equally active toward either phosphohistone or phosphocasein in the presence of 5 mM EDTA, 5 mM MnCl_2 , or 10 mM MgCl_2 (Table 11). As indicated in Table 11, the phosphatase S preparation had a specific activity of 2,000-3,000 units per mg protein when its activity toward phosphocasein or

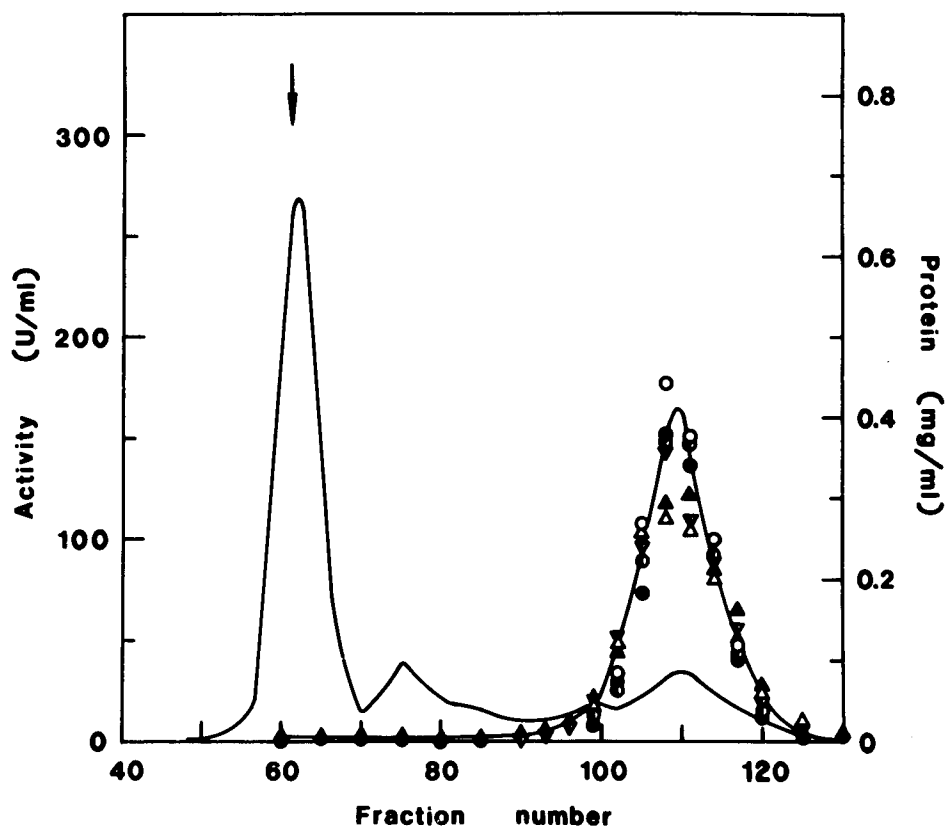


Fig. 4. Gel filtration of phosphatase S (the major active peak indicated in Fig. 3) on Sephadex G-100 column. The concentrated phosphatase S (4.8 ml, 2.6 mg/ml), the major peak of activity obtained from DEAE-Sephadex A-50 chromatography (Fig. 3), was filtered through a Sephadex G-100 column (2 x 98 cm) for elution with Buffer G. Fractions of 1.8 ml were collected. The enzymic activity toward phosphocasein in the presence of 5 mM EDTA (○), 5 mM MnCl_2 (◐), or 10 mM MgCl_2 (●), and the enzymic activity toward phosphohistone in the presence of 5 mM EDTA (△), 5 mM MnCl_2 (▽), or 10 mM MgCl_2 (▲) were determined with aliquots from the indicated fractions as described in the text. Protein concentrations were indicated by (—). The arrow refers to void volume.

phosphohistone was determined in the presence of 5 mM EDTA. This represented a 12,000 to 16,000-fold purification with approximately 30% recovery of the enzymic activity from crude extract. About 1 mg of enzyme protein was obtained from 1 kg of canine heart muscle by the present purification scheme. Phosphatase SR, the minor activity obtained by resolving the Peak III enzyme on DEAE-Sephadex column (Fig. 3) was also divalent cation-independent and exhibited similar substrate specificity to that of phosphatase S. The total activity of phosphatase SR recovered was about 10% as was that of phosphatase S.

Both phosphatase S and phosphatase SR had a Stokes radius of $24.5\overset{\circ}{\text{\AA}}$ and an $s_{20,w}$ value of 3.45 S, as determined by gel filtration on Sephadex G-100 and by sucrose density gradient ultracentrifugation, respectively. The molecular weight of phosphatase S calculated from Stokes radius and $s_{20,w}$ values was 34,800. This was in good agreement with the molecular weight value of 35,000 as determined by SDS-gel electrophoresis (cf. Polyacrylamide gel electrophoresis of phosphatase S section).

When assays were carried out in the presence of 5 mM EDTA, phosphorylase phosphatase activity was co-eluted with phosphohistone and phosphocasein phosphatase activities in DEAE-cellulose (Fig. 2), DEAE-Sephadex (Fig. 3), and

Sephadex G-100 (Fig. 4) chromatographies. The activity ratio of phosphorylase phosphatase to phosphohistone or to phosphocasein phosphatase remained relatively constant throughout the purification.

B) Phosphatases U-1 and U-2

Similar procedures were employed for further purification of Peaks I and II obtained from DEAE-cellulose chromatography of the ethanol precipitation fraction (Fig. 2). After chromatographing the Peak I enzyme on a DEAE-Sephadex column (elution profile not shown), fractions containing the Mg^{2+} - and Mn^{2+} -activated phosphocasein phosphatase activity were pooled, concentrated, and filtered through a Sephadex G-100 column. As shown in Fig. 5, gel filtration chromatography of the Peak I enzyme yielded a single peak of Mg^{2+} - and Mn^{2+} -activated phosphatase activity which preferentially dephosphorylated phosphocasein. The Stokes radius of this Mg^{2+} - and Mn^{2+} -activated enzyme derived from Peak I was estimated at $31\overset{O}{\text{\AA}}$. After chromatographing Peak II enzyme on a DEAE-Sephadex column, fractions containing the Mn^{2+} -activated phosphohistone phosphatase activity were pooled, concentrated, and filtered through a Sephadex G-100 column. As shown in Fig. 6, gel filtration chromatography of the Peak II enzyme yielded a single peak of Mn^{2+} -activated phosphatase activity which preferentially dephosphorylated

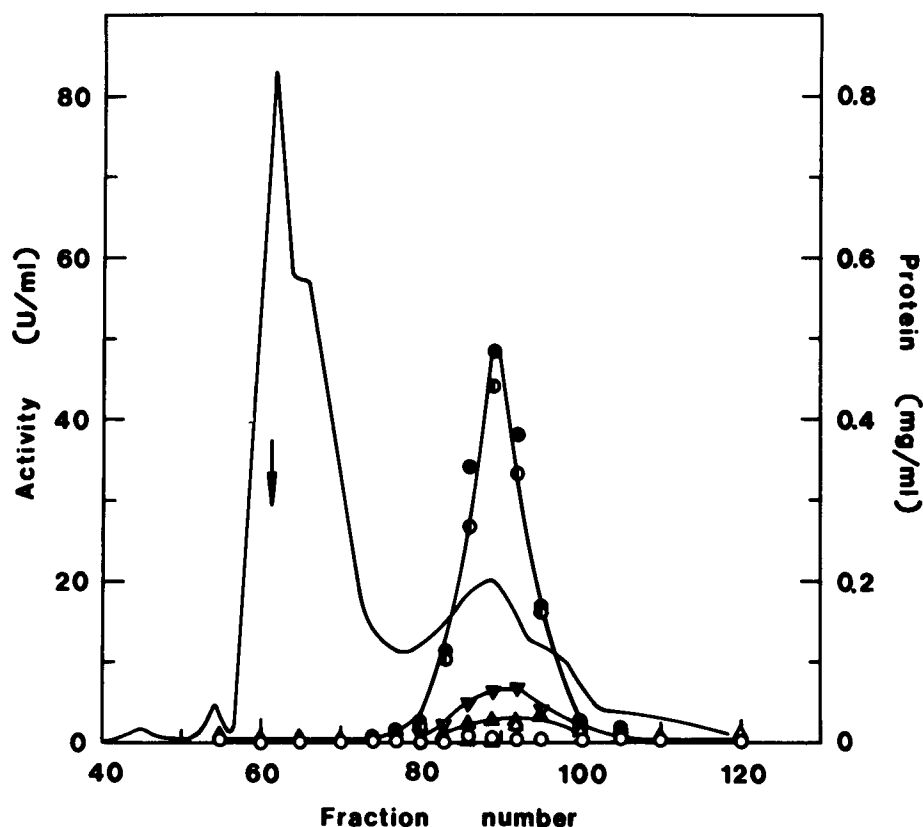


Fig. 5. Gel filtration of the Mg^{2+} -activated phosphocasein phosphatase (the enzyme derived from Peak I as indicated in Fig. 2) on Sephadex G-100 column. The Mg^{2+} - and Mn^{2+} -activated phosphocasein phosphatase (4.5 ml, 4.6 mg/ml) obtained from DEAE-Sephadex A-50 chromatography of Peak I (Fig. 2) was filtered through a Sephadex G-100 column (2 x 98 cm) for elution with Buffer G. Fractions of 1.8 ml were collected. The enzymic activity toward phosphocasein in the presence of 5 mM EDTA ($\bigcirc$), 5 mM MnCl_2 ($\bullet$), or 10 mM MgCl_2 ($\bullet$), and the enzymic activity toward phosphohistone in the presence of 5 mM EDTA ($\triangle$), 5 mM MnCl_2 (∇), or 10 mM MgCl_2 ($\blacktriangle$) were determined with aliquots from the indicated fractions as described in the text. Protein concentrations were indicated by (—). The arrow refers to void volume.

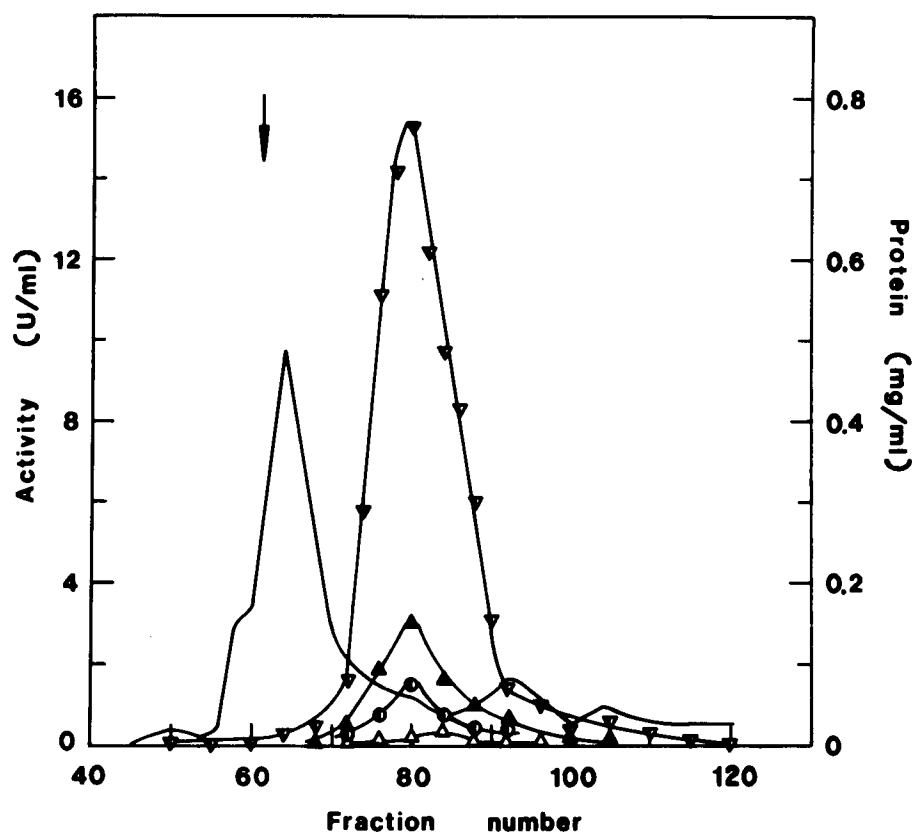


Fig. 6. Gel filtration of the Mn^{2+} -activated phosphohistone phosphatase (the enzyme derived from Peak II as indicated in Fig. 2) on Sephadex G-100 column. The Mn^{2+} -activated phosphohistone phosphatase (4.1 ml, 1.9 mg/ml) obtained from DEAE-Sephadex A-50 chromatography of Peak II (Fig. 2) was filtered through a Sephadex G-100 column (2 x 98 cm) for elution with Buffer G. Fractions of 1.8 ml were collected. The enzymic activity toward phosphocasein in the presence of 5 mM EDTA (○), 5 mM MnCl_2 (●), or 10 mM MgCl_2 (●•), and the enzymic activity toward phosphohistone in the presence of 5 mM EDTA (Δ), 5 mM MnCl_2 (▽), or 10 mM MgCl_2 (▲) were determined with aliquots from indicated fractions as described in the text. Protein concentrations were indicated by (—). The arrow refers to void volume.

phosphohistone. The Stokes radius of this Mn^{2+} -activated enzyme derived from Peak II was estimated as $39\text{\AA}$.

Both of the final purified enzymes derived from Peaks I and II, as indicated in Fig. 2, were inactive in the absence of a divalent cation (Table 11). The Peak I enzyme preferred phosphocasein as a substrate and was activated by 10 mM of Mg^{2+} or 5 mM Mn^{2+} to the same extent. By contrast, the Peak II enzyme preferred phosphohistone as a substrate and was activated by Mn^{2+} but not Mg^{2+} . The Peak I enzyme was an Mg^{2+} -activated phosphocasein phosphatase and was named phosphatase U-2. The Peak II enzyme was an Mn^{2+} -activated phosphohistone phosphatase and was named phosphatase U-1. The Mg^{2+} -activated phosphocasein phosphatase activity (phosphatase U-2) of the Peak I enzyme was purified approximately 1,200-fold with 6% recovery from the crude extract, while the Mn^{2+} -activated phosphohistone activity (phosphatase U-1) of the Peak II enzyme was purified 1,400-fold with 3.4% recovery. Both of phosphatases U-1 and U-2 were not homogeneous as judged by polyacrylamide gel electrophoresis.

The $s_{20,w}$ values of phosphatases U-1 and U-2 were estimated as 3.80 and 3.86, respectively. Using the $s_{20,w}$ and Stokes radius values obtained and assuming a partial specific volume of 0.725 ml/g, the molecular weights

of phosphatases U-1 and U-2 were calculated (719) as 61,000 and 49,300, respectively. The molecular weight, divalent cation-dependency, and substrate specificity of the purified phosphatases U-2 and U-1 are, respectively, the same as those of the Mg^{2+} -activated phosphocasein phosphatase and the Mn^{2+} -activated phosphohistone phosphatase, which have been isolated from canine heart by a procedure not involving the ethanol precipitation step (723). The activity ratio of Mn^{2+} -activated phosphorylase phosphatase to Mn^{2+} -activated phosphohistone phosphatase (phosphatase U-1) remained relatively constant throughout the purification. Besides, these two Mn^{2+} -activated phosphatase activities in phosphatase U-1 were co-migrated in polyacrylamide gel electrophoresis. Very little phosphorylase phosphatase activity could be detected in phosphatase U-2.

III. Polyacrylamide Gel Electrophoresis of Phosphatase S

Polyacrylamide gel electrophoresis of the purified phosphatase S preparation resulted in a single protein band which coincided with the enzymic activity toward phosphohistone (Fig. 7), phosphocasein, and phosphorylase a (not shown). Both protein stain and enzymic activity profile, however, were broad and showed a slight tailing on the side of the tracking dye front. The activity profile for dephosphorylation of phosphohistone showed a

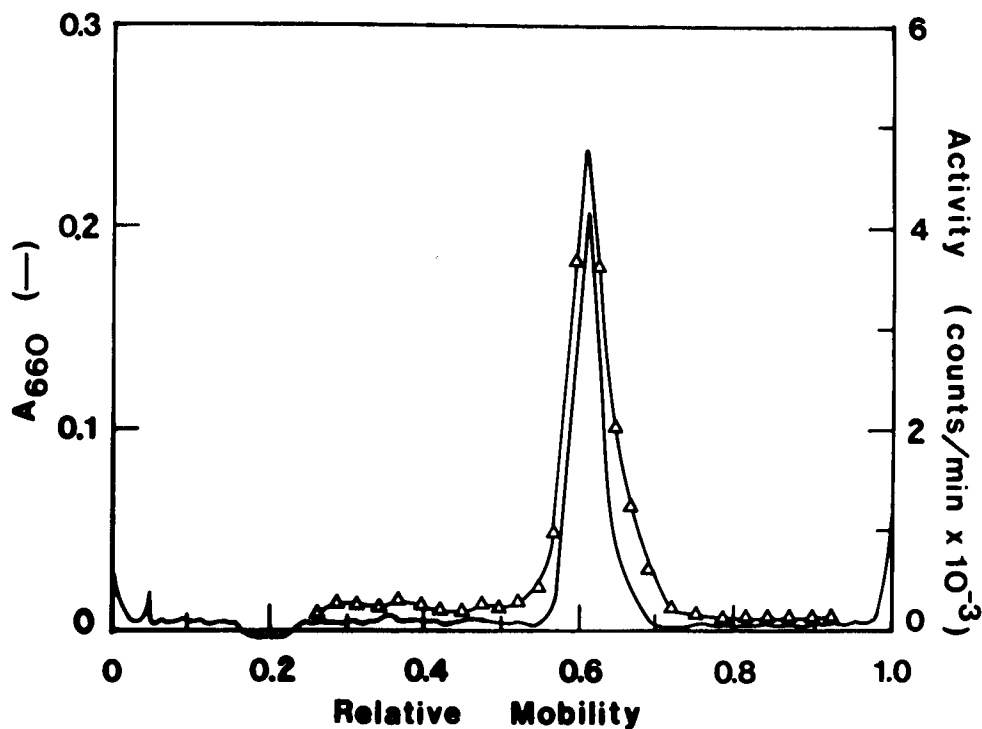


Fig. 7. Polyacrylamide gel electrophoresis of the purified phosphatase S. 10 μ g and 1.5 μ g of purified phosphatase S were separately loaded on two 7% polyacrylamide gels. Following electrophoresis, the gel loaded with 10 μ g of enzyme was stained with Coomassie Brilliant Blue and scanned at 660 nm (—); the gel loaded with 1.5 μ g of enzyme was sliced and assayed for the activity toward phosphohistone (Δ) in the presence of 5 mM EDTA as described in the text. Dye front was indicated by the wire.

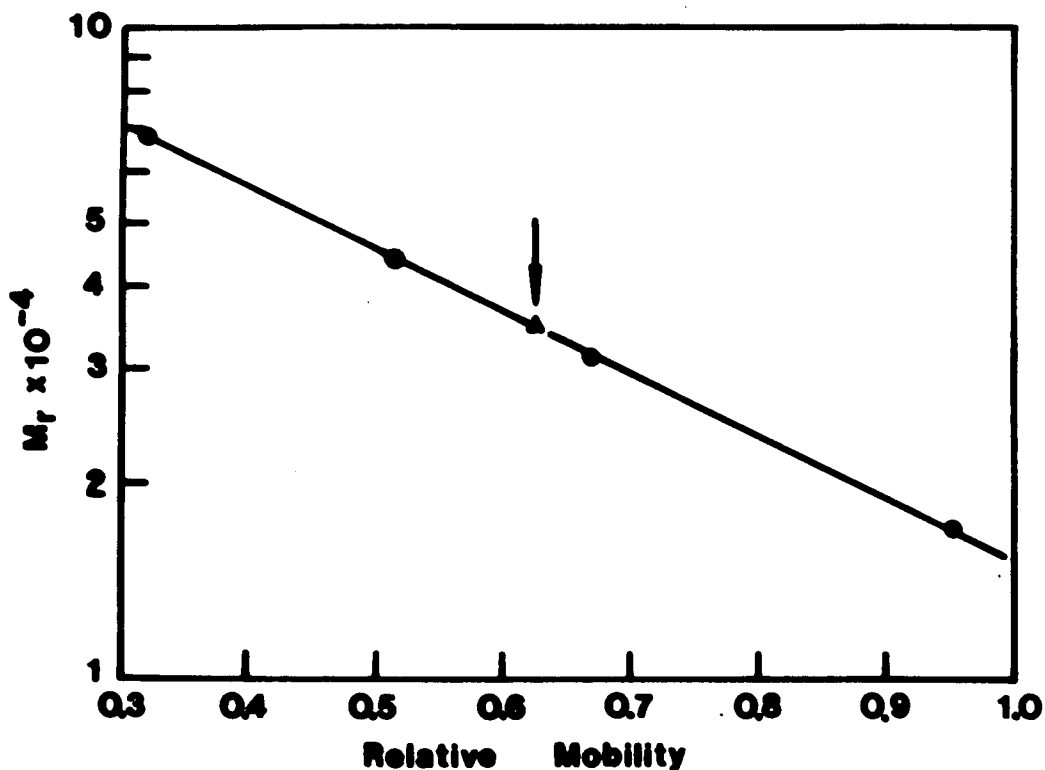


Fig. 8. Polyacrylamide gel electrophoresis of the purified phosphatase S in the presence of sodium dodecyl sulfate. 14 μ g of purified phosphatase S was loaded on a 10% polyacrylamide gel and the electrophoresis was carried out as described in the text. The single protein band corresponded to a molecular weight of 35,000 ($\blacktriangle$). The marker protein used were bovine serum albumin (68,000), ovalbumin (44,000), carbonic anhydrase (31,000), and myoglobin (17,000). Dye front was indicated by the wire.

more pronounced tailing than those for dephosphorylation of phosphorylase a and phosphocasein. The data suggest that the phosphatase S preparation might contain more than one active species with slightly different mobilities on gel electrophoresis. Gel electrophoresis of phosphatase SR resulted in three to four closely spaced peaks of phosphohistone phosphatase activity which coincided with two visible broad protein stain bands. Among these activity peaks, the one which migrated slowest had the same mobility as that of phosphatase S on gel electrophoresis. As shown in Fig. 8, gel electrophoresis of the phosphatase S preparation in the presence of sodium dodecyl sulfate showed a single protein band with a molecular weight corresponding to 35,000. SDS-gel electrophoresis of phosphatase SR also resulted in a single protein band with a mobility indistinguishable from that of phosphatase S.

IV. Gel Filtration of Phosphatases A, B, C, and U on Sephadex G-200 Column

The various fractions of phosphatase obtained from DEAE-cellulose chromatography without ethanol treatment (Fig. 1) were chromatographed separately on a Sephadex G-200 column previously equilibrated in Buffer G. The activity profiles of phosphatases A, B, C, and U determined by using phosphohistone as a substrate in the presence and

absence of 5 mM MnCl_2 are shown in Figs. 9A, 10A, 11A, and 12A, respectively. The data indicate that, when the fractions were assayed in the absence of Mn^{2+} , each phosphatase emerged from the gel filtration column as a single peak of activity. The activity profiles of phosphatases A, B, and C were not influenced by Mn^{2+} , whereas, in the case of phosphatase U, an additional peak of Mn^{2+} -activated enzyme emerged from the column after a minor peak of Mn^{2+} -insensitive activity which was the contamination of phosphatase B (Fig. 12A).

The fractions containing the major activity of each phosphatase were pooled after gel filtration chromatography and stored at 4° for further studies. The activity of phosphatase B was stimulated more than twofold by 2 mM ATP, while this nucleotide concentration inhibited the activity of phosphatase A about 50% and did not significantly affect phosphatase C activity. Only phosphatase U required Mn^{2+} for activity. In the presence of 5 mM Mn^{2+} , 2 mM ATP inhibited phosphatase U activity about 50%. Thus, the responses to ATP of these four enzyme fractions (568) did not change after gel filtration chromatography.

V. Dissociation of Phosphoprotein Phosphatases by Ethanol Treatment

Phosphatases A, B, C, and U, obtained from Sephadex G-200 chromatography, were each treated with ethanol, as

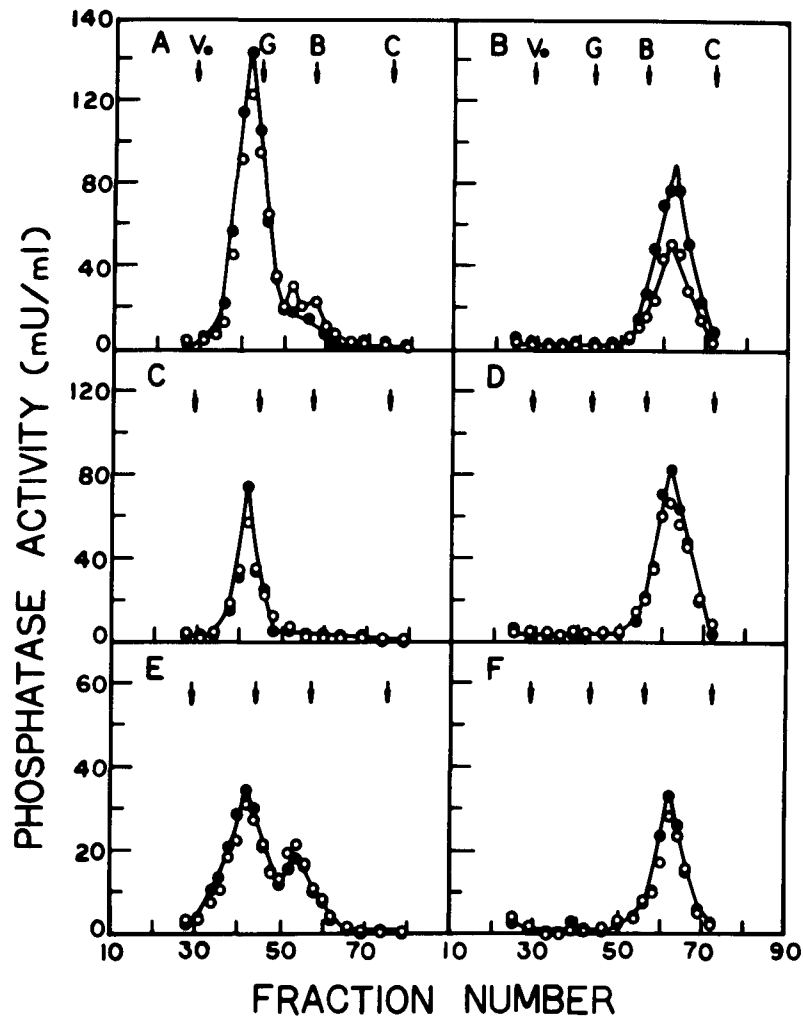


Fig. 9. Sephadex G-200 chromatography of phosphatase A and the ethanol-treated phosphatase A. Five ml phosphatase A (11.2 mg protein) obtained from DEAE-cellulose chromatography were applied on a Sephadex G-200 column (2.5 x 61 cm) pre-equilibrated with Buffer G. The column was eluted with the same buffer. Fractions of 3.8 ml were collected. Phosphatase activity toward phosphohistone (2A), phosphocasein (2C), and phosphorylase a (2E) was measured in the absence (●) or in the presence (○) of $MnCl_2$ (5 mM for phosphohistone and phosphocasein; 0.5 mM for phosphorylase a) as described in the text. The active fractions (30-48) were pooled and concentrated by membrane ultra-filtration to 6.2 ml. Three ml of the concentrated

Fig. 9 (Continued)

fraction were treated with ethanol as essentially that of Brandt et al (608,613), with minor modification. One volume of the enzyme was mixed with 5 volumes of 95% ethanol at room temperature, and the mixture was centrifuged immediately at 15,000 x g for 10 min. Phospho-protein phosphatase activity in the precipitate was extracted twice with 0.5 volume of Buffer G. The extracts were combined and dialyzed for 1-2 hours against 200 volumes of Buffer G. Five ml (0.47 mg protein) of the ethanol-treated phosphatase A (total volume = 6 ml) were chromatographed on the same Sephadex G-200 column for elution with the same buffer. Fractions of 3.8 ml were collected. Phosphatase activities toward phosphohistone (2B), phosphocasein (2D), and phosphorylase a (2F) were measured in the absence (●) and in the presence (○) of $MnCl_2$ as described above.

V_0 refers to void volume. G, B, and C indicate the elution volume of bovine γ -globulin, bovine serum albumin, and cytochrome c, respectively.

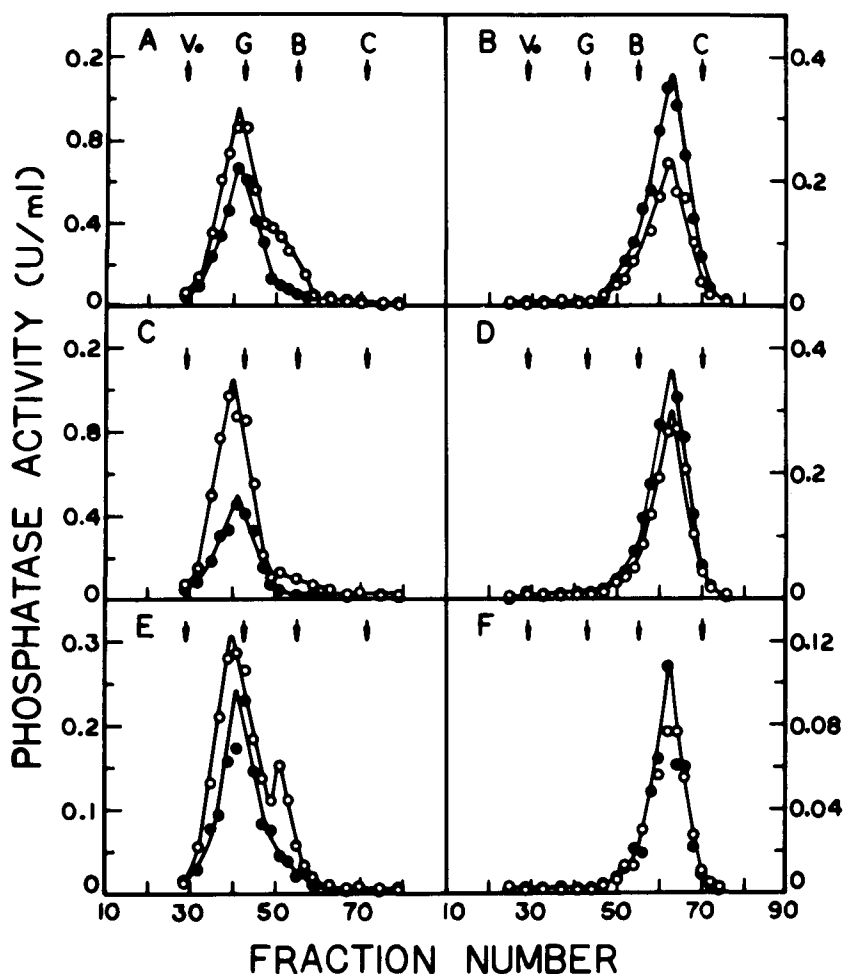


Fig. 10. Sephadex G-200 chromatography of phosphatase B and the ethanol-treated phosphatase B. Five ml of phosphatase B (15.2 mg protein) obtained from DEAE-cellulose chromatography were chromatographed on a Sephadex G-200 column (2.5 x 61 cm) and fractions collected were assayed for phosphohistone (3A), phosphocasein (3C), and phosphorylase a (3E) phosphatase activity in the absence (●) or in the presence (○) of $MnCl_2$ as described in the legend to Fig. 9. The active fractions (31-47) were pooled and concentrated to 9.1 ml. Three ml of the concentrated fraction were treated with ethanol, chromatographed on the Sephadex column and fractions collected were assayed for phosphohistone (3B), phosphocasein (3D), and phosphorylase a (3F) phosphatase activity in the

Fig. 10 (Continued)

absence (●) or in the presence (○) of MnCl_2 as described above.

V_o refers to void volume. G, B, and C indicate the elution volume of bovine γ -globulin, bovine serum albumin, and cytochrome c, respectively.

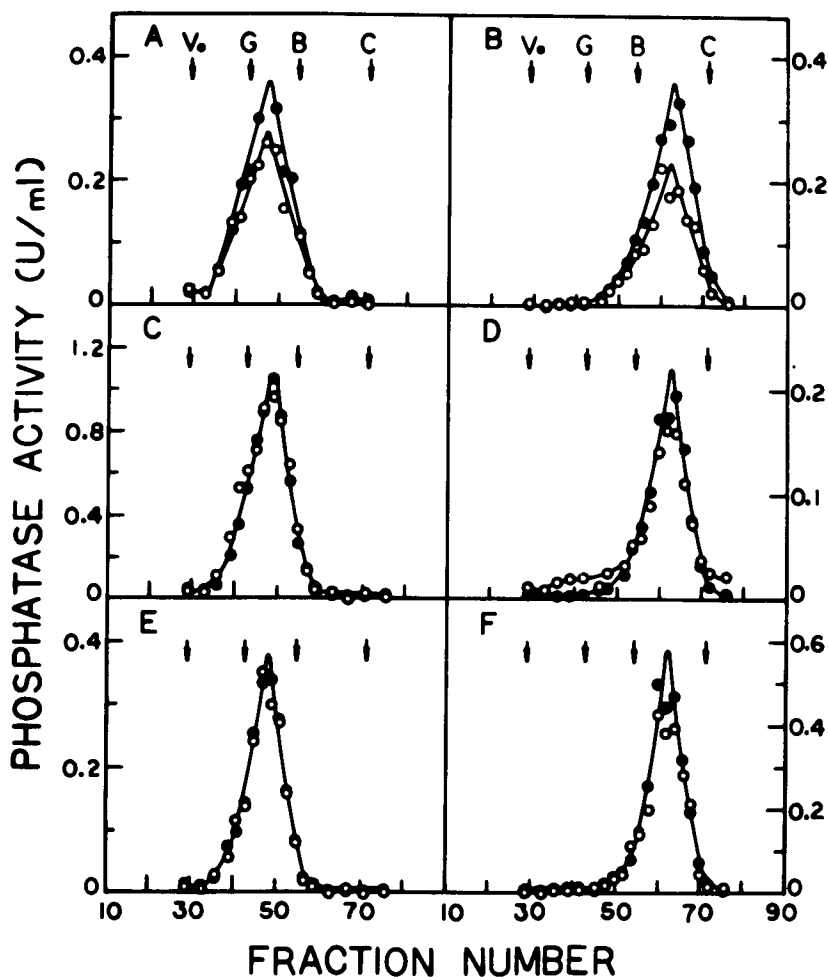


Fig. 11. Sephadex G-200 chromatography of phosphatase C and the ethanol-treated phosphatase C. Five ml phosphatase C (6.5 mg protein) obtained from DEAE-cellulose chromatography were chromatographed on a Sephadex G-200 column (2.5 x 61 cm) and fractions collected were assayed for phosphohistone (4A), phosphocasein (4C), and phosphorylase a (4E) phosphatase activity in the absence (●) or in the presence (○) of MnCl₂ as described in the legend to Fig. 9. The active fractions (35-58) were pooled and concentrated to 6 ml. Three ml of the concentrated fraction were treated with ethanol, chromatographed on the Sephadex G-200 column and fractions collected were assayed for phosphohistone (4B), phosphocasein (4D), and phosphorylase a (4F) phosphatase activity

Fig. 11 (Continued)

in the absence (●) and in the presence
(○) of MnCl_2 as described above.

V_0 refers to void volume. G, B, and C indicate
the elution volume of bovine γ -globulin, bovine
serum albumin, and cytochrome c, respectively.

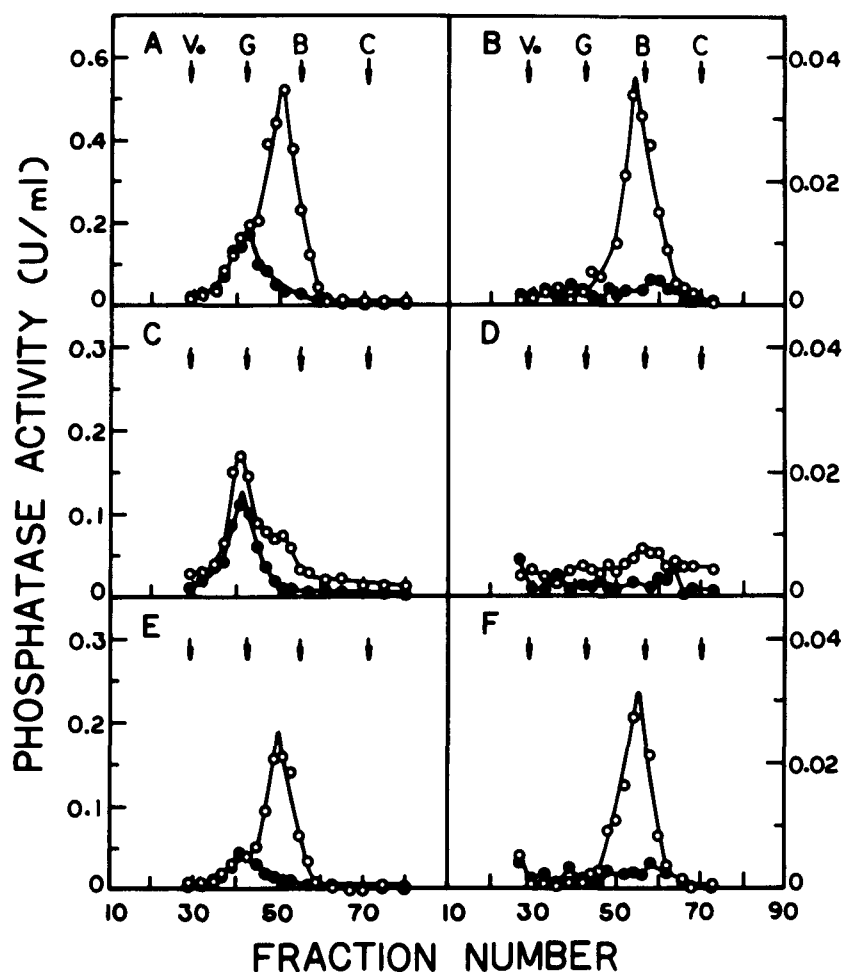


Fig. 12. Sephadex G-200 chromatography of phosphatase U and the ethanol-treated phosphatase U. Five ml of phosphatase U (15.6 mg protein) obtained from DEAE-cellulose chromatography were chromatographed on a Sephadex G-200 column and fractions collected were assayed for phosphohistone (5A), phosphocasein (5C), and phosphorylase α (5E) phosphatase activity in the absence (●) and presence (○) of $MnCl_2$ as described in the legend to Fig. 9. The active fractions (48-59) were pooled and concentrated to 6.1 ml. Three ml of the concentrated fraction were treated with ethanol, chromatographed on the Sephadex G-200 column and fractions collected were assayed for phosphohistone (5B), phosphocasein (5D), and

Fig. 12 (Continued)

phosphorylase a (5F) phosphatase activity in the absence (●) and in the presence (○) of MnCl_2 as described above.

V_0 refers to void volume. G, B, and C indicate the elution volume of bovine γ -globulin, bovine serum albumin, and cytochrome c, respectively.

described by Brandt et al. (608,613), which increased the activities of phosphatases A, B, and C toward phosphohistone in the absence of Mn^{2+} , by 110, 250, and 317 percent, respectively. In contrast, the activity of phosphatase U, measured in the presence of 5 mM Mn^{2+} , was decreased 20-30 percent after the ethanol treatment.

Chromatography of the ethanol-treated phosphatases on a Sephadex G-200 column previously equilibrated in Buffer G resulted in dissociation of phosphatases A, B, and C into a smaller molecular size activity. Figs. 9B, 10B, and 11B show the dissociation of the phosphatases A, B, and C, respectively. The Stokes radii for the ethanol-treated phosphatases A, B, and C, as determined by gel filtration, were all $24.5\overset{O}{A}$ (Fig. 13), and their $s_{20,w}$ values, as determined by sucrose density gradient, were all 3.45. Furthermore, the ethanol-treated phosphatases A, B, and C showed similar responses to Mn^{2+} and ATP, being inhibited about 50% by 2 mM ATP and not significantly affected by 5 mM $MnCl_2$. These data suggest that phosphatases A, B, and C consist of a common catalytic subunit, phosphatase S.

In contrast, chromatography of the ethanol-treated phosphatase U on Sephadex G-200 column (Fig. 12B) showed a single peak of Mn^{2+} -activated activity contained in an elution volume similar to that of the untreated enzyme

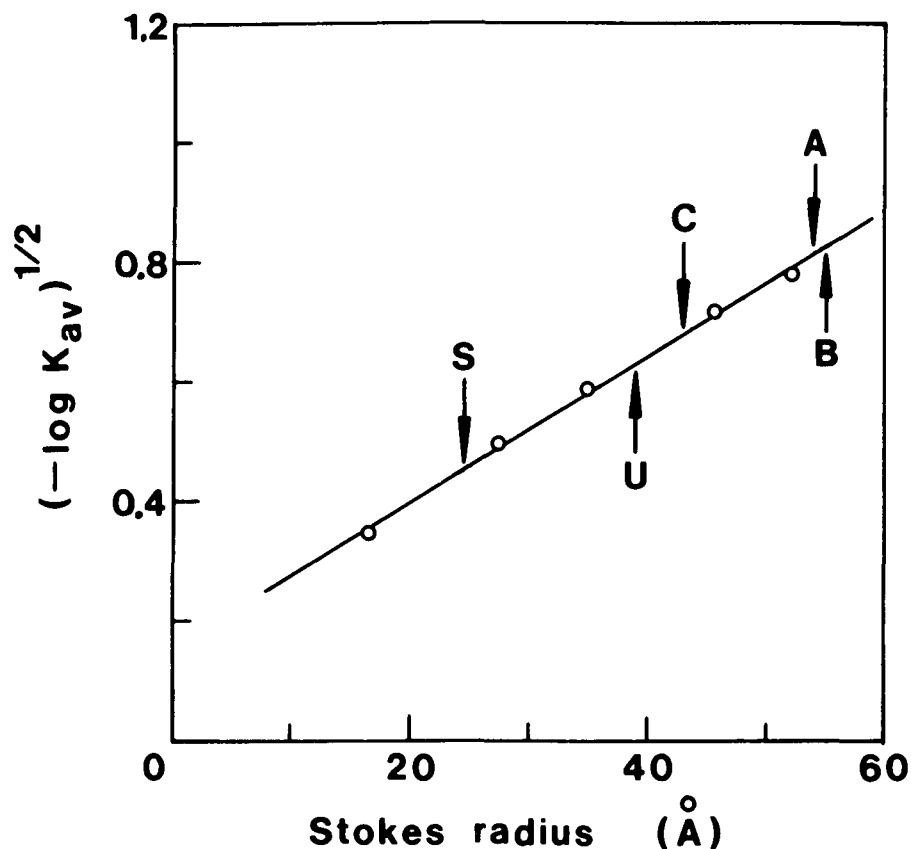


Fig. 13. Estimation of the Stokes radii of phosphatases by gel filtration on Sephadex G-200. The column (1.6 x 84 cm) was eluted with a buffer containing 50 mM Tris·HCl (pH 7.4) and 0.1 M KCl. Fractions of 1.2 ml were collected at a flow rate of 5 ml per hour. The column was calibrated with the following proteins (Stokes radius): bovine γ -globulin ($a_0 = 52\text{\AA}$), yeast alcohol dehydrogenase ($a = 46\text{\AA}$), bovine serum albumin ($a = 35\text{\AA}$), ovalbumin ($a = 28\text{\AA}$), and cytochrome c ($a = 17\text{\AA}$). The data are plotted according to the method of Siegel and Monty (719). A, phosphatase A; B, phosphatase B; C, phosphatase C; U, phosphatase U; S, phosphatase S.

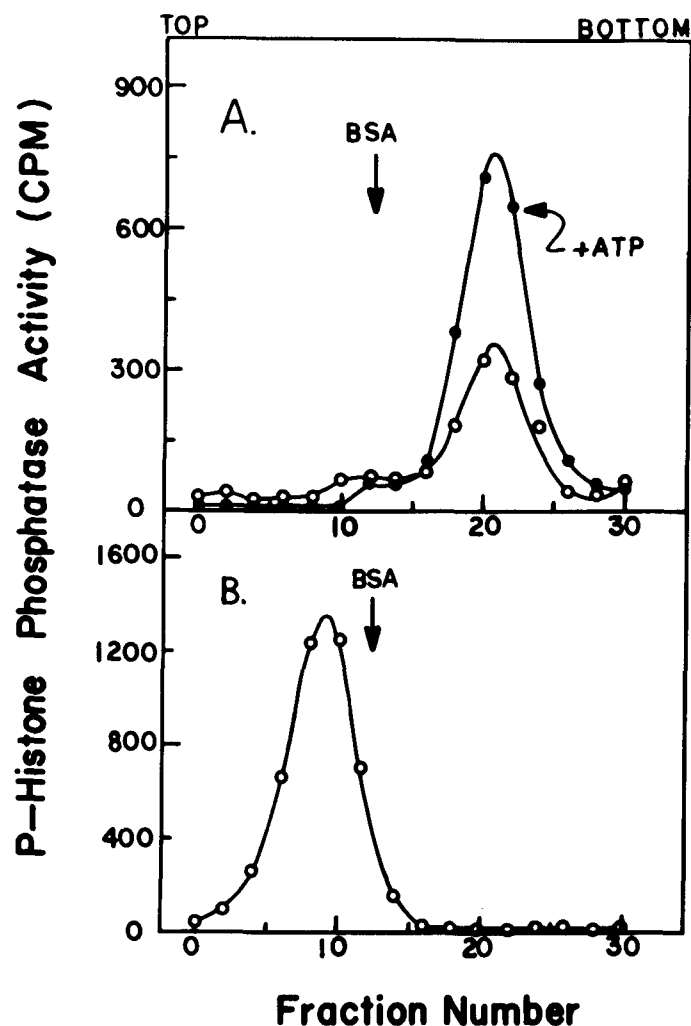


Fig. 14. Sucrose density gradient ultracentrifugation of phosphoprotein phosphatases B and S. Samples of 0.1 ml were layered onto sucrose gradient (5-20% in 20 mM Tris·HCl, pH 7.4) and centrifuged in a S.W. 50.1 rotor at 39,000 rpm for 18 hr at 4°. Fractions of 3 drops were collected from the bottom of the tubes. Phospho-histone phosphatase activity was measured in an incubation volume of 0.1 ml containing 50 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 0.1 M KCl, 10 μ M phosphohistone, in the presence (●) or the absence (○) of 2 mM ATP, and enzyme from the indicated fractions. Incubation was carried out at 30° for 10 min. The enzymic activity was expressed in counts/min. Figs. 14A

Fig. 14 (Continued)

and 14B show the sedimentation profile of phosphatases B and S, respectively. BSA indicates the sedimentation position of bovine serum albumin (4.41 S).

(compare Figs. 12A and 12B). The data indicate that ethanol treatment did not dissociate phosphatase U into the smaller molecular size, Mn^{2+} -insensitive enzyme obtained after similar treatment of phosphatases A, B, and C.

Aside from gel filtration, the dissociation of phosphatases A, B, and C may also be demonstrated by sedimentation. For example, sucrose density gradient ultracentrifugation of phosphatase B showed a peak of ATP-stimulated phosphohistone phosphatase activity with an $s_{20,w}$ value of 7.1 (Fig. 14A). The presence of 2 mM ATP in the sample as well as in the sucrose gradient did not change the sedimentation profile of phosphatase B. After the phosphatase B fraction was treated with ethanol, a peak of the enzymic activity with a $s_{20,w}$ value of 3.45 was observed (Fig. 14B).

In order to examine further the molecular forms and subunit composition of phosphohistone phosphatases, the 0-55% $(NH_4)_2SO_4$ fraction was treated with 80% ethanol at room temperature prior to fractionation. Two forms of phosphohistone phosphatase can also be directly separated from this ethanol-treated $(NH_4)_2SO_4$ fraction by Sephadex G-100 chromatography. The elution pattern obtained is shown in Fig. 15A. Peak GI is an Mn^{2+} -dependent phosphohistone phosphatase, while Peak GII is not dependent on Mn^{2+} for activity. The apparent molecular weights of Peaks GI

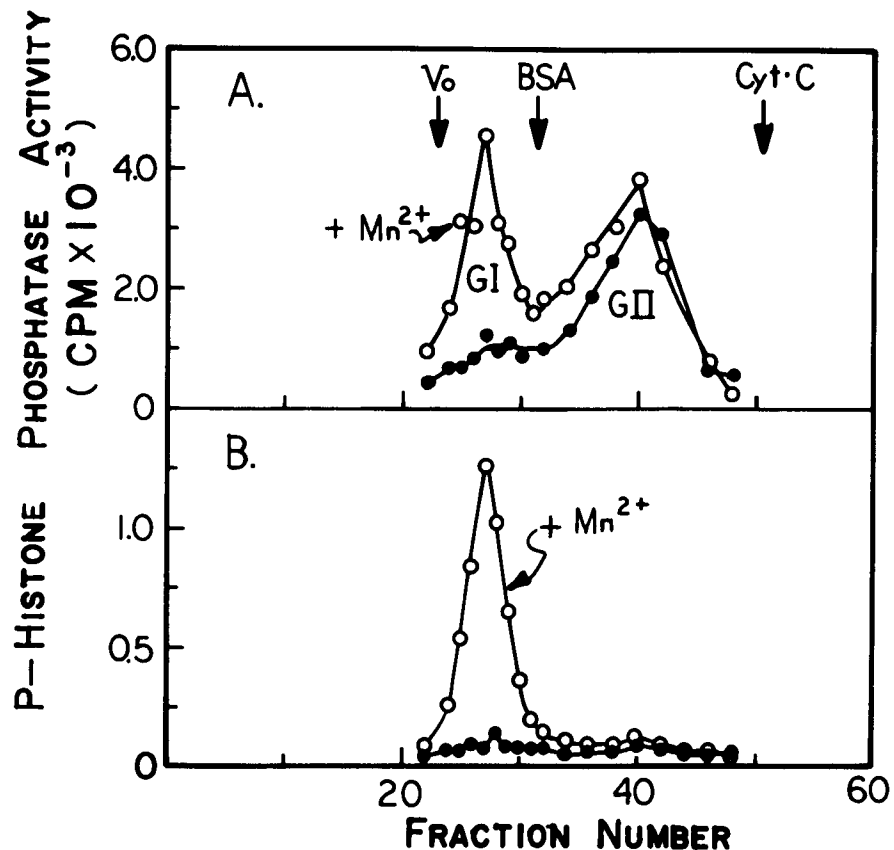


Fig. 15. Sephadex G-100 chromatography of the ethanol treated 55% $(NH_4)_2SO_4$ fraction and peak GI phosphoprotein phosphatase. (A) Two ml of the ethanol treated 55% $(NH_4)_2SO_4$ fraction was applied on a Sephadex G-100 column (2.5 x 60 cm) which was pre-equilibrated with Buffer A. The enzyme was eluted with the same buffer. Fractions of 4.5 ml were collected. Phosphohistone phosphatase was assayed in the presence ($\bigcirc$) or in the absence ($\bullet$) of 5 mM $MnCl_2$. (B) The active fractions (24-29) of peak GI as shown in Fig. 15A were pooled and treated with ethanol. The ethanol treated peak GI (total volume about 2 ml) was rechromatographed on the Sephadex G-100 column and assayed as described in Fig. 15A.

V_0 refers to void volume. BSA and cyt c indicate the elution volume of bovine serum albumin and cytochrome c, respectively.

GII are identical to those of phosphatases U and S obtained after the DEAE chromatography as described above. To examine the possibility that the Mn^{2+} -dependent phosphohistone phosphatase was a residual activity due to incomplete dissociation of the enzyme, peak GI was again treated with ethanol, and then rechromatographed on the same Sephadex G-100 column. As shown in Fig. 15B, a single, symmetrical peak of Mn^{2+} -dependent enzyme was eluted in the same position as Peak GI. In the absence of Mn^{2+} , no appreciable activity was detected in any fraction. The data indicated that the larger molecular size, Mn^{2+} -dependent phosphatase U cannot be dissociated by ethanol treatment to the smaller molecular size, Mn^{2+} -independent phosphatase S. Sephadex G-100 chromatography with 5 mM Mn^{2+} had no effect on the elution profile of the Mn^{2+} -dependent phosphatase U. The Sephadex G-100 elution profile and the insensitivity to Mn^{2+} of the phosphatase S (peak GII) were not affected by retreating with ethanol. These data indicate that peak GI was identical to phosphatase U, and peak GII was identical to the smaller molecular size enzyme derived from phosphatases A, B, or C. This smaller molecular size, ATP-inhibited, Mn^{2+} -insensitive enzyme, which appears to be the phosphatase S, a common catalytic subunit of several fractions of phosphoprotein phosphatase.

Although these two forms of phosphoprotein phosphatase (U and S) can be separated from the ethanol-treated $(\text{NH}_4)_2\text{SO}_4$ fraction by either DEAE-cellulose or gel filtration, they sediment with a similar velocity in sucrose density gradient centrifugation. As shown in Figs. 16A and 16B, phosphoprotein phosphatases U and S (peak GII) isolated by Sephadex G-100 chromatography, sedimented with an $s_{20,w}$ value of 3.8 and 3.5, respectively. Fig. 16C shows the sedimentation profile of a mixture of these two forms of the enzyme, indicating that they cannot be readily separated by sucrose density gradient centrifugation. Sedimentation in sucrose density gradient with 5 mM Mn^{2+} did not effect the sedimentation profile of the phosphatase U.

All fractions (Table 10) obtained by DEAE-cellulose chromatography without ethanol treatment catalyzed the dephosphorylation of phosphorylase a and phosphocasein, as well as phosphohistone, in the presence and absence of 5 mM MnCl_2 . These activities were co-eluted from the DEAE-column with phosphohistone phosphatase activity (data not shown).

Ethanol treatment of phosphatases A, B, and C also resulted in a two- to threefold increase of the enzyme activity toward phosphocasein and phosphorylase a. On the other hand, ethanol treatment of phosphatase U resulted in

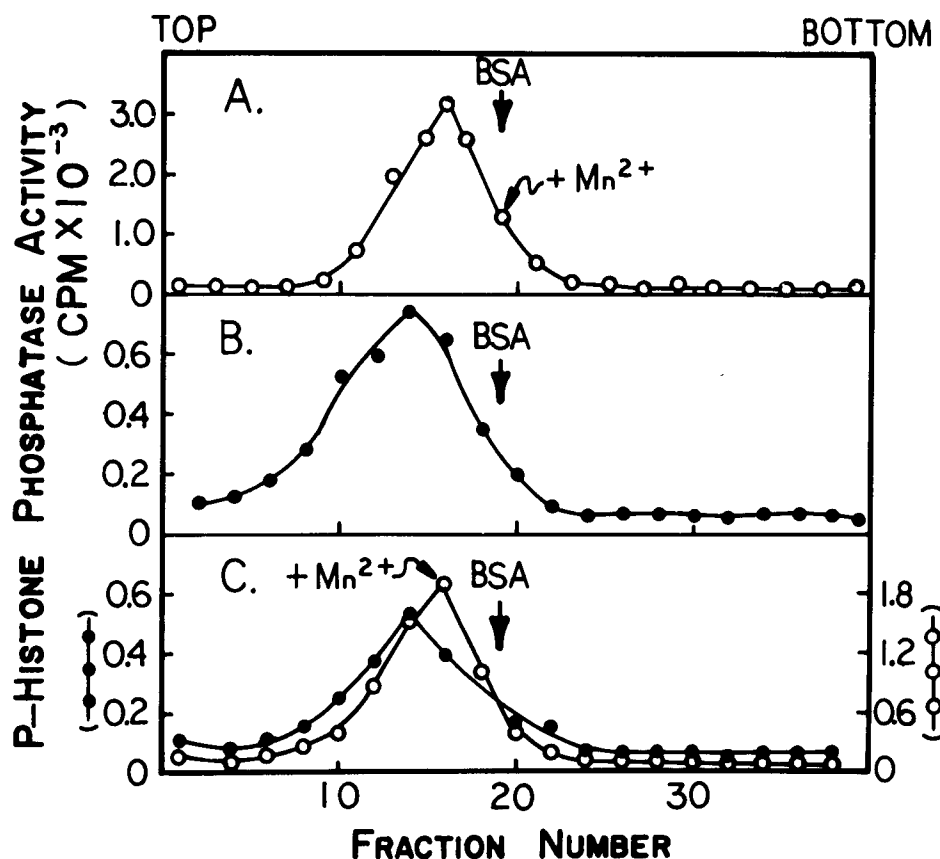


Fig. 16. Sucrose density gradient ultracentrifugation of phosphoprotein phosphatases U and S. The phosphohistone phosphatases U and S were isolated from the ethanol treated 55% $(\text{NH}_4)_2\text{SO}_4$ fraction by Sephadex G-100 chromatography as shown in Fig. 15. Samples of 0.1 ml were sedimented as described in the legend to Fig. 14. Fractions of 3 drops were collected from the bottom of the tubes and assayed for phosphohistone phosphatase activity in the presence (○) or absence (●) of 5 mM MnCl_2 . The sedimentation profile of phosphohistone phosphatases U and S, and the mixture of these two forms of phosphohistone phosphatase are shown in Figs. 16A, B, and C, respectively. Bovine serum albumin (BSA) was used as a marker (4.41 S).

a 20-30 percent decrease of the enzymic activity toward these two phosphoproteins. Chromatography of phosphatases A, B, C, and U, either before or after treating with ethanol, on Sephadex G-200 column showed that phosphorylase and phosphocasein phosphatase were co-eluted with phosphohistone phosphatase activity (Figs. 9-12). As shown in Figs. 12C and 12D, the Mn^{2+} -activated phosphatase, however, was much less active toward phosphocasein than toward phosphohistone and phosphorylase a, indicating that phosphocasein was a poor substrate for this enzyme preparation.

The catalytic properties of the Mn^{2+} -activated phosphatase (phosphatase U) have been described by Li and Hsiao (570). The observation that $MgCl_2$ can markedly activate the enzymic activity toward only phosphocasein but not phosphohistone or phosphorylase a suggested that the fraction U may contain an Mg^{2+} -activated phosphocasein phosphatase. As discussed in previous sections, the divalent cation dependent phosphoprotein phosphatases can be further separated into two different enzymic activities: an Mg^{2+} - and Mn^{2+} -activated phosphocasein phosphatase (phosphatase U-2) and an Mn^{2+} -activated phosphohistone phosphatase (phosphatase U-1) which also dephosphorylates phosphorylase a.

The mechanism of ATP-stimulation on phosphatase B and

the properties change associated with the dissociation of phosphatase B by ethanol to its putative catalytic subunit, phosphatase S, have been studied by Li and Hsiao (569).

VI. Physical Properties of Phosphoprotein Phosphatases

The Stokes radii for phosphatases A, B, C, U-1, and U-2, as determined by gel filtration, were 54, 55, 43, 39, and 31Å, respectively (Fig. 13). The sedimentation coefficient ($s_{20,w}$) for phosphatases A, B, C, U-1, and U-2 as determined by sucrose density gradient centrifugation, were 7.0, 7.1, 5.4, 3.8, and 3.86, respectively (see Figs. 14A and 14B for phosphatases B and S, respectively).

The molecular weight and frictional ratio for various fractions of phosphoprotein phosphatases were calculated according to Siegel and Monty (719), using Stokes radius and $s_{20,w}$ values obtained and assuming a partial specific volume of 0.725 for the enzymes. Axial ratio was estimated according to Schachman (720). The results are summarized in Table 12. The frictional and axial ratios suggest that phosphatase S is a globular protein.

VII. Catalytic Properties of Phosphatase S

The catalytic properties of the purified phosphatase S were similar to those of the catalytic subunit of $M_r=34,800$ prepared from phosphatases A ($M_r=156,000$), B ($M_r=161,000$), and C ($M_r=95,000$) by the treatment with 80% ethanol at

TABLE 12Physical Properties of Canine Heart Phosphoprotein PhosphataseIsozymes

Phosphatases	Stokes radius Å	$S_{20,w}$	M_r	Fractional ratio	Axial ratio
A	54	7.0	156,000	1.520	9
B	55	7.1	161,000	1.532	9
C	43	5.4	95,600	1.425	7
S (catalytic subunit of A, B, and C)	24.5	3.45	34,800	1.137	4
U-1 (Mn ²⁺ -activated)	39	3.8	61,000	1.501	9
U-2 (Mg ²⁺ -activated)	31	3.86	49,300	1.281	5.5

room temperature as described in previous dissociation study and as reported by Li and Hsiao (569).

A) Substrate specificity

As shown in Table 13, the purified enzyme exhibited a broad substrate specificity and was about equally active toward phosphorylase a, phosphorylated cyclic AMP-dependent protein kinase, myosin, histone, and casein. The broad substrate nature of phosphatase S is similar to those phosphoprotein phosphatases of molecular weights in the range of 30-35,000 purified from rabbit liver (608,616) and bovine heart (520). Cyclic AMP and cyclic GMP at concentrations ranging from μM to mM showed no significant effect on the enzymic activity toward phosphohistone, phosphocasein, and phosphorylase a. The enzymic activity toward the phosphorylated cyclic AMP-dependent protein kinase of bovine heart, however, was greatly stimulated by cyclic AMP. In the absence of the cyclic nucleotide, phosphatase S exhibited much less activity than in its presence. In this respect, the property of phosphatase S was the same as that of the phosphoprotein phosphatase purified from bovine heart (520). The enzyme showed no significant hydrolytic activity toward the low molecular

TABLE 13
Substrate Specificity of Phosphatase S

<u>SUBSTRATE</u>	<u>RELATIVE ACTIVITY (%)</u>	
Phosphohistone	100	
Phosphocasein	120	
Phosphorylase <u>a</u>	105	
[³² P]myosin (1 μ M/10 μ M)	10	(170 ^b)
[³² P]cyclic-AMP dependent Protein Kinase (5 μ M/10 μ M)	46	

^aAssays were performed in the presence of 5 mM EDTA as described in the text. The relative activities of different substrates were compared to the phosphohistone phosphatase activity. The concentration of substrate (in terms of [³²P]) were as follows: 10 μ M phosphohistone, 10 μ M phosphocasein, 10 μ M phosphorylase, 1 μ M myosin and 5 μ M protein kinase.

^bIf 1 μ M of phosphohistone was used as substrate, the relative activity toward myosin was 170%.

weight phosphoesters* such as phosphoserine, phosphothreonine, inorganic pyrophosphate, β -glycerophosphate, glucose-6-phosphate, glucose-1-phosphate, and ATP.

B) Conversion of phosphorylase activity from a to b

Besides hydrolyzing ^{32}Pi from labeled phosphorylase a, the phosphatase S may also show phosphorylase phosphatase activity by catalyzing the conversion of phosphorylase from a to b. As shown in Fig. 17, the phosphatase S converted phosphorylase from an AMP-independent form (phosphorylase a) to an AMP-dependent form (phosphorylase b).

C) pH profile

Measuring the enzymic activity in the standard assay mixture in the absence of EDTA, Mg^{2+} , or Mn^{2+} , as described under "Experimental Procedures", phosphatase S exhibited a bell-shaped pH profile with a pH optimum between 6.5-8 for either phosphohistone (Fig. 18A) or phosphorylase a (Fig. 18B) and with a pH optimum between 7-8.5 for phosphocasein (Fig. 18A).

D) Effects of divalent cations

As shown in Table 14, when phosphohistone or phosphocasein was used as a substrate, inclusion of 5 mM EDTA,

*However, recent study indicates that an alkaline phosphatase activity, which catalyzed the hydrolyzation of p-nitrophenyl phosphate and β -naphthyl phosphate and required a proper divalent cation (e.g., Mg^{2+}) and a sulfhydryl compound to express its activity, was co-purified with phosphatase S. Li, H.-C.; Hsiao, K.-J., and Sampathkumar, S., manuscript submitted for publication, 1978.

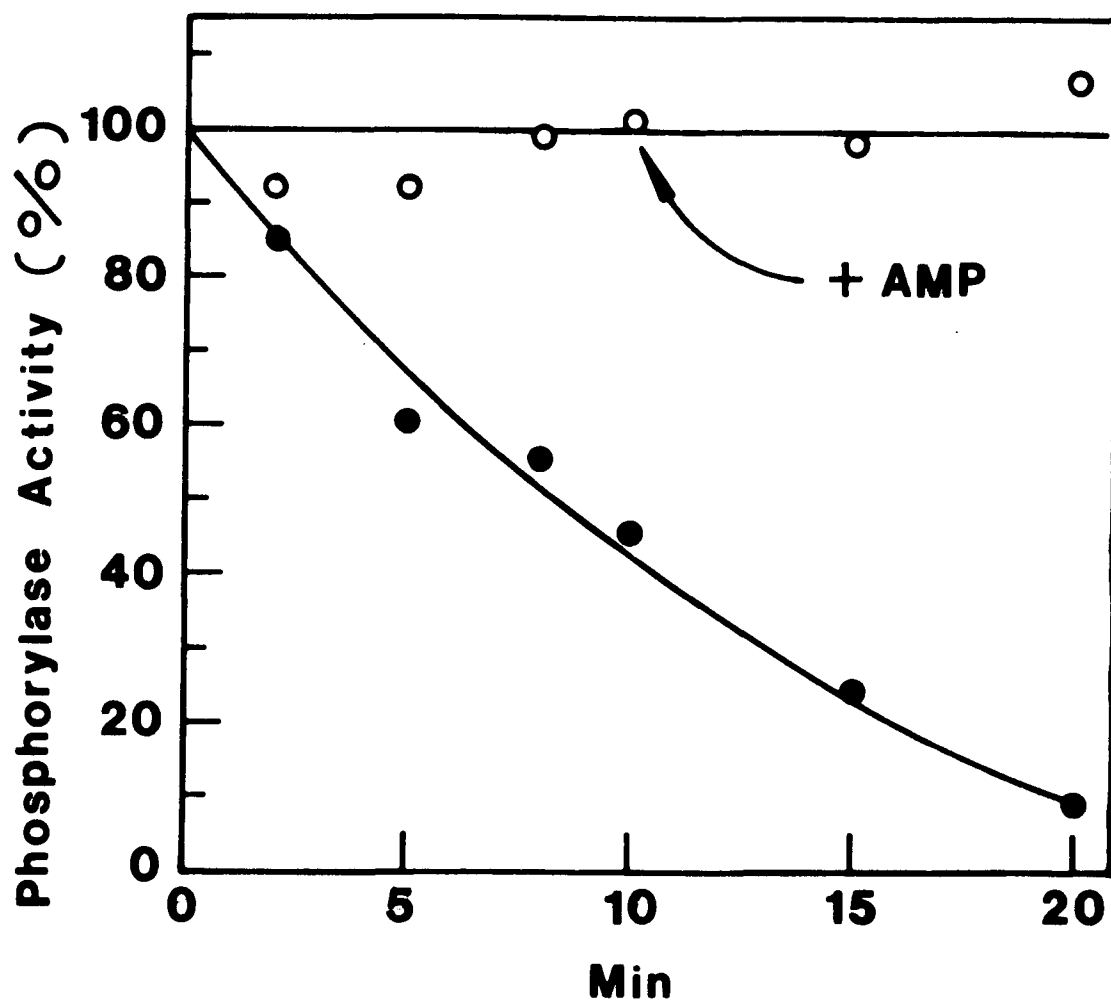


Fig. 17. Time course of conversion of phosphorylase activity from $\underline{a}$ to $\underline{b}$ by phosphatase S.
 0.1 mg phosphorylase $\underline{a}$ and 0.262 μg phosphatase S was incubated in 0.5 ml reaction mixture at 30° as described in the text. Aliquots of 50 μl were withdrawn from the incubation mixture and put into ice cold stopping buffer at indicated time to terminate the reaction. 50 μl of the diluted mixture was immediately analyzed for phosphorylase activity in the absence (●) or in the presence (O) of 1 mM AMP.

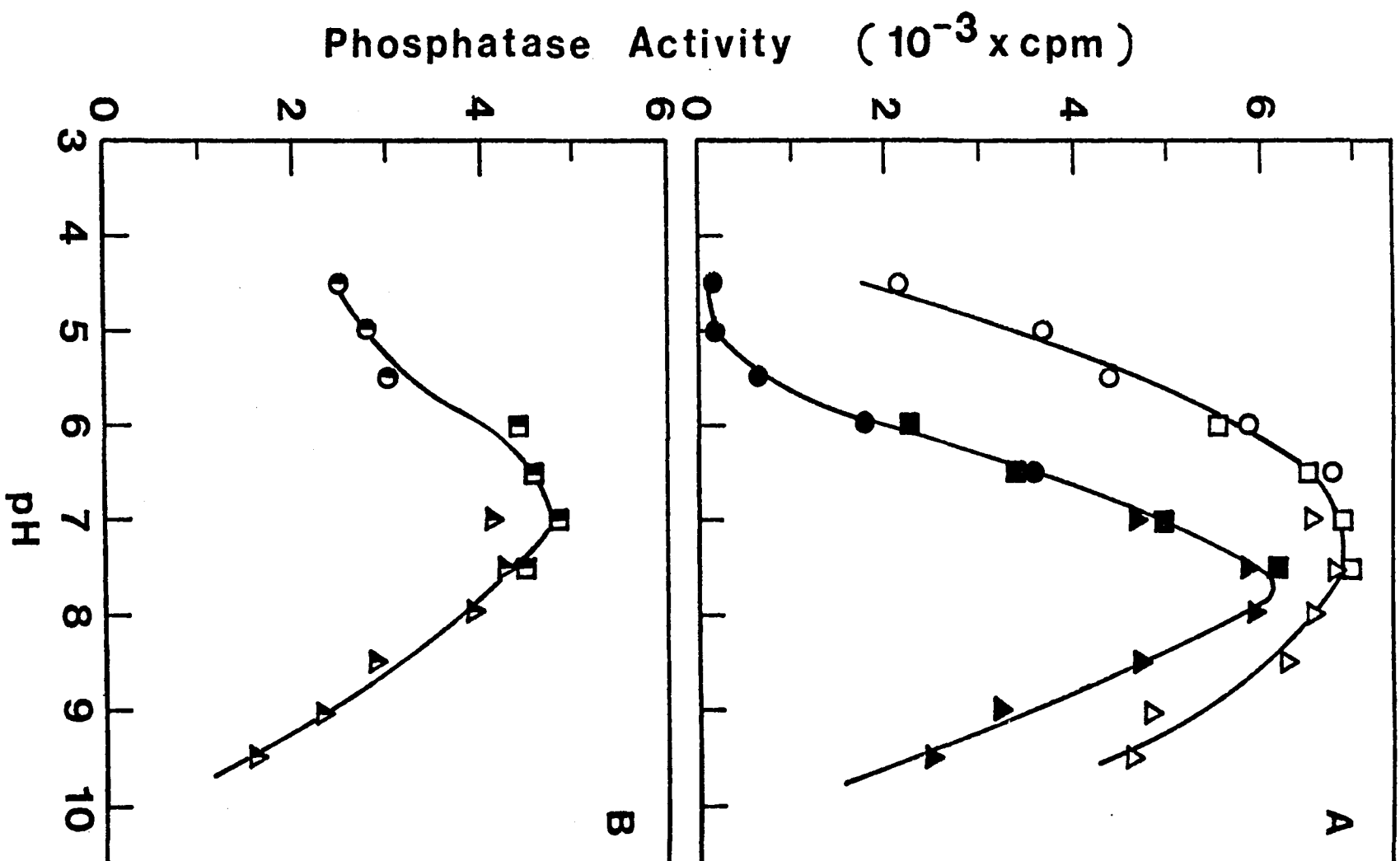


Fig. 18

Fig. 18. Effect of pH on the activity of phosphatase S.
 The enzymic activity was measured in an incubation volume of 50 μ l containing 50 mM buffer, 1 mM dithiothreitol, 0.1 M KCl, 2.5 ng phosphatase S, and 10 μ M phosphohistone ($\circ, \Delta, \square$), phosphocasein ($\bullet, \blacktriangle, \blacksquare$), or phosphorylase ($\odot, \blacktriangle, \blacksquare$). Incubation was carried out at 30° for 10 min. The following buffers at indicated pH were used: 2-(N-morpholino) ethane sulfonate (MES) ($\circ, \bullet, \odot$); imidazole ($\square, \blacksquare, \blacksquare$); and Tris·HCl ($\Delta, \blacktriangle, \blacktriangle$).

TABLE 14

Effects of Divalent Cations on the Activity of Phosphatase S in Reaction Mixtures

Addition (5 mM)	Relative Activity		
	Phosphorylase <u>a</u>	Phosphocasein	Phosphohistone
none	100	100	100
EDTA	91	104	90
EGTA	96	111	86
MgCl ₂	39	106	89
MnCl ₂	64	132	107
CaCl ₂	35	100	93
CoCl ₂ ^b	66	91	102
FeCl ₂	>1	>1	1
ZnCl ₂	>1	1	2
CuCl ₂	10	>1	4

^aThe phosphoprotein phosphatases activities were measured as described under "Experimental Procedures" with a 50 μ l-assay mixture containing 50 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 0.1 M KCl, 2.6 ng phosphatase S, 10 μ M phosphoprotein and 5 mM various divalent cations as indicated. The activity is expressed relative to that without added divalent cations.

^bAssayed in the absence of dithiothreitol.

Mg^{2+} or Ca^{2+} in the assay mixture did not significantly affect the enzymic activity. The same concentration of Mn^{2+} and Co^{2+} slightly stimulated the enzymic activity. The extent of Mn^{2+} and Co^{2+} stimulation varied slightly from preparation to preparation, and usually the stimulation was less than onefold. The presence of 5 mM Mg^{2+} , Ca^{2+} , Mn^{2+} , or Co^{2+} in the assay mixture, however, inhibited the enzymic activity toward phosphorylase a. The inhibition may be due to the formation of a metal ion-phosphorylase a complex which is a poor substrate for the phosphatase (570). Zn^{2+} , Cu^{2+} , and Fe^{2+} were potent inhibitors. Addition of 5 mM Zn^{2+} , Cu^{2+} , or Fe^{2+} to the assay mixture completely abolished the enzymic activity toward all phosphoproteins. Including 1, 2, and 5 mM MnCl_2 in the assay mixture (without 0.1 M KCl) inhibited the phosphorylase phosphatase activity 8%, 23%, and 47%, respectively.

E) Effects of nucleotides and related compounds

The activity of the purified phosphatase S responded to ATP and other related compounds similar to that of the catalytic subunit derived from phosphatase B as described by Li and Hsiao (569). When phosphohistone or phosphocasein was used as a substrate, inclusion of 2 mM of ATP, GTP, UTP, CTP, AMP- $(\text{CH}_2)\text{P}$, or AMP-P $(\text{CH}_2)\text{P}$ in the assay mixture containing no divalent cation inhibited the

TABLE 15

Effects of Various Compounds on the Activity of Phosphatase S in
Reaction Mixtures

Addition	mM	Relative Activity		
		Phosphorylase <u>a</u>	phosphocasein	phosphohistone
none	-	100	100	100
Adenine	2	122	107	99
Adenosine	2	114	95	96
AMP	2	8	91	85
ADP	2	10	49	76
ATP	2	38	29	55
GTP	2	44	35	53
UTP	2	50	48	61
CTP	2	44	41	51
AMP(CH ₂)P	2	30	39	64
AMP-P(CH ₂)P	2	9	46	26
Cyclic AMP	0.2	85	109	94
Cyclic GMP	0.2	109	101	95
NaF	20	40	36	79
KPi	20	20	21	55
Pyrophosphatae	2	10	2	39
Tripolyphosphate	2	29	23	51
Trimetaphosphate	2	72	62	92
Polyphosphate (n=15)	2	8	3	5

The phosphoprotein phosphatases activities were measured as described in Table 14 with various compounds at the concentration as indicated. The activity is expressed relative to that without added effectors.

enzymic activity about 40-70%. The same concentration of ADP inhibited the enzymic activity to a lesser extent, while adenine, adenosine, and AMP showed no significant effect (Table 15). The presence of equimolar concentration of Mg^{2+} or Mn^{2+} diminished the ATP inhibitory effect (not shown). When phosphorylase a was used as substrate, in contrast to phosphohistone and phosphocasein, the AMP or ADP inhibited the enzymic activity about 90%. The inhibition presumably may be due to effector-substrate interaction, which make the phosphorylase a a poor substrate for phosphatase S (582).

2 mM of various polyphosphates, 20 mM phosphate, and 20 mM NaF inhibited the enzymic activity toward all substrates tested (Table 15). Among them, pyrophosphate and polyphosphate (n=15) were more potent than the others. Subsequent studies indicated that nucleoside triphosphates and pyrophosphate inhibited phosphatase S activity by removing an essential divalent cation(s) from the active site of the enzyme.

KCl was originally included in the incubation mixture for optimizing the assay condition and eliminating salt effects of various effectors, when phosphohistone was used as a substrate (569). However, elimination of KCl from assay mixture increased enzymic activity toward phosphorylase a

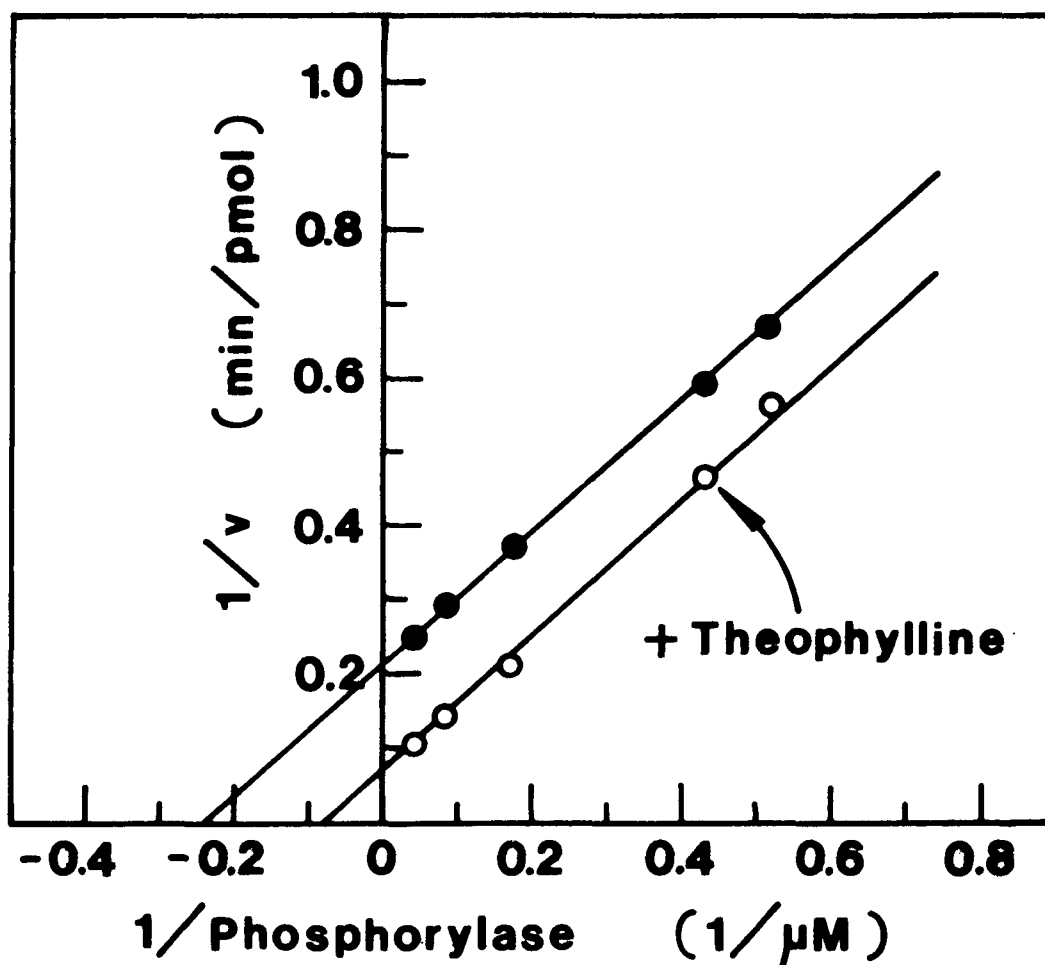


Fig. 19. Effects of phosphorylase α concentration on the activity of phosphatase S. The reaction was carried out in an incubation volume of 50 μ l containing 50 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 4.4 ng phosphatase S, various concentrations of phosphorylase α (in term of ^{32}P), and in the absence (●) or in the presence (○) of 5 mM theophylline as indicated. After incubated at 30° for 10 min, $^{32}\text{P}_i$ hydrolyzed was determined as described in the text. The data were present in double-reciprocal plot.

about two- to threefold. Therefore, KCl was eliminated from reaction mixture for phosphorylase phosphatase assay in subsequent studies. Assaying without KCl did not significantly change the effects of various compounds on phosphorylase phosphatase activity as described above.

F) Michaelis-Menten constants

The enzyme exhibited a Michaelis-Menten type of saturation kinetics and the apparent K_m values for phosphohistone, phosphocasein, and phosphorylase a were estimated as 27, 16, and 4 μ M, respectively. In the presence of 5 mM theophylline, both K_m and V for phosphorylase a were increased about 2.5-fold (Fig. 19).

VIII. Mechanism of the Action of ATP, Pyrophosphoryl Compounds and Divalent Cations on Phosphatase S

A) Inhibitory effects of ATP and pyrophosphoryl compounds on phosphatase S

Although it was found that ATP (Figs. 1B and 14A) and various related compounds stimulated phosphatase B activity toward phosphohistone (569). The stimulatory effect was contributed by ATP-phosphohistone interaction rather than ATP-enzyme interaction (569). Preincubation of either phosphatase B or S with 2 mM ATP at 30° resulted in a progressive inactivation of the enzymic activity toward phosphohistone (Fig. 20), phosphorylase a, and phosphocasein (not shown). The ATP inactivation was rapid, causing a loss

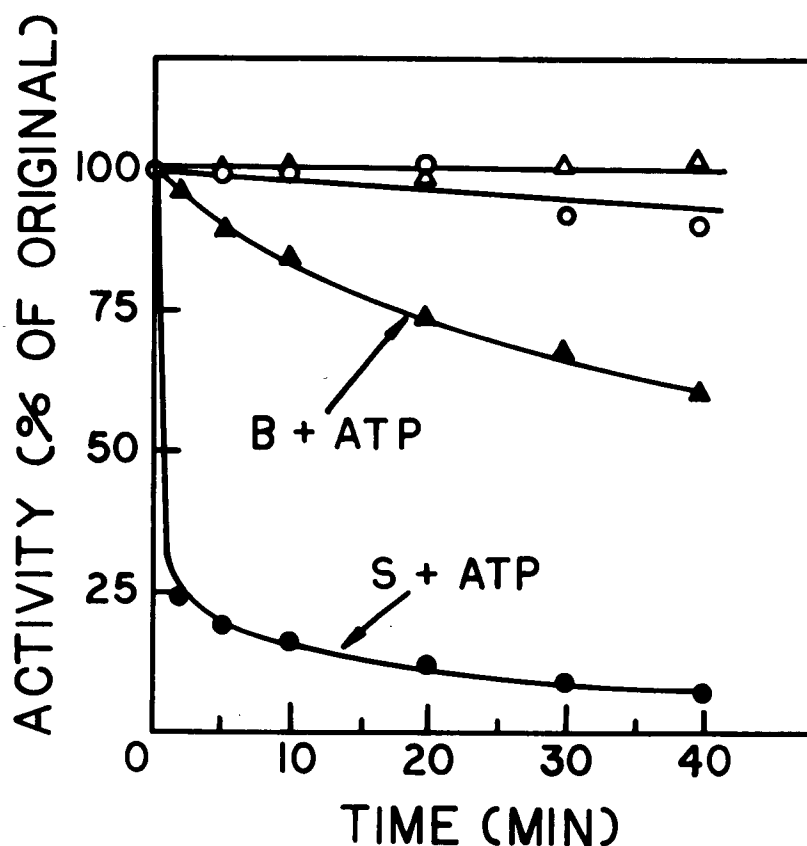


Fig. 20. Inactivation of phosphatases B and S by ATP as a function of time. The reactions were carried out at 30° in a volume of 0.2 ml containing 20 mM Tris·HCl, pH 7.4, 0.2% bovine serum albumin, and 14 µg of phosphatase B in the absence (Δ) or in the presence ($\blacktriangle$) of 2 mM ATP; 2.3 µg of phosphatase S (preparation of ethanol treated phosphatase B) in the absence of ($\circ$) or in the presence of ($\bullet$) of 1 mM ATP. Aliquots of 2 and 4 µl were analyzed for remaining phosphatase B and S activities, respectively, at indicated time. The enzymic activity was measured in an incubation volume of 0.1 ml containing 50 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 0.1 M KCl, 10 µM phosphohistone, and the treated phosphatase as described in the text.

of about 90% of the phosphatase S activity during a 10 min preincubation, whereas 2 mM ATP inactivated phosphatase B activity by 15% in 10 min (Fig. 20). The inhibitory effect of ATP on enzyme was further studied by using purified phosphatase S.

As shown in Fig. 21, the concentration of ATP for half maximum inactivation by preincubation was about 0.1-0.2 mM, when either phosphorylase a or phosphocasein was used as a substrate for measuring the enzymic activity. When the enzymic activity was measured in the presence of Mn^{2+} , the ATP-inactivated enzyme appeared to be reactivated (Fig. 21).

The ability of various ATP analogues and chelating agents to inactivate phosphatase S was examined. As shown in Table 16, all nucleoside di- and tri-phosphates as well as pyrophosphate were capable of inactivating the enzyme. GTP, CTP, UTP, and (β , γ -methylene) adenosine 5'-triphosphate were as effective as ATP, while ADP was less effective than the nucleotide triphosphates. Among all compounds examined, pyrophosphate (PPi) was the most effective inactivator. On the other hand, inorganic phosphate (Pi), AMP, adenosine adenine, EDTA, EGTA, 1,10-phenanthroline, and 8-hydroxy-quinoline were not effective. In the presence of EDTA, the ATP inactivation effect was not potentiated. In the presence of equimolar concentration of $MgCl_2$ (Fig. 21B) or

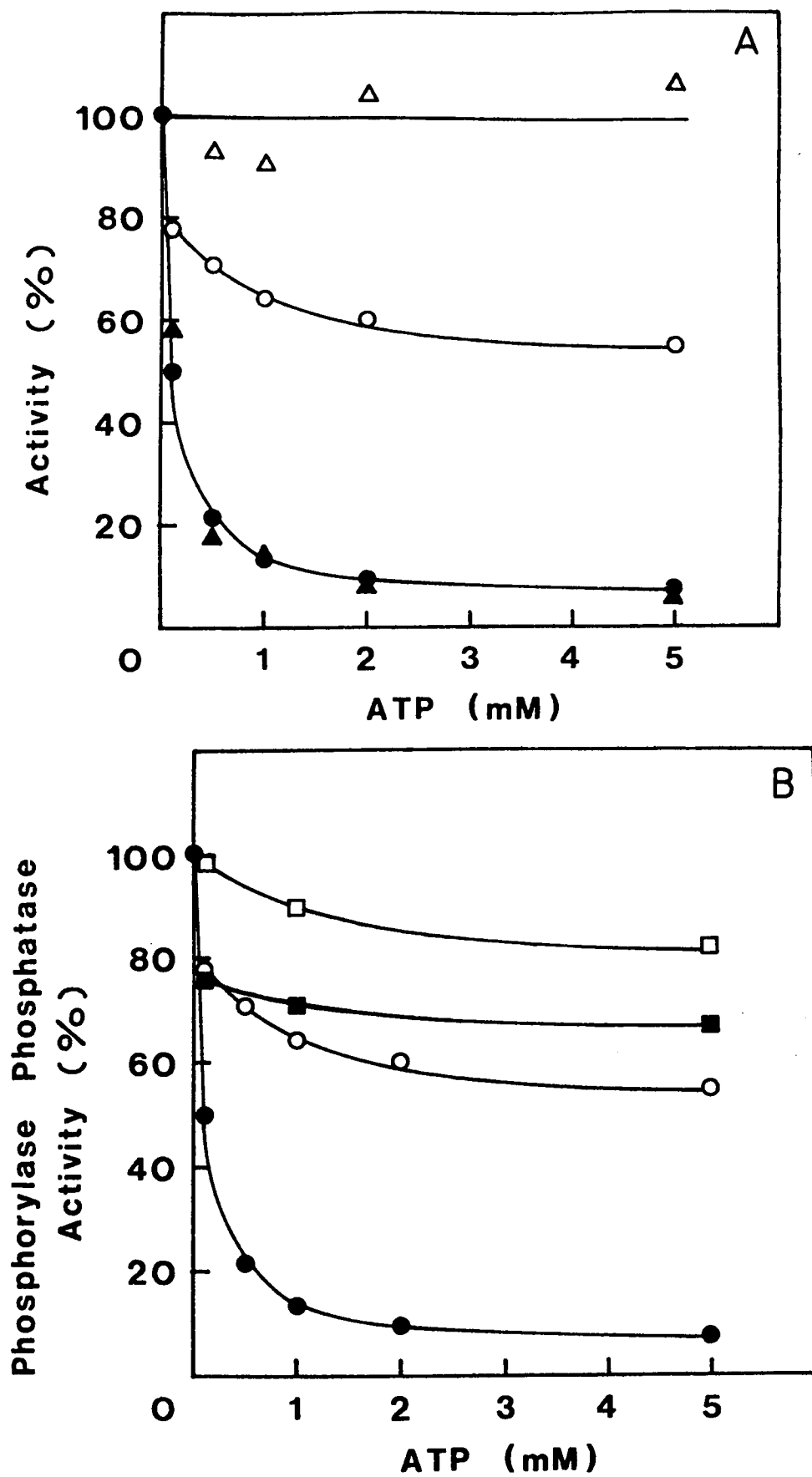


Fig. 21

Fig. 21. Effect of ATP concentrations on the activity of phosphatase S. Phosphatase S (1.6 μ g) was pre-incubated at 30° in a volume of 50 μ l containing 50 mM Tris·HCl, pH 7.4, 2 mg/ml bovine serum albumin with various concentrations of ATP as indicated. After 5 min, 2 ml of ice cold dilution buffer (20 mM Tris·HCl, pH 7.4, and 2 mg/ml bovine serum albumin) was added to each preincubation mixture to terminate the reaction. 10 μ l of the diluted preincubation mixture was immediately analyzed for phosphorylase phosphatase activity in the absence (●) or in the presence (○) of 0.5 mM MnCl_2 , and phosphocasein phosphatase activity in the absence (▲) or in the presence (△) of 5 mM MnCl_2 as described in the text. In Fig. 21B, the phosphatase S was preincubated in the presence (□, ■) or in the absence (○, ●) of equal molar MgCl_2 besides ATP. And phosphorylase phosphatase activity was measured in the absence (●, ■) or in the presence (○, □) of 0.5 mM MnCl_2 as described in the text.

TABLE 16

Effects of Various Compounds on the Activity of Phosphatase S
in Preincubation Mixtures

Addition	mM	Relative Activity	
		Phosphorylase <u>a</u>	Phosphocasein
none	-	100	100
Adenine	0.2	94	114
Adenosine	0.2	94	112
AMP	0.2	68	128
ADP	0.2	59	87
ATP	0.2	42	48
GTP	0.2	25	45
CTP	0.2	57	52
UTP	0.2	59	52
AMP-P(CH ₂)P	0.2	55	66
ATP + MgCl ₂	0.2 + 0.2	85	91
ATP + MnCl ₂	0.2 + 0.2	121	114
ATP + P _i	0.2 + 20	95	98
P _i	20	96	95
PP _i	0.2	18	12
EDTA	2.0	102	102
EGTA	2.0	112	124
8-Hydroxyquinoline	2.0	98	111
1,10-Phenanthroline	2.0	96	88

Phosphatase S (0.66 µg) was preincubated at 30°C in a volume of 25 µl containing 40 mM Tris·HCl, pH 7.4, 1.6 mg/ml bovine serum albumin and various compounds at the concentration indicated. After 5 min, 2 ml of ice cold dilution buffer (20 mM Tris·HCl, pH 7.4 and 2 mg/ml bovine serum albumin) was added to each preincubation mixture to terminate the reaction. 10 µl of the diluted preincubation mixture was immediately analyzed for phosphorylase phosphatase or phosphocasein phosphatase in the absence of divalent cations as described under "Experimental Procedures".

MnCl_2 (not shown), the ATP inactivation effect was almost abolished. Although preincubating the enzyme with Pi did not result in any inactivation of the enzyme (Table 16), direct addition of Pi into the assay mixture inhibited the enzymic activity (Table 15). The data indicated that Pi acted as a reversible inhibitor for phosphatase S. It was found that Pi could protect the enzyme from ATP inactivation (Table 16). When the enzyme was preincubated with 0.5 mM ATP, increasing Pi concentrations in the preincubation mixture decreased the ATP inactivation effect (Fig. 22). The Pi concentration for decreasing 50% of the ATP inactivation effect was about 5 mM.

During the course of preincubating phosphatase S with ATP, either in the absence or in the presence of equimolar concentration of Mg^{2+} or Mn^{2+} , no enzymic hydrolysis of ATP to ADP and Pi could be detected. Cyclic AMP and cyclic GMP, at concentrations ranged from 1 μM to 1 mM showed no effect on ATP inactivation of the enzyme.

When the enzymic activity was measured in the presence of 1 mM Mn^{2+} , the inactivated enzyme resulting from preincubation with either nucleotides or PPI (Table 16) exhibited similar activity as that of the control enzyme preincubated in the absence of any effectors (data not shown). It appears that ATP, other nucleotide di- and

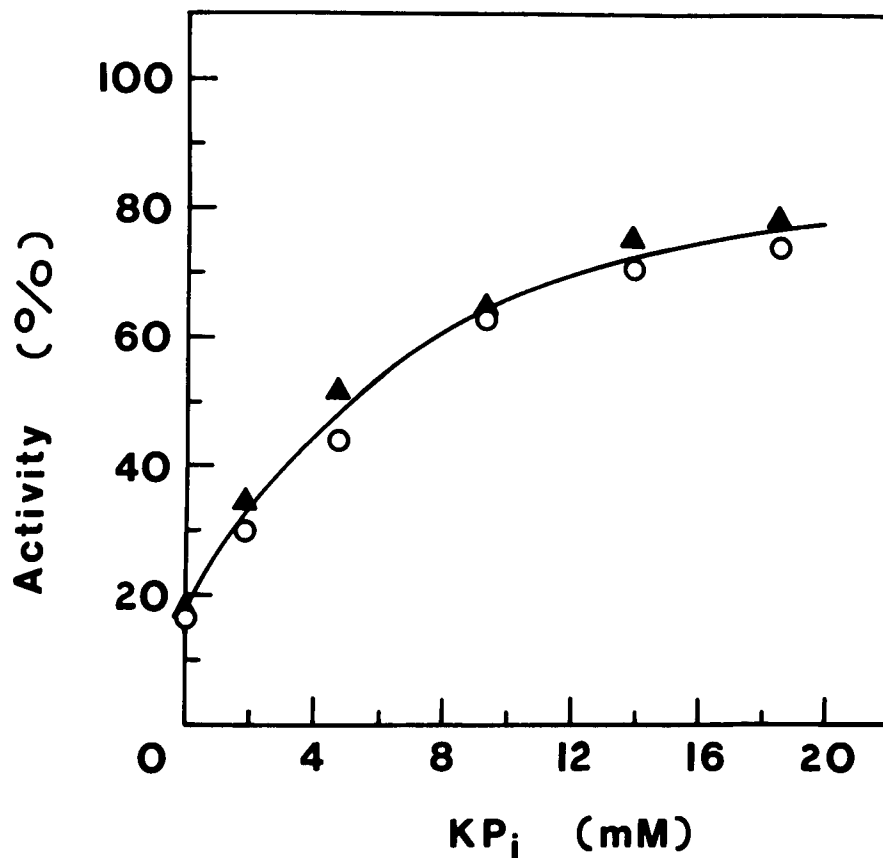


Fig. 22. Effect of orthophosphate concentrations on the ATP inactivation of phosphatase S. Phosphatase S (0.66 μ g) was preincubated at 30° in a volume of 25 μ l containing 40 mM Tris·HCl, pH 7.4, 1.6 mg/ml bovine serum albumin, 0.46 mM ATP, and different concentrations of potassium phosphate (pH 7.4) as indicated. After 5 min, 2 ml of ice cold dilution buffer (20 mM Tris·HCl, pH 7.4, and 2 mg/ml bovine serum albumin) was added to each preincubation mixture to terminate the reaction. An aliquot (10 μ l) of the diluted preincubation mixture was immediately analyzed for phosphorylase phosphatase (instead of 0.1 M KCl, 5 mM theophylline was included in the assay mixture) (O) or phosphocasein phosphatase (▲) in the absence of divalent cations as described in

Fig. 22 (Continued)

the text. The activities were expressed in terms of percentage of the control experiments in which the preincubation mixtures contained neither ATP nor orthophosphate.

triphosphate and PPi inactivated phosphatase S by converting it to a divalent cation-dependent form.

B) Preparation of divalent cation-dependent phosphatase S (apophosphatase S)

A divalent cation-dependent form of phosphatase S free from the converting agent has been prepared by the following procedures. Phosphatase S (0.66 mg) was incubated at 30° in a 6 ml reaction mixture containing 5 mM ATP, 50 mM Tris·HCl, pH 7.4, 10 mM 2-mercaptoethanol and 20% glycerol. After 5-10 min incubation, the reaction mixture was filtered through a Sephadex G-25 column (1.6 x 90 cm) equilibrated with a buffer containing 20 mM Tris·HCl, pH 7.4, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 M KCl and 10% glycerol. The column was eluted with the same buffer. The enzyme eluted from the column at the void volume was divalent cation dependent and was separated from ATP (Fig. 23). The enzyme (fractions 45-58) was collected, concentrated by membrane ultrafiltration (PM 10, Amicon), and dialyzed extensively against a buffer containing 20 mM Tris·HCl, pH 7.4, and 50% glycerol. The enzyme so prepared was divalent cation-dependent, contained less than 0.05 mole ATP per mole of enzyme protein as determined by using either [γ -³²P] or [γ -¹⁴C]ATP as a converting agent. The enzyme had the same Stokes radius (25⁰Å) as that of the

Fig. 23

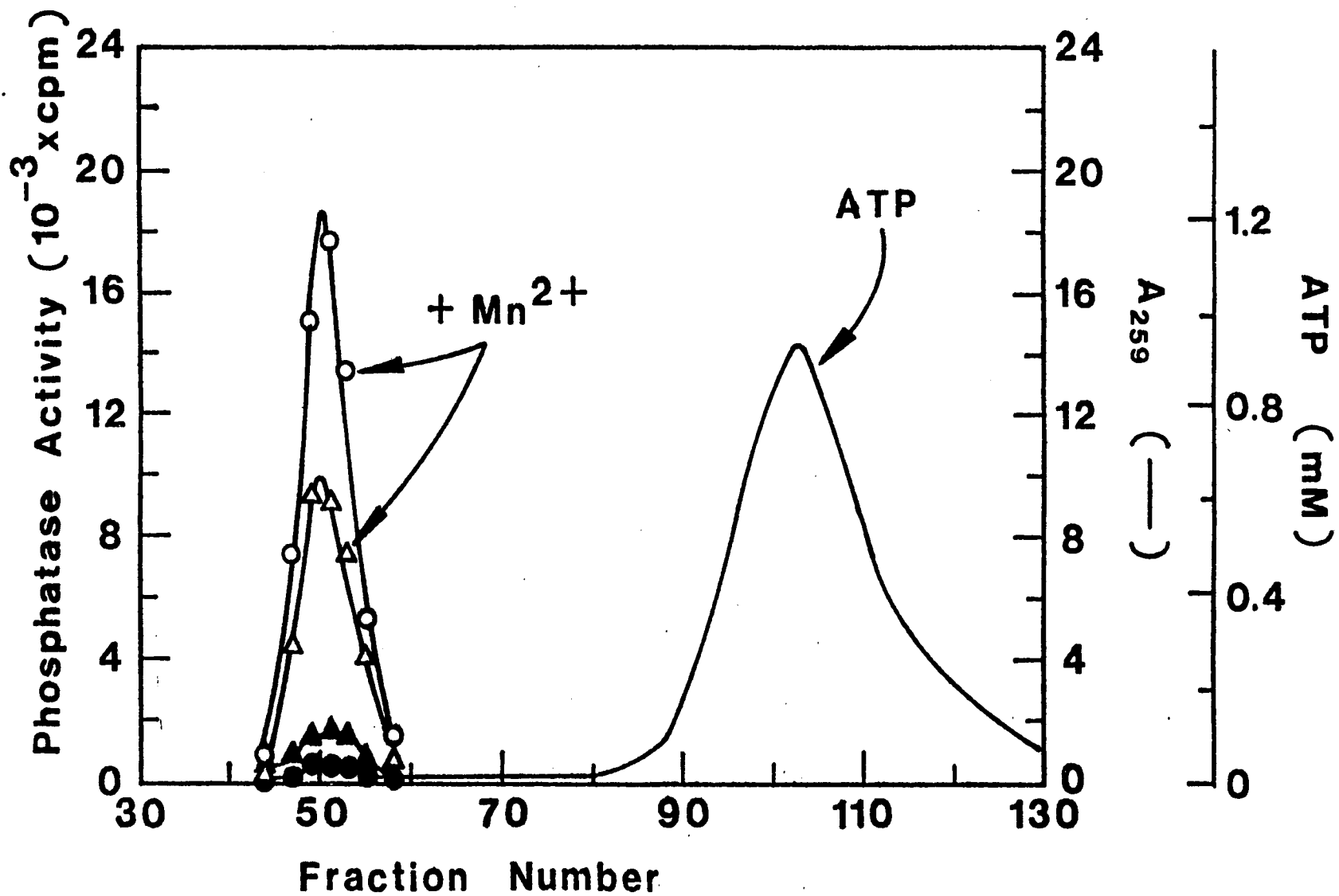


Fig. 23. Separation of apophosphatase S from ATP by Sephadex G-25 chromatography. The phosphatase S was preincubated with 5 mM ATP at 30° for 5 min as described in the text. The reaction mixture was applied to a Sephadex G-25 column (1.6 x 96 cm) pre-equilibrated with Buffer G plus 1 mM EDTA. The column was eluted with the same buffer and fractions of 1.6 ml were collected. The phosphatase activity toward phosphohistone (▲, Δ) and phosphocasein (●, ○) were measured in the absence (▲, ●) or in the presence (Δ, ○) of 5 mM MnCl₂ with aliquots of 1 μl from the indicated fractions. ATP was determined by measuring absorbance at 259 nm (——).

original phosphatase S as determined by chromatographing the enzyme on a calibrated Sephadex G-100 column (Table 12). This divalent cation-dependent form was arbitrarily designated as apophosphatase S in the following presentation. The total enzymic activity recovered in the apophosphatase S preparation was about 120-150% as that of the original phosphatase S when both of the enzymes were measured in the presence of the Mn^{2+} . Apophosphatase S could also be prepared by using 5 mM inorganic pyrophosphate instead of 5 mM ATP as a converting agent by the procedure described above.

C) Effects of divalent cations on apophosphatase S

When the apophosphatase S activity toward phosphorylase a and phosphocasein was preincubated in the presence of 2 mM MnCl_2 , CoCl_2 , ZnCl_2 , CaCl_2 , FeCl_2 , CuCl_2 , HgCl_2 , BaCl_2 , CdSO_4 , NiSO_4 , or $\text{Pb}(\text{CH}_3\text{OO})_2$, it was found that only MnCl_2 and CoCl_2 ($\text{Co}^{2+} > \text{Mn}^{2+}$) could effectively stimulate the enzymic activity (Table 17). ZnCl_2 and NiCl_2 exhibited a slight stimulatory effect (less than 5% as that of Co^{2+}). The other metal ions examined were not effective. When the enzymic activity was measured in the presence of 1 mM EDTA, the activation effect of Co^{2+} was increased slightly and the activation effect of Mn^{2+} was decreased (Table 17). Similar results were obtained when phosphohistone was used as a substrate.

TABLE 17

Effects of Divalent Cations on the Activity of Apophosphatase S

Effectors	Activity (pmol/min)			
	Phosphorylase <u>a</u>		Phosphocasein	
	-	EDTA	-	EDTA
EDTA	0.1	0.2	0.1	0.3
MnCl ₂	2.8	0.5	4.4	2.4
CoCl ₂	9.2	10.8	8.3	9.3
MgCl ₂	0.2	0.2	0.4	0.3
CaCl ₂	0.2	0.2	0.2	0.2
ZnCl ₂	0.1	0.3	0.1	0.1
NiSO ₄	0.7	0.2	0.4	0.2
CdSO ₄	0.2	0.2	0.2	0.1
BaCl ₂	0.1	0.2	0.1	0.1
HgCl ₂	0.1	0.1	0.2	>0.1
Pb(CH ₃ COO) ₂	0.1	0.2	0.1	0.1
CuCl ₂	0.2	0.2	0.1	>0.1
FeCl ₂	0.1	0.2	0.1	0.2

0.44 μ g of apophosphatase S was preincubated in a volume of 22 μ l containing 50 mM Tris·HCl, pH 7.4, 1 mg/ml bovine serum albumin and 2.3 mM various effectors as indicated. The preincubation was carried out at 0°C for 15 min and stopped by adding 1 ml of ice cold buffer containing 20 mM Tris·HCl, pH 7.4 and 2 mg/ml bovine serum albumin. Aliquots (10 μ l) of diluted preincubation mixture were analyzed for phosphatase activity. The phosphorylase phosphatase activity was measured in an assay mixture of 50 μ l containing 50 mM Tris·HCl, pH 7.4, 5 mM theophylline and 10 μ M [³²P]phosphorylase a (in term of ³²P) and in the absence or presence of 1 mM EDTA as indicated. After incubated at 30°C for 10 min, the ³²P_i released was determined as described under "Experimental Procedures". For measuring phosphocasein phosphatase activity, 0.1 M KCl and 10 μ M [³²P]casein were used instead of theophylline and phosphorylase a.

The stimulatory effect of Mn^{2+} and Co^{2+} were further studied. As shown in Fig. 24, preincubation of apophosphatase S with Mn^{2+} or Co^{2+} at 0° resulted in activation of the enzyme. The Mn^{2+} activation reaction was almost instantaneous and reached a maximum within 10 min. Longer preincubation caused a gradual decrease in the enzymic activity. In comparison, Co^{2+} activated the enzyme at a slower rate. The enzymic activity increased with time and reached a plateau after about 20 min preincubation. The enzymic activity obtained by Co^{2+} activation was about 2-3 times higher than that obtained by Mn^{2+} after 10 min preincubation. When the enzyme was preincubated with a mixture of Mn^{2+} and Co^{2+} , the enzyme was activated to the same extent as if the enzyme was preincubated with Mn^{2+} alone. Furthermore, the time course of activation showed the same characteristics as that of Mn^{2+} . The data indicated that the Mn^{2+} and the Co^{2+} activation effects were not additive and that the Co^{2+} activation effect was totally inhibited in the presence of Mn^{2+} .

The effects of Mn^{2+} and Co^{2+} concentrations on the activation of apophosphatase S are shown in Figs. 25 and 26, respectively. Both Mn^{2+} and Co^{2+} saturation curves exhibited sigmoidal kinetics. The Mn^{2+} and Co^{2+} concentrations for half maximum activation are about 100-250

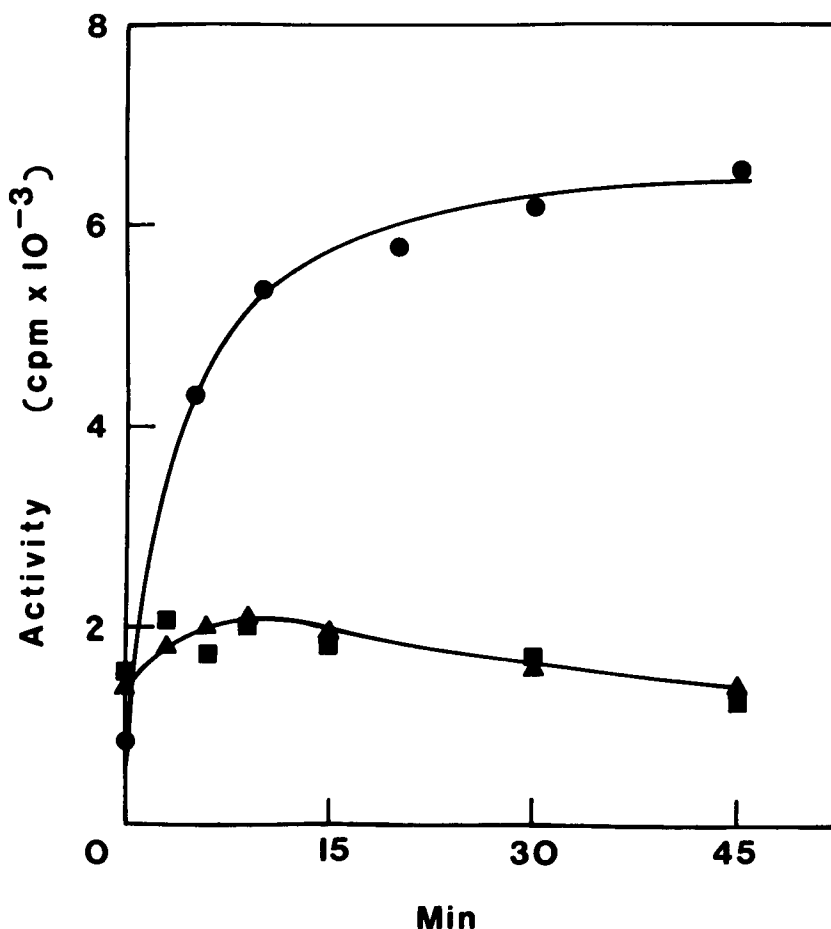


Fig. 24. Time course of activation of apophosphatase S by MnCl₂ and CoCl₂. Apophosphatase S (0.44 μ g) was preincubated at 0° in a volume of 20 μ l containing 50 mM Tris·HCl, pH 7.4, 1 mg/ml bovine serum albumin and 0.5 mM MnCl₂ (▲); 0.1 mM CoCl₂ (●); or 0.5 mM MnCl₂ plus 0.1 mM CoCl₂ (■). The reaction was initiated by the addition of the divalent cations. At the indicated time intervals, aliquots of 2 μ l were withdrawn from the preincubation mixture and mixed with 100 μ l ice cold dilution buffer (20 mM Tris·HCl, pH 7.4, and 2 mg/ml bovine serum albumin) to terminate the reaction. 10 μ l of the diluted preincubation mixture was immediately analyzed for phosphorylase phosphatase activity in the absence of added divalent cations as described in Table 17.

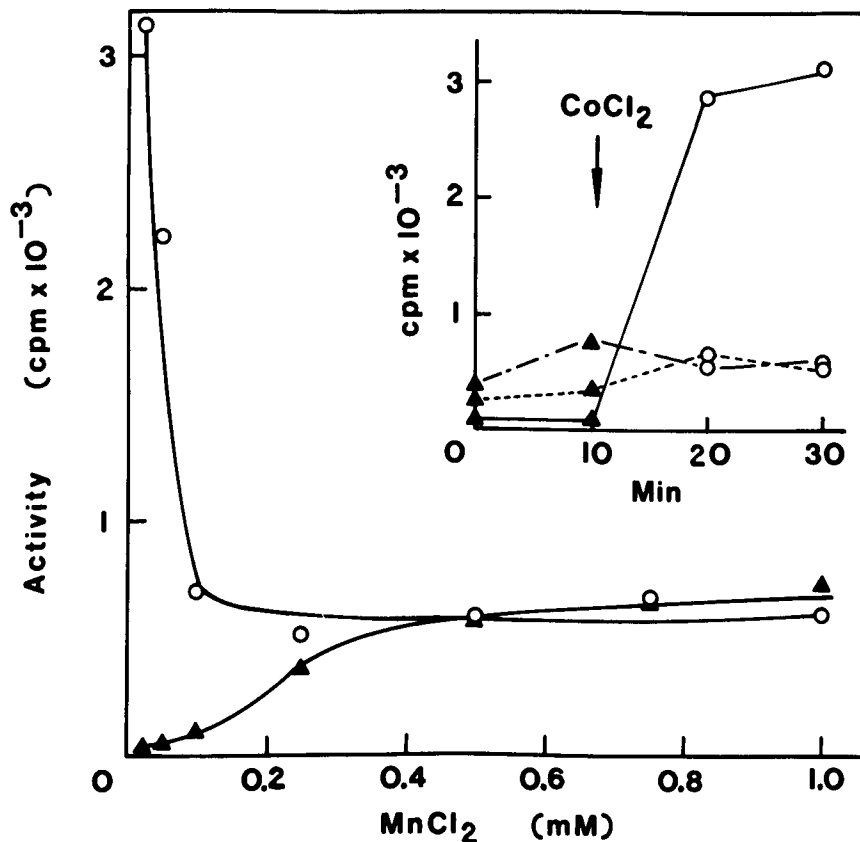


Fig. 25. Effects of MnCl_2 concentrations on the activation of apophosphatase S and on the subsequent activation of the enzyme by CoCl_2 . Apophosphatase S (0.44 μg) was preincubated at 0° in a volume of 20 μl containing 50 mM Tris·HCl, pH 7.4, 1 mg/ml bovine serum albumin and different concentrations of MnCl_2 as indicated. After 10 min, aliquots of 2 μl were withdrawn from the preincubation mixture and mixed with 100 μl of ice cold dilution buffer (20 mM Tris·HCl, pH 7.4, and 2 mg/ml bovine serum albumin) to terminate the reaction. 10 μl of the diluted preincubation mixture was immediately analyzed for phosphorylase phosphatase activity (▲) in the absence of added divalent cations as described in Table 17.

After withdrawing 2 μl from the 20 μl preincubation mixtures for assaying the enzymic activity as described above, 2 μl of 2 mM CoCl_2 was added to the remaining preincubation mixtures and the

Fig. 25 (Continued)

preincubation was carried out for an additional 20 min at 0°. An aliquot (2 μ l) of the preincubation mixtures containing 0.2 mM CoCl_2 was then withdrawn and mixed with the dilution buffer as described above. 10 μ l of the diluted preincubation mixture containing CoCl_2 was then analyzed for phosphorylase phosphatase activity ($\bigcirc$) in the absence of added divalent cations. The insert indicates the time course of the same experiment. Apophosphatase S was first preincubated with different concentrations of MnCl_2 (0.025 mM, ———; 0.25 mM, -----; and 1.0 mM, ---) and aliquots were analyzed for the enzymic activity at time intervals as indicated. After withdrawing an aliquot at 10 min incubation with MnCl_2 , CoCl_2 was added to the remaining preincubation mixture and aliquots were analyzed for the enzymic activity at time intervals as indicated.

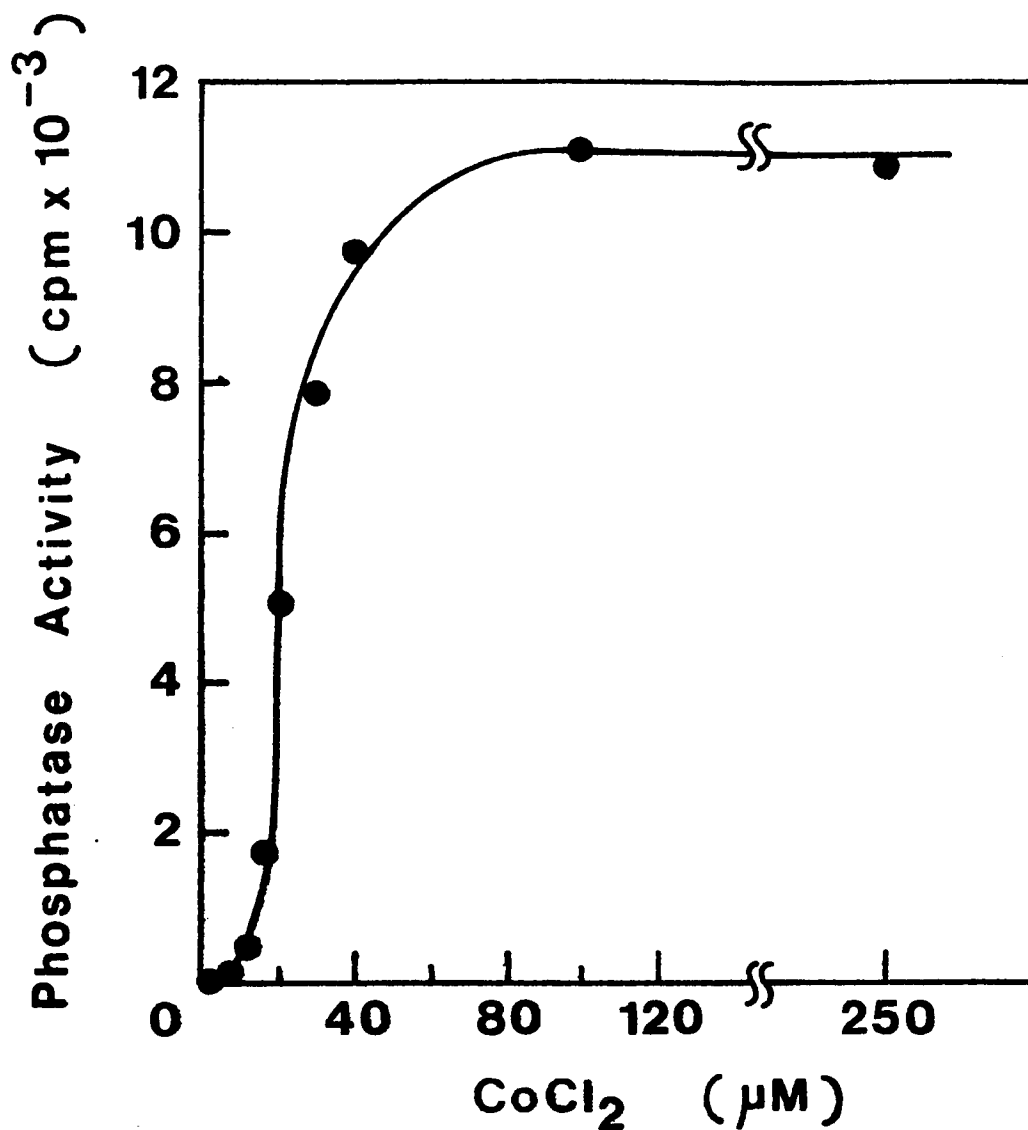


Fig. 26. Effects of CoCl_2 concentrations on the activation of apophosphatase S. Apophosphatase S ($0.2 \mu\text{g}$) was preincubated in a volume of $20 \mu\text{l}$ containing 50 mM Tris·HCl, pH 7.4, 1 mg/ml bovine serum albumin, and different concentrations of CoCl_2 (●) for 20 min at 30° as indicated. The reactions were initiated by adding CoCl_2 and terminated by 1 ml of ice cold dilution buffer (20 mM Tris·HCl, pH 7.4, and 2 mg/ml bovine serum albumin) to the preincubation mixture. $10 \mu\text{l}$ of the diluted preincubation mixture was immediately analyzed for phosphorylase phosphatase activity in the absence of added divalent cations, as described in Table 17.

and 20-40 μM , respectively. At all concentrations examined, Co^{2+} activated the enzymic activity to a greater extent than Mn^{2+} . The Hill coefficients (assumed enzymic activity proportion to divalent cation bound; i.e., $[\text{M}^{2+}]_{\text{bound}} \propto v$ and $n[\text{P}]_{\text{total}} \propto V$) for Mn^{2+} and Co^{2+} were estimated as 1.3 and 3.5, respectively.

The inhibitory effect of Mn^{2+} on Co^{2+} activation of the apophosphatase S was further examined by preincubating the enzyme with different concentrations of Mn^{2+} at 0° for 10 min followed by incubating the enzyme with saturating concentration of Co^{2+} (0.2 mM). As shown in Fig. 25, the Co^{2+} -activated enzymic activity was decreased as the Mn^{2+} concentrations used to preincubate the enzyme were increased. When the Mn^{2+} concentration was higher than 0.2 mM, the Co^{2+} -activation effect was almost completely abolished and the enzyme showed only the level of Mn^{2+} -activated activity. When the enzyme was first fully activated by Co^{2+} , subsequent incubation of the Co^{2+} -treated enzyme with Mn^{2+} did not decrease the enzymic activity to the level of the Mn^{2+} -treated enzyme. Preincubation of the enzyme with mM concentrations of Mg^{2+} or Ca^{2+} did not affect the activation of the enzyme by Mn^{2+} or Co^{2+} . Preincubation of the enzyme with 0.2 mM Zn^{2+} at 0° for 10 min, however, completely abolished both the Mn^{2+} and Co^{2+} activation

effects. The Mn^{2+} and Co^{2+} activation effects were the same when either phosphorylase a or phosphocasein was used as a substrate.

Cyclic AMP and cyclic GMP, at concentrations ranging from μM to mM , did not show any effect on Mn^{2+} or Co^{2+} activation of the apophosphatase S. ATP, at concentrations equal to or lower than those of the tested divalent cations (Mg^{2+} , Ca^{2+} , Mn^{2+} , or Co^{2+}), did not enhance the effectiveness of these metal ions to activate the apoenzyme.

$[^{32}\text{P}]$ myosin could also be dephosphorylated by apophosphatase S and the activity was dependent on addition of Mn^{2+} but not Mg^{2+} .

D) Preparation of Mn^{2+} -reactivated and Co^{2+} -reactivated apophosphatase S

Apophosphatase S (0.11 mg) was incubated at 0° in a 0.75 ml reaction mixture containing 50 mM Tris·HCl, pH 7.4, 20% glycerol, and 2 mM of MnCl_2 or CoCl_2 . After a 10 min incubation, the reaction mixture was filtered through a Sephadex G-25 column (1.6 x 96 cm), presaturated with buffer G. The column was eluted with the same buffer. The enzyme eluted from the column at void volume was completely separated from the bulk of divalent cation in the preincubation mixture. Both the Mn^{2+} - and the Co^{2+} -treated enzyme exhibited substantial activity when the

enzymic activity was measured in the absence of added divalent cation in the assay mixture (Fig. 27). The activity of the Mn^{2+} -treated enzyme, however, was stimulated about twofold and inhibited by 40-70% by addition of 5 mM Mn^{2+} and 5 mM EDTA, respectively, into the assay mixture (Fig. 27A). Preincubation of the Mn^{2+} -treated enzyme with 2 mM ATP resulted in rapid inactivation of the enzymic activity. These observations indicated that the Mn^{2+} activation was associated with loose binding of Mn^{2+} to the enzyme (see also Table 17). Presumably some of these bound Mn^{2+} had been removed from the enzyme by Sephadex G-25 filtration processes and some of the remaining bound Mn^{2+} could be further removed by either EDTA or ATP. By contrast, the Co^{2+} -treated enzyme eluted from the gel filtration column was stimulated 10-20% and inhibited 10-20% by addition of 5 mM EDTA (Fig. 27B) and 1 mM Co^{2+} (not shown), respectively, into the assay mixture. Preincubation of the Co^{2+} -treated enzyme with 2 mM ATP at 30° for 10 min did not effect the enzymic activity at all. These observations indicated that Co^{2+} activation was associated with tight binding of Co^{2+} to the enzyme. The active fractions obtained from Sephadex G-25 chromatography were pooled, concentrated by membrane ultrafiltration (PM 10, Amicon), and dialyzed extensively against a buffer containing 20 mM Tris-HCl, pH 7.4, and

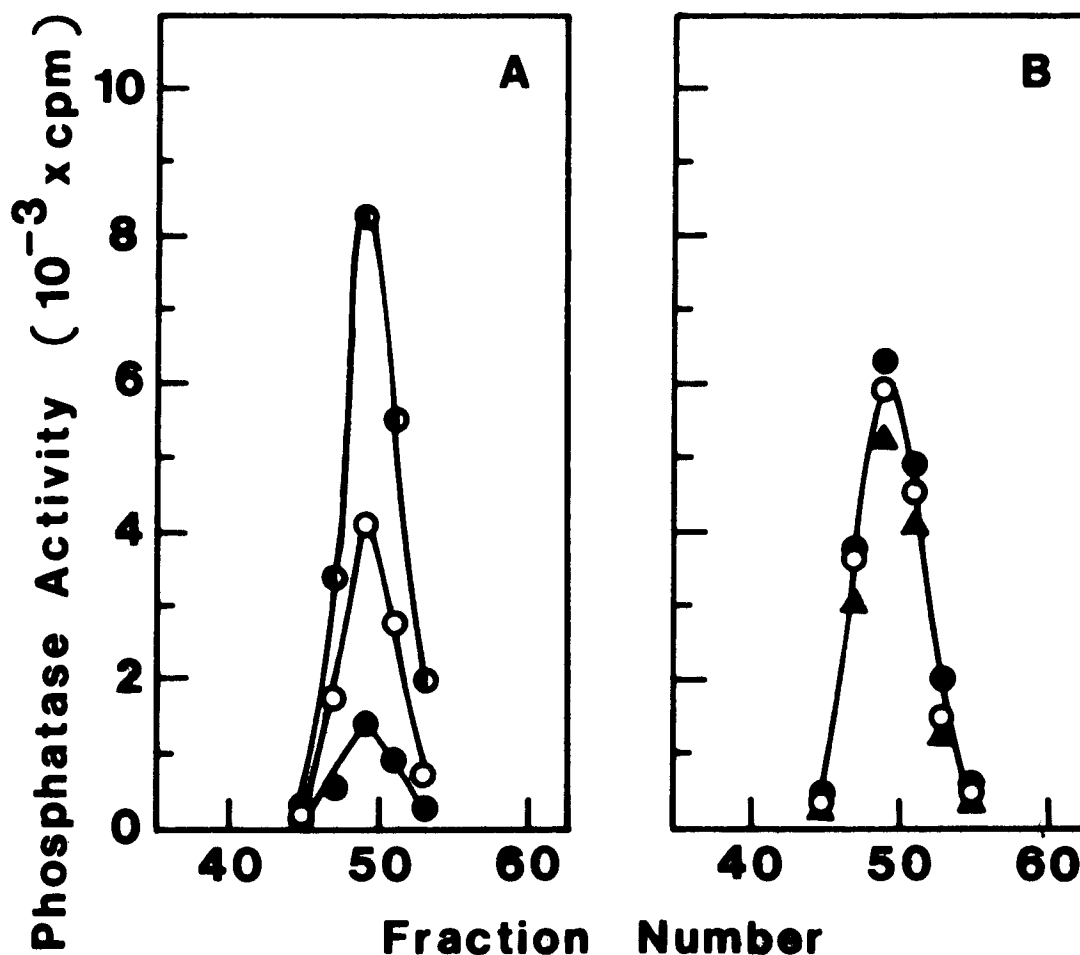


Fig. 27. Sephadex G-25 gel filtration of MnCl_2 (A) and CoCl_2 (B) reactivated apophosphatase S. 0.11 mg of apophosphatase S was reactivated at 0° for 10 min in a 0.75 ml reaction mixture containing 50 mM Tris·HCl, pH 7.4, 20% glycerol, and 2 mM of MnCl_2 (panel A) or CoCl_2 (panel B). The two divalent cation reactivated enzymes were filtrated through a Sephadex G-25 column (1.6 x 96 cm) with Buffer G separately. Fractions of 1.6 ml were collected. Phosphatase activity toward phosphocasein was measured in the absence (○) or in the presence of 5 mM MnCl_2 (●), 5 mM EDTA (●) or 0.2 mM CoCl_2 (▲) as described in the text with 1- μ l aliquots from the indicated fractions.

50% glycerol. Both the Mn^{2+} - and the Co^{2+} -treated enzyme had the same Stokes radius ($25\overset{\text{O}}{\text{\AA}}$) as that of apophosphatase S.

As shown in Table 18, the Co^{2+} -treated enzyme, after being subjected to gel filtration, membrane filtration, and extensive dialysis, remained fully active when the enzymic activity was measured in the absence of divalent cation. By contrast, the Mn^{2+} -treated enzyme had lost most of its divalent cation independence after being subjected to the same separation processes. The isolated Mn^{2+} -treated enzyme showed low activity in the absence of added divalent cation and its activity was stimulated several fold by either Mn^{2+} or Co^{2+} . In contrast to apophosphatase S and the original phosphatase S, the Mn^{2+} -treated enzyme was stimulated by Co^{2+} to about the same extent as by Mn^{2+} . It appeared that the Mn^{2+} -treated enzyme, after being subjected to various separating processes, still contained a tightly bound Mn^{2+} which was not essential for the enzymic activity but could prevent the enzyme from being fully activated by Co^{2+} .

Table 18 also showed that the specific activity of apophosphatase S was higher than that of the original phosphatase S when the enzymic activity was measured in the presence of added Mn^{2+} or Co^{2+} . The data suggested that the ATP treatment might have removed from the enzyme a small

TABLE 18
Specific Activity of Phosphatase S and Its Derivatives

Enzyme	Specific Activity (unit/mg)								
	Phosphorylase <u>a</u>			Phosphocasein			Phosphohistone		
	0	MnCl ₂ (1mM)	CoCl ₂ (0.5mM)	0	MnCl ₂ (5mM)	CoCl ₂ (0.5mM)	0	MnCl ₂ (5mM)	CoCl ₂ (0.5mM)
Phosphatase S	1060	1056	1422	570	929	1141	881	996	1016
Apophosphatase S	44	1466	2833	13	1553	2373	59	808	1124
Co ²⁺ -reactivated Apophosphatase S	3040	2279	2813	3979	2203	2533	2040	1488	1749
Mn ²⁺ -reactivated Apophosphatase S	123	721	710	222	810	808	137	449	406

Phosphatase S, apophosphatase S, the Co²⁺-reactivated and the Mn²⁺-reactivated apophosphatase S were prepared as described in the text. The enzymic activities towards phosphorylase a, phosphocasein and phosphohistone were determined as described in Table 17 in the absence and presence of MnCl₂ or CoCl₂ at concentrations as indicated.

molecular weight component, probably a divalent cation, which was inhibitory to Mn^{2+} and Co^{2+} activation. The activity of the Co^{2+} -treated enzyme was several times higher than that of the original phosphatase S when the enzymic activity was measured in the absence or in the presence of added Mn^{2+} or Co^{2+} . The specific activity of the Co^{2+} -treated enzyme was about the same as that of apophosphatase S measured in the presence of added Co^{2+} , showing that the enzyme was preserved after the processes of separating unbound Co^{2+} from the enzyme. By contrast, the specific activity of the Mn^{2+} -treated enzyme was about half as that of apophosphatase S when the enzymic activity was measured in the presence of added Mn^{2+} , indicating that part of the enzyme was inactivated during the processes of separating the unbound Mn^{2+} from the enzyme.

E) Properties of apo-, Mn^{2+} -reactivated, and Co^{2+} -reactivated phosphatase S

The original phosphatase S as well as the apoenzyme and the metal ion reactivated enzymes exhibited Michaelis-Menten kinetics. When the enzymic activity was measured in the absence of added divalent cation, the K_m values of the original phosphatase S, the isolated Mn^{2+} - and Co^{2+} -treated enzymes for phosphorylase a were estimated as 5, 4, and 8 μM , respectively (Fig. 28). In the presence of 5 mM theophylline, both K_m and V (not shown) of Mn^{2+} - and Co^{2+} -treated enzymes

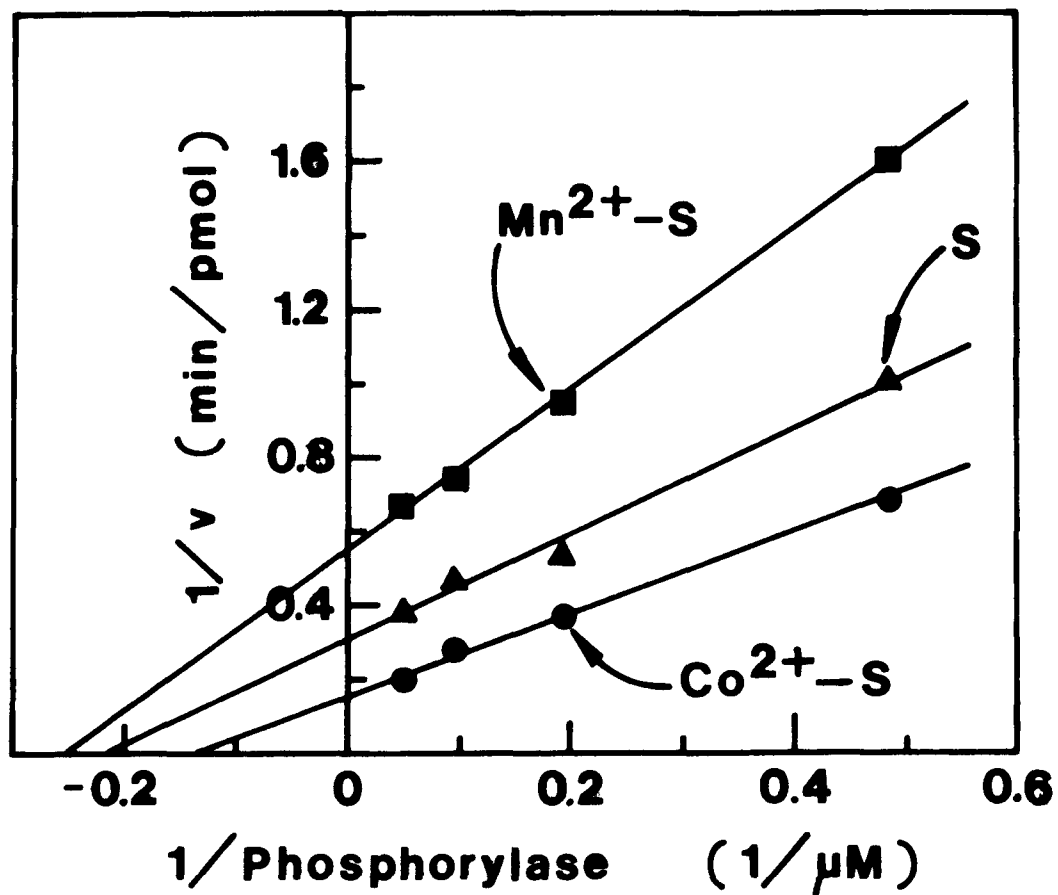


Fig. 28. Effects of phosphorylase a concentration on the activities of phosphatase S and its derivatives. The enzymic activities of phosphatase S (4.4 ng; ▲), Co^{2+} -activated apophosphatase S (2.1 ng; ●) and Mn^{2+} -activated apophosphatase S (15 ng; ■) were determined with various concentrations of phosphorylase a in the absence of added divalent cations as described in the legend to Fig. 19. The data were present in double-reciprocal plot.

for phosphorylase a were increased as it did on phosphatase S (Fig. 19).

Both Co^{2+} - and Mn^{2+} -treated enzymes catalyzed the dephosphorylation of $[\text{}^{32}\text{P}]$ myosin. Co^{2+} -treated apophosphatase S was not stimulated by additional divalent cation, but the Mn^{2+} -treated enzyme was stimulated fourfold by addition of 5 mM MnCl_2 , when myosin was used as a substrate. These are in good agreement when other phosphoproteins were used as substrate (Table 18).

As described previously, after filtration on Sephadex G-25 column, the Mn^{2+} -treated apophosphatase S but not the Co^{2+} -treated enzyme, could be inactivated by preincubating with ATP. When the Mn^{2+} -treated enzyme was further purified by membrane ultrafiltration and extensive dialysis, the isolated enzyme lost most of its divalent cation independence (Table 18), and the residual Mn^{2+} -independent activity became resistant to ATP inactivation in a similar manner as the isolated Co^{2+} -treated enzyme. When the isolated Mn^{2+} - and Co^{2+} -treated enzymes were preincubated separately with 5 mM ATP at 30° for 5 min, the divalent cation independent activity of the Mn^{2+} -treated enzyme was decreased about 10%, while the activity of the Co^{2+} -treated enzyme was not changed (Fig. 29). Including phosphoproteins in the preincubation mixture did not

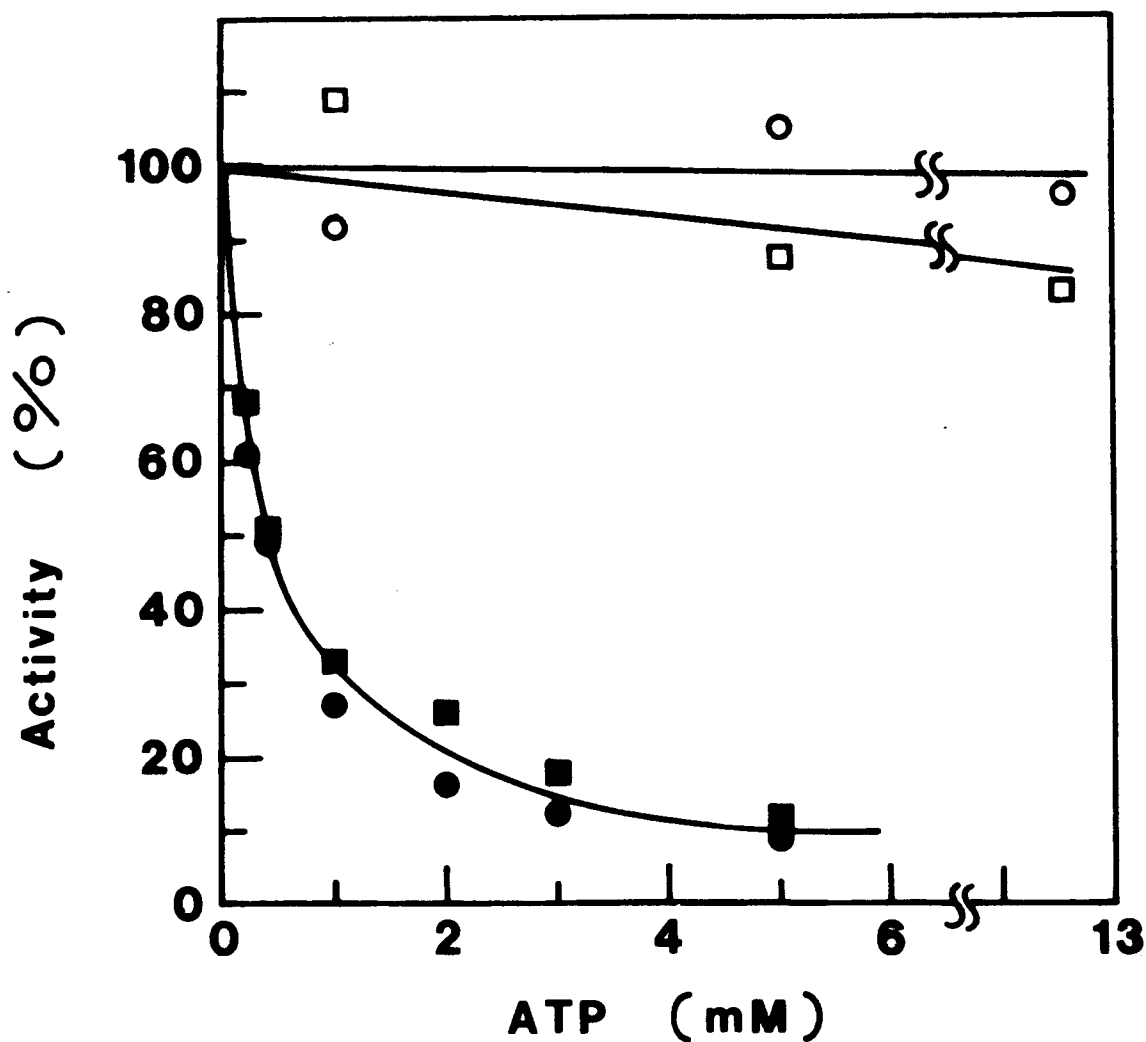


Fig. 29. Effect of ATP concentration on the activity of Mn²⁺-activated and Co²⁺-activated apophosphatase S. The Mn²⁺-activated (0.6 μ g, □) and Co²⁺-activated (0.16 μ g, ○) apophosphatase S was preincubated at 30° with various concentrations of ATP as described in the legend to Fig. 21. After 5 min, 0.5 ml of ice cold dilution buffer was added to each preincubation mixture. 10 μ l of the diluted mixture was immediately analyzed for phosphocasein phosphatase activity in the absence of ATP as described in Table 17. The Mn²⁺-activated (■) and Co²⁺-activated (●) apophosphatase S preincubated in the absence of ATP were analyzed for phosphocasein phosphatase activity in the presence of various concentrations of ATP as indicated.

increase the ability of ATP to inactivate the Co^{2+} -treated enzyme. Including ATP in the assay mixture, however, inhibited both Mn^{2+} -treated and Co^{2+} -treated to similar extent (Fig. 29). The concentration of ATP for half maximum inactivation was about 0.3-0.4 mM.

As shown in Fig. 30, the thermostability of apophosphatase S was much lower than that of either the phosphatase S or the Co^{2+} -treated enzyme. The isolated Mn^{2+} -treated enzyme was also very labile. When 5 mM of Mn^{2+} was included in the incubation mixture, both apophosphatase S and the isolated Mn^{2+} -treated enzyme became as stable as the original phosphatase S to heat denaturation (data not shown). These data indicated that the divalent cations were not only essential for the catalytic activity but also played an important role in stabilizing the enzyme.

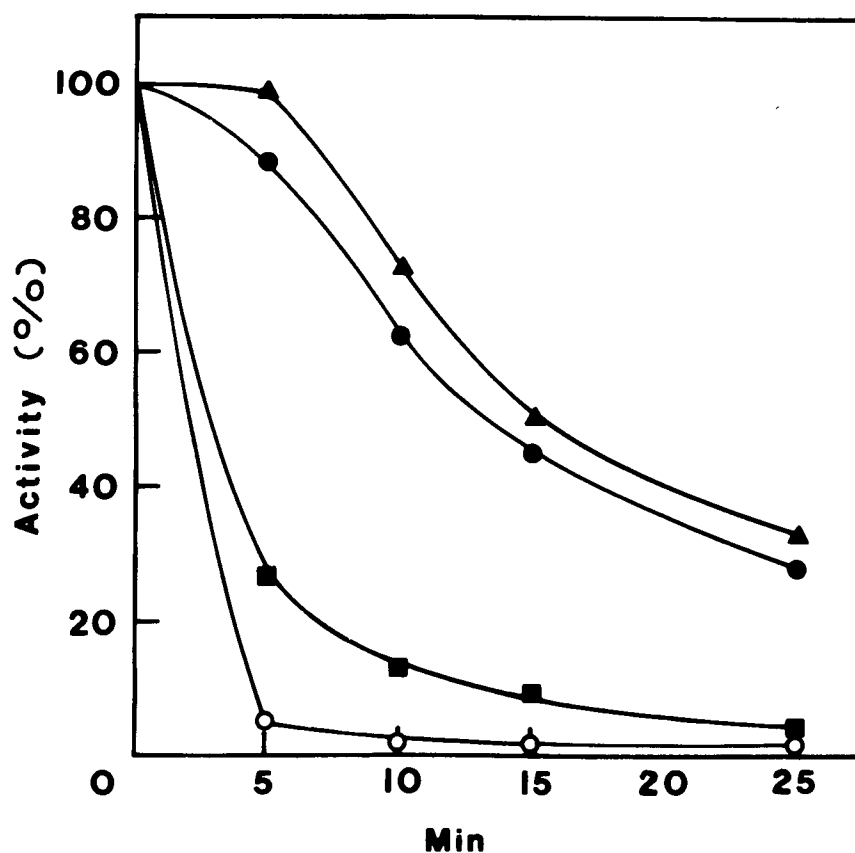


Fig. 30. Thermal stability of phosphatase S and its derivatives. Phosphatase S (0.22 μg , ●), apophosphatase S (0.22 μg , ○), the purified Co^{2+} -activated apophosphatase S (0.11 μg , ▲) and the purified Mn^{2+} -activated apophosphatase S (1.2 μg , ■) were incubated at 45° in a volume of 1 ml containing 20 mM Tris·HCl, pH 7.4, and 2 mg/ml bovine serum albumin. At indicated time intervals, 10 μl were withdrawn from the incubation mixture and analyzed for phosphorylase phosphatase activity. The enzymic activities of apophosphatase S were measured in the presence of 0.2 mM CoCl_2 as described in Table 17. The enzymic activities of other phosphatases were measured in the absence of added divalent cations.

DISCUSSION

I. Isozymes and Dissociation of Phosphoprotein Phosphatases from Canine Heart

The present findings indicate that phosphoprotein phosphatase activity in a canine heart extract arises from four molecular forms of the enzyme that can be separated by DEAE-cellulose chromatography. These are designated as phosphatase A ($M_r=156,000$), B ($M_r=161,000$), C ($M_r=95,600$), and U ($M_r=61,000$). ATP inhibited phosphatase A, stimulated phosphatase B, and showed no significant effect on phosphatase C when phosphohistone was used as the substrate. In contrast to the other three fractions of the enzyme, phosphatase U requires Mn^{2+} for activity. The different responses of these four fractions of the enzyme to ATP and Mn^{2+} indicate that they possess different regulatory properties which could reflect distinct roles for each enzyme in the control of protein dephosphorylation by the cell.

Phosphatases A, B, and C were dissociated by treating with ethanol into a single catalytic subunit (phosphatase S) having a molecular weight of 34,800. This value is in agreement with those reported for ethanol-treated phosphorylase phosphatase isolated from rabbit liver and other

sources (520,595,608,616). Phosphatase U, a Mn^{2+} -activated enzyme is neither dissociated nor activated by treating with ethanol. Chromatography of the ethanol-treated 0-55% $(NH_4)_2SO_4$ fraction of canine heart extract on Sephadex G-100 (Fig. 15) also demonstrated that the multiple forms of the enzyme are reduced to two forms, namely, phosphatases U and S. The data suggest that the multiple forms of the enzyme in canine heart consist of at least two basic molecular forms.

Further studies on phosphatase U reveal that this fraction can be resolved by successive gel filtration chromatography into two enzymes (U-2, $M_r=49,300$ and U-1, $M_r=61,000$) with different substrate specificity and divalent cation requirement (723). Phosphatases A, B, and C are active in the absence of divalent cation, exhibit a broad substrate specificity and can be activated and dissociated by treating with 80% ethanol at room temperature into a common catalytic subunit of $M_r=34,800$. By contrast, both enzyme species derived from phosphatase U are divalent cation-dependent and cannot be activated nor dissociated by the ethanol treatment. These data indicate that the highly purified phosphatase S represents the catalytic subunit of phosphatases A, B, and C. Furthermore, the Mg^{2+} -activated phosphocasein phosphatase (U-2) ($M_r=49,300$)

and the Mn^{2+} -activated phosphohistone phosphatase (U-1) ($M_r=61,000$) prepared from 0-55% $(\text{NH}_4)_2\text{SO}_4$ fraction by ethanol precipitation appear to be identical to the two divalent cation-dependent enzymes with narrow substrate specificity separated from the phosphatase U preparation described by Li and Hsiao previously (723). The following points may be deduced from all these data obtained from the studies of cardiac phosphoprotein phosphatases:

(1) Both phosphatases with high and low substrate specificity exist in cardiac muscle. These enzymes may play different roles in the regulation of protein dephosphorylation in the cell.

(2) The multiple forms of phosphoprotein phosphatases isolated from canine heart may be divided into two classes. Class I isozymes include several molecular forms of divalent cation-independent, nonspecific phosphatases which appear to consist of a common catalytic subunit of $M_r=35,000$ with broad substrate specificity. An important feature of Class I isozymes is that they can be activated with concomitant dissociation into a smaller catalytic entity. These observations suggest that the Class I isozymes may exist in interconvertible forms: the holoenzymes with low activity and the catalytic subunit with high activity. An unknown metabolite(s) may be involved in the regulation

of Class I isozymes by mediating their interconversion. Class II isozymes include two divalent cation-dependent phosphatases with narrow substrate specificity. The catalytic entities of the Class II isozymes are different from those of the Class I isozymes.

(3) The broad substrate specificity property of Class I isozymes and their catalytic subunit, phosphatase S, is similar to that of the multiple molecular forms of phosphorylase a phosphatase, glycogen synthetase phosphatase, other phosphoprotein phosphatases isolated from various sources (563,564,571-576,602,615,622,648,652), and the cyclic AMP-dependent protein kinase phosphatase isolated from bovine heart (520). Thus, Class I isozymes represent a general phosphoprotein phosphatase which can dephosphorylate most of the known physiological phosphoproteins.

(4) The observations that the Class I and Class II phosphatases exhibit different requirements for divalent cation and that the Class II isozymes show different divalent cation specificities suggest that the metal ions may play an important role in the regulation of protein dephosphorylation in the cell.

Other laboratories have reported that the multiple forms of phosphoprotein phosphatases isolated from various

tissues can be dissociated by either ethanol (520,608,613) or 2-mercaptoethanol (572-574,615) treatment to yield a single, lower molecular weight form concomitant with activation of enzymic activity. Whether the Class I isozymes, phosphatases A, B, and C, consisted of an oligomer of phosphatase S or a combination of phosphatase S with other proteins is not known, but Brandt et al. (613) proposed that the multiple forms of phosphoprotein phosphatase consist of an enzyme-inhibitory protein complex which contains a single catalytic subunit of molecular weight 32,000. Purification of the ethanol-activated phosphorylase phosphatase to homogeneous state (608) and isolation of heat-stable inhibitory protein(s) (609) from rabbit liver were subsequently described. Huang and Glinsmann (578) have reported that a partially purified phosphorylase phosphatase from rabbit skeletal muscle contains heat-stable inhibitory proteins. These observations suggest that phosphatases A, B, and C isolated from canine heart may consist of an enzyme-inhibitory protein complex.

Although phosphatases U and S can be clearly separated by gel filtration (Fig. 15), they sediment with a similar velocity in sucrose density gradient centrifugation (Fig. 16).

These observations may be valuable information for the studies of molecular species of phosphoprotein phosphatases in other tissues.

II. Purification of Phosphoprotein Phosphatases from Canine Heart

An important feature of the present purification procedure (Scheme 3) is the activation and purification which results from precipitation with 80% ethanol at room temperature. This was originally described by Brandt et al. (608,613) for purification of rabbit liver phosphorylase phosphatase and was modified by Chou et al. (520) for purification of a phosphoprotein phosphatase of bovine heart. The present purification procedure, however, leads to the isolation of three of phosphoprotein phosphatase species ($M_r=35,000$, $49,300$, and $61,000$), while the methods of Brandt et al. (608) and Chou et al. (520) lead to the isolation of a single species ($M_r=30-32,000$). The present data indicate that at least these basic catalytic entities of phosphoprotein phosphatase which differ from one another in substrate specificity and divalent cation dependency can be isolated from canine cardiac muscle. Similar to those phosphoprotein phosphatases of molecular weights in the range of $30-35,000$ purified from rabbit liver (608,616), bovine heart (520), and rabbit skeletal

muscle (595), the purified phosphatase S exhibits a broad substrate specificity and can dephosphorylate phosphorylase a, phosphorylated histone, casein, cyclic AMP-dependent protein kinase, and myosin light chain. By contrast, the two divalent cation-dependent enzymes exhibit a narrow substrate specificity. Phosphoprotein phosphatase activities which are relatively specific for the inhibitory component of troponin (602), myosin light chain (548), α and β -subunit of phosphorylase kinase (587), glycogen synthetase (621,632), and phosphocasein (626) have been recently reported.

Morgan et al. (222) have isolated a myosin light-chain phosphatase with a molecular weight about 70,000 from rabbit white skeletal muscle. The myosin light-chain phosphatase was inactivated upon dialysis and the activity could be restored by Mg^{2+} , Mn^{2+} , or Co^{2+} . The enzyme was highly specific for myosin P-light chain and was not active toward phosphorylated glycogen synthetase, phosphorylase kinase, histone, casein, troponin, and phosphorylase a. It appears to be that, besides this specific enzyme, the myosin light-chain can also be dephosphorylated by the general phosphatase S isolated from canine heart.

Recently, Chou et al. (520) have reported the purification of a phosphoprotein phosphatase from bovine heart,

with a procedure involving precipitation of the enzyme with 70% ethanol at room temperature. Although the purified bovine heart enzyme shows two protein bands with phosphatase activity after polyacrylamide gel electrophoresis, this enzyme preparation migrated as a single protein band corresponding to a molecular weight of 31,000 after polyacrylamide gel electrophoresis in sodium dodecyl sulfate. These data indicate that the bovine heart phosphoprotein phosphatase preparation contains two active species with the same molecular weight. In the case of the canine heart enzyme, the Peak III enzyme was resolved by DEAE-Sephadex chromatography into a major active fraction (phosphatase S) and a minor active fraction (phosphatase SR) (Fig. 3). Both phosphatase S and SR preparations, however, had the same molecular weight (35,000) as determined by SDS-gel electrophoresis, gel filtration chromatography and sucrose density gradient ultracentrifugation. Polyacrylamide gel electrophoresis of phosphatase S preparation resulted in a single protein band which coincided with the enzymic activity (Fig. 7). Phosphatase SR, however, showed several minor active peaks with higher mobility than that of phosphatase S in gel electrophoresis. The data indicate that, in addition to a major active phosphoprotein phosphatase of $M_r=35,000$, several minor active species with similar

molecular weight exist. A slight modification of the charge group(s) on phosphatase S may account for the presence of these minor phosphatase activities which show similar molecular weights and catalytic properties but different properties in ion exchange chromatography and polyacrylamide gel electrophoresis as those of phosphatase S. Further investigations are required to clarify the interrelationship among these isozymes. Khandelwal et al. (616) have reported the isolation of two phosphoprotein phosphatases from rabbit liver. Both of these two enzymes exhibit a broad substrate specificity. They are, however, slightly different in molecular weights (phosphatase I, $M_r=30,500$ and phosphatase II, 34,000). The molecular weight and the broad substrate specificity property of the canine heart phosphatase S are similar to those of phosphatase II obtained from rabbit liver (616).

It has been reported by Li and Hsiao (569) that nucleoside phosphates and salts stimulate the phosphatase B activity by forming complex with phosphohistone. Li and Hsiao (570) have also demonstrated that $MnCl_2$, besides acting as an activator for the Mn^{2+} -activated phosphohistone phosphatase, can modulate kinetic parameters of the dephosphorylation reaction by interacting with phosphohistone and phosphorylase a. Chou et al. (520) have reported that cyclic

AMP enhances phosphatase activity toward protein kinase by dissociating the protein kinase holoenzyme. The present finding that cyclic AMP stimulates phosphatase S activity toward protein kinase gives further support to the previous proposition (569,570) that modification at substrate level by various metabolites represents an important mechanism for the regulation of phosphoprotein phosphatase activity.

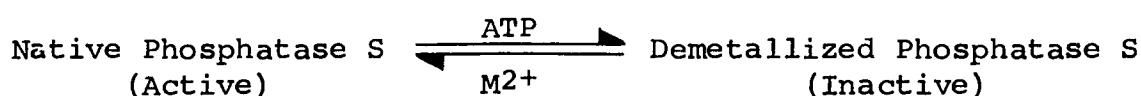
III. The Role of ATP and Divalent Cations in the Regulation of Class I Isozymes of Cardiac Phosphoprotein Phosphatases

Many studies on ATP and divalent cations regulation of phosphoprotein phosphatases in crude extracts, partially purified and purified preparations of different tissues have been reported (Table 8). In spite of all these efforts, the role of ATP and divalent cations on the regulation of phosphoprotein phosphatases remains ambiguous. The reasons are primary because (a) the isozyme species of phosphoprotein phosphatase has not been clearly defined, (b) both nucleotides and divalent cations can modify the enzymic reaction by interacting with either the enzyme itself or with the phosphoprotein substrate. For example, isolation of divalent cation-dependent and -independent phosphoprotein phosphatases from cardiac and skeletal muscle has been reported (570,572). Studies indicate that interactions of ATP or divalent cation with different

phosphoprotein substrates can result in either stimulation or inhibition of the dephosphorylation reaction catalyzed by a given phosphoprotein phosphatase (569,608). Thus, for carrying out a proper study of the mechanism of ATP and divalent cation effects on a phosphoprotein phosphatase reaction, one has to use a well defined, highly purified enzyme preparation and be aware of the various effects of substrate-effector interactions on the enzymic reaction.

Phosphatase S, the enzyme used in the studies on the role of ATP and divalent cations in the regulation of enzymic activity, has been highly purified and its relationship with other isozyme species in the extract of cardiac muscle has been well defined. ATP and divalent cations (Mg^{2+} , Ca^{2+} , Mn^{2+} , and Co^{2+}) can inhibit phosphatase S activity (Table 14), presumably by complexing with phosphorylase a and making it an unfavorable substrate. In order to study the direct interactions of ATP and divalent cations with the enzyme protein, preincubation of these effectors with phosphatase S followed by diluting the preincubation mixture and measuring the enzymic activity has been carried out. The results clearly demonstrate that ATP inactivates phosphatase S by converting it to a divalent cation-dependent form. The ATP-inactivated enzyme can be reactivated by either Mn^{2+} or Co^{2+} but not other divalent

cations (Table 17). These results indicate that phosphatase S is a metalloenzyme which can exist in two interconvertible forms: the metallized form (active) and the demetallized form (inactive). ATP and divalent cations (Mn^{2+} or Co^{2+}) can mediate the interconversions between the active and inactive forms.



Since the effective concentrations of ATP and Mn^{2+} are in the range of one tenth millimolar, it appears that this novel regulatory mechanism of ATP and divalent cations on the phosphoprotein phosphatase may be of physiological importance. ATP inactivates a large molecular form of phosphoprotein phosphatase (B) which contains phosphatase S as a catalytic subunit (Fig. 20). The other larger molecular forms of the Class I isozymes can also be inactivated by ATP and that the ATP inhibitory effect is diminished in the presence of Mn^{2+} . These observations indicate that ATP and divalent cations may regulate the activity of either the large molecular forms or the catalytic subunit of Class I isozymes by the same mechanism as described above.

The activities of phosphoprotein phosphatase in crude extract of cardiac muscle as well as the partially purified

large molecular forms of Class I isozyme are stimulated by Mn^{2+} (568-570). The extent of Mn^{2+} stimulation varied from preparation to preparation. Usually, the Mn^{2+} stimulating activities of the large molecular form isozymes decrease as the purification processes proceed. Aside from the dephosphorylation of phosphoproteins by other Mn^{2+} -activated phosphatases (570,572), the Mn^{2+} -stimulating activity in crude extracts and further purified enzyme fractions may be due to the fact that the Class I isozymes can exist in divalent cation-dependent and -independent forms. The metal-dependent form is much less stable than the independent form (Fig. 30). This may account for the decrease in the ability of Mn^{2+} to stimulate the enzyme activity during purification of the Class I isozymes.

As shown in Table 16, it appears that only those compounds which contain a pyrophosphoryl moiety are capable of inactivating phosphatase S. EDTA and other non-phosphoryl chelating agents cannot inactivate the enzyme. These data suggest that the essential divalent cation(s) may be buried in the active site of the enzyme. ATP and other pyrophosphoryl compounds, due to their structural similarity to the phosphoprotein substrates and their ability to chelate divalent cations, can readily enter the phosphoryl group binding site to remove the essential metal ion(s). EDTA

and other non-phosphoryl chelating agents fail to cause inactivation of phosphatase S because they cannot readily enter the active site to exert their chelating function. These interpretations are consistent with the facts that ATP inactivation effect can be suppressed by Mg^{2+} (Fig. 21B). Although Mg^{2+} cannot reactivate the ATP-inactivated enzyme its suppression of the ATP action may be explained by its complexing with the nucleotide and thus abolishing its ability to chelate the essential metal ion(s) on the enzyme. Inorganic phosphate, which is a product of the enzymic reaction and has been shown to be a reversible inhibitor (Tables 15 and 16), may exert its action by competing with ATP for the phosphoryl binding site on the enzyme.

As shown in Fig. 20, ATP inactivated phosphatase B (a large molecular form) much slower than phosphatase S (its catalytic subunit). It is possible that, when the Class I isozymes exist in the large molecular forms, their catalytic sites are blocked due to aggregation or the binding of inhibitory proteins. Therefore, the entrance of ATP to the phosphoryl binding site(s) is hindered. This may explain why the large molecular forms of Class I isozymes are not only less active than their common catalytic subunit, phosphatase S, but also more stable to ATP inactivation.

The present data have excluded the possibility that the inactivation of phosphatase S is a result of phosphorylation or adenylation of the enzyme by ATP. The present data also indicate that the inactivation processes is not accompanied with hydrolysis of ATP to ADP and Pi. The ATP inactivated enzyme has the same Stokes radius as that of the original phosphatase S, indicating that the formation of the divalent cation-dependent forms of the enzyme is not due to aggregation of phosphatase S to a larger molecular form. Since the isolated apophosphatase S does not contain ATP, the possibility that this enzyme form exists as an enzyme-ATP complex has been excluded. The present data, however, do not exclude the possibility that ATP and its derivatives inactivate the enzyme by interacting with the enzyme other than at the active site. Such interaction may cause a conformational change in the enzyme molecule, and lead to a release of the bound divalent cation(s) essential for the enzymic function.

Evidence indicates that the Mn^{2+} -activated apophosphatase S contains two distinct classes of the divalent cation: a loosely bound and a tightly bound Mn^{2+} . The loosely bound Mn^{2+} activation effect is unstable to various processes for separating the metal ion from the enzyme protein. Furthermore, the activity of the Mn^{2+} -activated

enzyme can be inactivated by EDTA (Table 17, Fig. 27A), ATP, and pyrophosphate. The tightly bound Mn^{2+} contributes little to the catalytic function but is inhibitory to the Co^{2+} activation effect. Thus, after subjecting to various processes for separating Mn^{2+} from the enzyme protein, the resulting Mn^{2+} -activated enzyme has lost most of its divalent cation-independent activity. The enzyme, however, is still insensitive to Co^{2+} activation (Table 18). Furthermore, the residual divalent cation-independent activity of the isolated Mn^{2+} -activated enzyme can not be readily inactivated by preincubating with EDTA, ATP (Fig. 29), or pyrophosphate. As shown in Fig. 25, the Mn^{2+} concentration for complete inhibition of the Co^{2+} activation effect is about equal to the Mn^{2+} concentration for half maximum activation of the apophosphatase S. These data are also consistent with the interpretation that the apoenzyme possesses a high and low affinity site for Mn^{2+} . Binding of Mn^{2+} to the high affinity site resulted in inhibition of Co^{2+} activation effect while the binding of Mn^{2+} to the low affinity site is mainly responsible for the activation of the enzyme. The Mn^{2+} saturation curve for activation of apophosphatase S is sigmoidal (Fig. 25). The Hill coefficient for Mn^{2+} is estimated as 1.3. These

data indicate that cooperative binding of more than one mole of Mn^{2+} per mole of enzyme is accompanied with the Mn^{2+} activation reaction.

In contrast to the Mn^{2+} -activated apophosphatase S, the Co^{2+} -activated enzyme remains in a divalent cation-independent form after subjecting to gel filtration chromatography (Fig. 27B) and extensive dialysis (Table 18). The Co^{2+} -activated enzyme cannot be again converted to a divalent cation-dependent form by preincubating with EDTA or ATP (Fig. 29). These observations indicate that the enzyme binds Co^{2+} very tightly. Although Co^{2+} exhibits higher affinity to the enzyme than Mn^{2+} , it appears that the rate of Mn^{2+} binding to either the high or low affinity site of the enzyme is faster than that of Co^{2+} binding to the enzyme. This statement is supported by the observation that Mn^{2+} activates the apoenzyme almost instantly, while the rate of Co^{2+} activation is relatively slow (Fig. 24). Thus, when the apoenzyme is exposed to a mixture of Mn^{2+} and Co^{2+} , Mn^{2+} binds to the enzyme before Co^{2+} does. As a result, Co^{2+} activation effect is inhibited. Since the activity of the isolated Co^{2+} -activated enzyme is only slightly inhibited by Mn^{2+} (Table 18), it appears that once Co^{2+} is bound, the metal ion cannot be readily displaced

from the enzyme by Mn^{2+} . The Co^{2+} activation of apophosphatase S exhibited sigmoidal kinetics (Fig. 26). The Hill coefficient for Co^{2+} is about 3.5, indicating that cooperative binding of about 4 mole of Co^{2+} per pmole of enzyme is accompanied with the activation reaction. Further studies involving direct measurements of the metal ion binding to the enzyme correlating with the activation of the enzymic activity are required to clarify the mechanism of Mn^{2+} and Co^{2+} activation of apophosphatase S.

Both the Mn^{2+} - and the Co^{2+} -activated enzyme have the same Stokes radius as that of the apoenzyme and the original phosphatase S, indicating that the interconversions between the metallized form and the demetallized form do not involve aggregation or dissociation of the enzyme molecule. The properties of the Mn^{2+} - and Co^{2+} -activated apophosphatase S are different from those of the original phosphatase S. The Mn^{2+} -activated enzyme is less stable than the original phosphatase S to various processes for separating divalent cations from the enzyme protein. On the other hand, the Co^{2+} -activated enzyme is so stable that it cannot be inactivated again by preincubating with ATP. In addition, the Co^{2+} -activated enzyme is much more active than the original phosphatase S. Thus, the present data do not answer the question of what is the essential

divalent cation(s) in phosphatase S. Direct measurement of the metal ion content in phosphatase S prepared in the absence of added divalent cations may answer this question. Based on the observations that (a) Mn^{2+} activation is reversible, (b) the activity of the apoenzyme as well as the isolated Mn^{2+} -activated apoenzyme measured in the presence of Mn^{2+} is similar to that of phosphatase S, and (c) the K_m value of the Mn^{2+} -activated apoenzyme for phosphorylase a is about the same as that of phosphatase S, one may argue that Mn^{2+} is more likely the essential divalent cation(s) in phosphatase S.

Kato et al. (575) have reported that a partially purified skeletal muscle phosphoprotein phosphatase can be inactivated by ATP, GTP, or ITP to a similar extent at the same concentration. The inactivated enzyme can be reactivated by Mg^{2+} in the presence of ATP and by Mn^{2+} or Co^{2+} in the absence of the nucleotide. Among these metal ions, Mn^{2+} is the most effective activator. They proposed that the phosphoprotein phosphatase is a metalloenzyme. Recently, Khatra and Soderling (724) have reported that a partially purified phosphoprotein phosphatase from skeletal muscle can be inactivated by ATP, F^- , and inorganic phosphate. The inactivated enzyme can be reactivated completely by

Mn^{2+} and partially by $\text{Co}^{2+} > \text{Zn}^{2+} > \text{Mg}^{2+}$. In general, these observations and interpretations are in good agreement with the present studies on the highly purified preparation of cardiac muscle phosphatase S. In contrast to their observations, the apophosphatase S can be reactivated only by Co^{2+} and Mn^{2+} but not by other divalent cations examined. Furthermore, Co^{2+} is the more effective activator. Although Mg^{2+} can block ATP inactivation (Fig. 21B). Mg^{2+} , either in the absence or in the presence of ATP cannot reactivate the apoenzyme nor does it affect the Mn^{2+} or Co^{2+} activation effect. The reasons for these discrepancies are not known. Since partially purified enzyme preparations have been used by other laboratories for the studies of ATP and divalent cation actions, it is possible that these preparations contain different isozyme species which respond differently than the cardiac muscle phosphatase S to divalent cations. Recently, Yan and Graves (725) have reported that the catalytic subunit of liver phosphoprotein phosphatase purified according to Brandt *et al.* (608) can be inactivated by incubation with 0.5 mM EDTA and 0.5 mM pyrophosphate. EDTA alone has no effect on the enzyme but pyrophosphate or ATP does. The inactivated enzyme can be reactivated completely by Mn^{2+} or Co^{2+} but not other divalent cation

tested (e.g., Mg^{2+}). Binding of the $^{60}\text{Co}^{2+}$ was proportional to the amount of apoenzyme used. They suggested that the phosphoprotein phosphatase is a metalloenzyme. Their results on liver phosphoprotein phosphatase are in very good agreement with the results described here for the Class I isozymes of cardiac phosphoprotein phosphatase.

REFERENCES

1. Holzer, H. & Duntze, W. (1971) *Ann. Rev. Biochem.* 40, 345-374
2. De Flora, A. (1968) *Ital. J. Biochem.* 17, 363-438
3. Segal, H. L. (1973) *Science* 180, 25-32
4. Shalteil, S., ed. (1975) *Metabolic Interconversions of Enzymes 1975*, Springer-Verlag, New York
5. Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds. (1974) *Metabolic Interconversions of Enzymes 1973*, Springer-Verlag, New York
6. Hujing, F. & Lee, E. Y. C., eds. (1973) *Protein Phosphorylation in Control Mechanism*, Academic Press, New York
7. Rubin, C. S. & Rosen, O. M. (1975) *Ann. Rev. Biochem.* 44, 831-887
8. Nimmo, H. G. & Cohen, P. (1977) *Adv. Cyclic Nucleotide Res.* 8, 145-266
9. Krebs, E. G. (1972) *Curr. Top. Cell. Regul.* 5, 99-133
10. Greengard, P. (1978) *Science* 199, 146-152
11. Adler, S. P., Purich, D. & Stadtman, E. R. (1975) *J. Biol. Chem.* 250, 6264-6272
12. Robison, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP*, Academic Press, New York
13. Kuo, J. F. & Greengard, P. (1969) *Proc. Natl. Acad. Sci. U.S.A.* 64, 1349-1355
14. Rall, T. W., Sutherland, E. W. & Berthet, J. (1957) *J. Biol. Chem.* 224, 463-475
15. Sutherland, E. W. & Rall, T. W. (1957) *J. Amer. Chem. Soc.* 79, 3608
16. Bell, R. M. & Koshland, D. E., Jr. (1971) *Science* 172, 1253-1256
17. Lipmann, F. A. & Levene, P. A. (1932) *J. Biol. Chem.* 98, 109-114
18. Lipmann, F. A. (1933) *Biochem. Z.* 262, 3-8
19. De Verdier, C.-H. (1953) *Acta Chem. Scand.* 7, 196-200
20. Plimmer, R. H. A. & Bayliss, W. M. (1906) *J. Physiol.* 33, 439-461

21. Plimmer, R. H. A. (1941) *Biochem. J.* 35, 461-469
22. Hultquist, D. E., Moyer, R. W. & Boyer, P. D. (1966) *Biochemistry* 5, 322-331
23. Hultquist, D. E. (1968) *Biochim. Biophys. Acta* 153, 329-340
24. Zetterqvist, O. (1967) *Biochim. Biophys. Acta* 141, 533-539
25. Chen, C.-C., Smith, D. L., Bruegger, B. B., Halpern, R. M. & Smith, R. A. (1974) *Biochemistry* 13, 3785-3789
26. Chen, C.-C., Bruegger, B. B., Kern, C. W., Lin, Y. C., Halpern, R. M. & Smith, R. A. (1977) *Biochemistry* 16, 4852-4855
27. Smith, L. S., Kern, C. W., Halpern, R. M. & Smith, R. A. (1976) *Biochem. Biophys. Res. Commun.* 71, 459-465
28. Black, S. & Wright, N. G. (1955) *J. Biol. Chem.* 213, 27-38
29. Lipmann, F. & Tuttle, L. C. (1944) *J. Biol. Chem.* 153, 571-582
30. Perlmann, G. E. (1955) *Adv. Prot. Chem.* 10, 1-30
31. Spronk, A. M., Yoshida, H. & Wood, H. G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 4415-4419
32. Switzer, R. L. (1968) *Biochem. Biophys. Res. Commun.* 32, 320-325
33. Cooper, R. A. & Kornberg, H. L. (1967) *Biochem. J.* 105, 49c-50c
34. Taborsky, G. (1974) *Adv. Prot. Chem.* 28, 1-210
35. Harold, F. M. (1966) *Bacteriol. Rev.* 30, 772-794
36. Li, H.-C. & Brown, G. G. (1973) *Biochem. Biophys. Res. Commun.* 53, 875-881
37. Levinson, S. L., Jacobs, L. H., Krulwich, T. A. & Li, H.-C. (1975) *J. Gen. Microbiol.* 88, 65-74
38. Kuo, J. F. & Greengard, P. (1969) *J. Biol. Chem.* 244, 3417-3419
39. Khandelwal, R. L., Spearman, T. N. & Hamilton, I. R. (1973) *FEBS Lett.* 31, 246-250
40. Mercier, J.-C., Grosclaude, F. & Ribadeau-Dumas, B. (1971) *Eur. J. Biochem.* 23, 41-51
41. Ribadeau-Dumas, B., Brignon, G., Grosclaude, F. & Mercier, J.-C. (1972) *Eur. J. Biochem.* 25, 505-514

42. Mercier, J.-C., Uro, J., Ribadeau-Dumas, B. & Grosclaude, F. (1972) *Eur. J. Biochem.* 27, 535-547
43. Fiat, A.-M., Alais, C. & Jolles, P. (1972) *Eur. J. Biochem.* 27, 408-412
44. Milstein, C. (1968) *Biochem. J.* 110, 127-134
45. Shainkin, R. & Perlmann, G. E. (1971) *J. Biol. Chem.* 246, 2278-2284
46. Clark, R. C. (1977) *Int. J. Biochem.* 8, 121-126
47. Cook, W. H. (1961) *Nature(London)* 190, 1173-1175
48. Francois, C. J., Glimcher, M. J. & Krane, S. M. (1967) *Nature(London)* 214, 621-622
49. Veis, A. & Perry, A. (1967) *Biochemistry* 6, 2409-2416
50. Lee, S. L., Veis, A. & Glonek, T. (1977) *Biochemistry* 16, 2971-2979
51. Papas, A., Seyer, J. M. & Glimcher, M. J. (1977) *FEBS Lett.* 79, 276-280
52. Seyer, J. M. & Glimcher, M. J. (1977) *Biochim. Biophys. Acta* 493, 441-451
53. Spector, A. R. & Glimcher, M. J. (1973) *Biochim. Biophys. Acta* 303, 360-362
54. Rhodes, M. B., Bennett, N. & Feeney, R. E. (1959) *J. Biol. Chem.* 234, 2054-2060
55. Kawasaki, H., Sato, H. & Suzuki, M. (1971) *Biochem. J.* 125, 495-505
56. Furneaux, P. J. S. (1970) *Biochim. Biophys. Acta* 215, 52-56
57. Glimcher, M. J., Francois, C. J., Richards, L. & Krane, S. M. (1964) *Biochim. Biophys. Acta* 93, 585-602
58. Krane, S. M., Stone, M. J., Francois, C. J., Glimcher, M. J. (1965) *Biochim. Biophys. Acta* 111, 562-564
59. Mellander, O. (1939) *Biochem. Z.* 300, 240
60. Waugh, D. F. & Von Hippel, P. H. (1956) *J. Amer. Chem. Soc.* 78, 4576-4582
61. Mecham, D. K. & Olcott, H. S. (1949) *J. Amer. Chem. Soc.* 71, 3670-3679
62. Alderton, G. & Fevold, H. L. (1945) *Arch. Biochem.* 8, 415-419

63. Osborne, T. B. & Campbell, G. F. (1900) J. Amer. Chem. Soc. 22, 422-450
64. Flavin, M. (1954) J. Biol. Chem. 210, 771-784
65. Rabinowitz, M. & Lipmann, F. (1960) J. Biol. Chem. 235, 1043-1050
66. Rosenstein, R. W. & Taborsky, G. (1970) Biochemistry 9, 649-657
67. Carmichael, D. J., Veis, A. & Wang, E. T. (1971) Calc. Tiss. Res. 7, 331-344
68. Seyer, J. M. & Glimcher, M. J. (1971) Biocnim. Biophys. Acta 236, 279-291
69. Rahmsdorf, H.-J., Pai, S.-H., Ponta, H., Herrlich, P., Roskoski, R., Jr., Schweiger, M. & Studier, F. W. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 586-589
70. Zillig, W., Fujiki, H., Blum, W., Janekovic, D., Schweiger, M., Rahmsdorf, H.-J., Ponta, H. & Hirsch-Kauffmann, M. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2506-2510
71. Pai, S.-H., Ponta, H., Rahmsdorf, H.-J., Hirsch-Kauffmann, M., Herrlich, P. & Schweiger, M. (1975) Eur. J. Biochem. 55, 299-304
72. Pai, S.-H., Rahmsdorf, H.-J., Ponta, H., Hirsch-Kauffmann, M., Herrlich, P. & Schweiger, M. (1975) Eur. J. Biochem. 55, 305-314
73. Rahmsdorf, H.-J., Herrlich, P., Pai, S.-H., Schweiger, M. & Wittmann, H. G. (1973) Mol. Gen. Genetics 127, 259-271
74. Ewald, R. & Gualerzi, C. (1977) Biochem. Biophys. Res. Commun. 77, 1517-1524
75. Martelo, O. J., Woo, S. L. C., Reimann, E. M. & Davie, E. W. (1970) Biochemistry 9, 4807-4813
76. Thang, M. N. & Meyer, F. (1971) FEBS Lett. 13, 345-348
77. Traugh, J. A. & Traut, R. R. (1972) Biochemistry 11, 2503-2509
78. Lai, M. M. C. (1976) Virology 74, 287-301
79. Erikson, E., Brugge, J. S. & Erikson, R. L. (1977) Virology 80, 177-185
80. Hatanaka, M., Twiddy, E. & Gilden, R. V. (1972) Virology 47, 536-538
81. Hizi, A. & Joklik, W. K. (1977) Virology 78, 571-575

82. Tsiapalis, C. M. (1977) J. Virol. 21, 843-848
83. Tsiapalis, C. M. (1977) Nature(London) 266, 27-31
84. Strand, M. & August, J. T. (1971) Nature New Biol. 233, 137-140
85. Silberstein, H. & August, J. T. (1973) J. Virol. 12, 511-522
86. Lee, S. G., Miceli, M. V., Jungmann, R. A. & Hung, P. P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2945-2949
87. Pal, B. K. & Roy-Burman, P. (1975) J. Virol. 15, 540-549
88. Pal, B. K., McAllister, R. M., Gardner, M. B. & Roy-Burman, P. (1975) J. Virol. 16, 123-131
89. Sokol, F., Clark, H. F. & Wiktor, T. J. & McFalls, M. (1974) J. Gen. Virol. 24, 433-445
90. Sokol, F. & Clark, H. F. (1973) Virology 52, 246-263
91. Imblum, R. L. & Wagner, R. R. (1974) J. Virol. 13, 113-124
92. Moyer, S. A. & Summers, D. F. (1974) J. Virol. 13, 455-465
93. Roux, L. & Kolakofsky, D. (1974) J. Virol. 13, 545-547
94. Lamb, R. A. (1975) J. Gen. Virol. 26, 249-263
95. Krystal, G., Winn, P., Millward, S. & Sakuma, S. (1975) Virology 64, 505-512
96. Tan, K. B. & Sokol, F. (1974) J. Virol. 13, 1245-1253
97. Waite, M. R. F., Lubin, M., Jones, K. J. & Rose, H. R. (1974) J. Virol. 13, 244-246
98. Paoletti, E. & Moss, B. (1972) J. Virol. 10, 417-424
99. Rosemond, H. & Moss, B. (1973) J. Virol. 11, 961-970
100. Kleiman, J. H. & Moss, B. (1975) J. Biol. Chem. 250, 2420-2429
101. Kleiman, J. H. & Moss, B. (1975) J. Biol. Chem. 250, 2430-2437
102. Gravell, M. & Cromeans, T. L. (1972) Virology 48, 847-851
103. Silberstein, H. & August, J. T. (1976) J. Biol. Chem. 251, 3176-3184
104. Silberstein, H. & August, J. T. (1976) J. Biol. Chem. 251, 3185-3190
105. Tan, K. B. & Sokol, F. (1972) J. Virol. 10, 985-994
106. Tan, K. B. & Sokol, F. (1973) J. Virol. 12, 696-703
107. Tao, M. & Doerfler, W. (1972) Eur. J. Biochem. 27, 448-452

108. Jeng, Y.-H., Wold, W. S. M., Sugawara, K., Gilead, Z. & Green, M. (1977) *J. Virol.* 22, 402-411
109. Linné, T., Jörnvall, H. & Philipson, L. (1977) *Eur. J. Biochem.* 76, 481-490
110. Russell, W. C., Skehel, J. J., Machado, R. & Pereira, H. G. (1972) *Virology* 50, 931-934
111. Randall, C. C., Rogers, H. W., Downer, D. N. & Gentry, G. A. (1972) *J. Virol.* 9, 216-222
112. Rubenstein, A. S., Gravel, M. & Darlington, R. (1972) *Virology* 50, 287-290
113. Tan, K. B. (1975) *Virology* 64, 566-570
114. Sen, A., Larson, D. & Todara, G. J. (1977) *Biochem. Biophys. Res. Commun.* 79, 518-524
115. Greengard, P. & Kebabian, J. W. (1974) *Fed. Proc.* 33, 1059-1067
116. Kalix, P., McAfee, D. A., Schorderet, M. & Greengard, P. (1974) *J. Pharmacol. Exp. Therp.* 188, 676-687
117. Greengard, P., Kebabian, J. W. & McAfee, D. A. (1973) *5th Int. Congr. Pharm.* 5, 207-217
118. Williams, M. & Rodnight, R. (1974) *Brain Res.* 77, 502-506
119. Dunkley, P. R., Holmes, H. & Rodnight, R. (1976) *Biochem. J.* 157, 661-666
120. Dunkley, P. R., Holmes, H. & Rodnight, R. (1977) *Biochem. J.* 163, 369-378
121. Ueda, T., Maeno, H. & Greengard, P. (1973) *J. Biol. Chem.* 248, 8295-8305
122. Ueda, T. & Greengard, P. (1977) *J. Biol. Chem.* 252, 5155-5163
123. Uno, I., Ueda, T. & Greengard, P. (1977) *J. Biol. Chem.* 252, 5164-5174
124. Krueger, B. K., Forn, J. & Greengard, P. (1977) *J. Biol. Chem.* 252, 2764-2773
125. Delorenzo, R. J. (1976) *Biochem. Biophys. Res. Commun.* 71, 590-597
126. Carnegei, P. R., Kemp, B. E., Dunkley, P. R. & Murray, A. W. (1973) *Biochem. J.* 135, 569-572

127. Steck, A. J. & Appel, S. H. (1974) J. Biol. Chem. 249, 5416-5420
128. Miyamoto, E. & Kakiuchi, S. (1974) J. Biol. Chem. 249, 2769-2777
129. Miyamoto, E. (1975) J. Neurochem. 24, 503-512
130. Miyamoto, E. & Kakiuchi, S. (1975) Biochim. Biophys. Acta 384, 458-465
131. Dunkley, P. R. & Carnegie, P. R. (1974) Biochem. J. 141, 243-255
132. Carnegie, P. R., Dunkley, P. R., Kemp, B. E. & Murray, A. W. (1974) Nature(London) 249, 147-149
133. Wald, G. (1968) Science 162, 230-239
134. Bownds, D., Dawes, J., Miller, J. & Stahlman, M. (1972) Nature New Biol. 237, 125-127
135. Kühn, H. & Dreyer, W. J. (1972) FEBS Lett. 20, 1-6
136. Frank, R. N., Cavanagh, H. D. & Kenyon, K. R. (1973) J. Biol. Chem. 248, 596-609
137. Kühn, H., Cook, J. H. & Dreyer, W. J. (1973) Biochemistry 12, 2495-2502
138. Weller, M., Virmaux, N. & Mandel, P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 381-385
139. Kuhn, H. (1974) Nature(London) 250, 588-590
140. Steck, T. L. (1974) J. Cell Biol. 62, 1-19
141. Guthrow, C. E., Jr., Allen, J. E. & Rasmussen, H. (1972) J. Biol. Chem. 247, 8145-8153
142. Roses, A. D. & Appel, S. H. (1973) J. Biol. Chem. 248, 1408-1411
143. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617
144. Sheetz, M. P. & Singer, S. J. (1977) J. Cell Biol. 73, 638-646
145. Birchmeier, W. & Singer, S. J. (1977) J. Cell Biol. 73, 647-659
146. Shapiro, D. L. & Marchesi, V. T. (1977) J. Biol. Chem. 252, 508-517
147. Avruch, J. & Fairbanks, G. (1974) Biochemistry 13, 5507-5514

148. Rubin, C. S. & Rosen, O. M. (1973) Biochem. Biophys. Res. Commun. 50, 421-429
149. Rudolph, S. A. & Greengard, P. (1974) J. Biol. Chem. 249, 5684-5687
150. Kant, J. A. & Steck, T. L. (1973) J. Biol. Chem. 248, 8457-8464
151. Fairbanks, G. & Avruch, J. (1974) Biochemistry 13, 5514-5521
152. Hosey, M. M. & Tao, M. (1977) J. Biol. Chem. 252, 102-109
153. Rubin, C. S. (1975) J. Biol. Chem. 250, 9044-9052
154. Chang, K.-J. & Cuatrecasas, P. (1974) J. Biol. Chem. 249, 3170-3180
155. Constantopoulos, A. & Najjar, V. A. (1973) Biochem. Biophys. Res. Commun. 53, 794-799
156. Layne, P., Constantopoulos, A., Judge, J. F. X., Rauner, R. & Najjar, V. A. (1973) Biochem. Biophys. Res. Commun. 53, 800-805
157. Najjar, V. A. & Constantopoulos, A. (1973) Mol. Cell. Biochem. 2, 87-93
158. Shlatz, L. & Marinetti, G. V. (1971) Biochem. Biophys. Res. Commun. 45, 51-56
159. Layne, P. P. & Najjar, V. A. (1977) Arch. Biochem. Biophys. 180, 328-333
160. Leibach, F. & Pillion, D. (1975) FEBS Lett. 50, 121-124
161. Brinkly, F., King, N., Milikin, E., Wright, R. K., O'Neal, C. H. & Wundram, I. J. (1968) Science 162, 1009-1011
162. Casnellie, J. E. & Greengard, P. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1891-1895
163. DeLorenzo, R. J., Walton, K. G., Curran, P. F. & Greengard, P. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 880-884
164. Liu, A. Y.-C. & Greengard, P. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3869-3873
165. Liu, A. Y.-C. & Greengard, P. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 568-572
166. Malkinson, A. M., Krueger, B. K., Rudolph, S. A., Casnellie, J. E., Haley, B. E. & Greengard, P. (1975) Metabolism 24, 331-341

167. Lemay, A., Deschenes, M., Lemaire, S., Proirier, G., Poulin, L. & Labrie, F. (1974) *J. Biol. Chem.* 249, 323-328
168. Labrie, F., Lemaire, S., Poirier, G., Pelletier, G. & Boucher, R. (1971) *J. Biol. Chem.* 246, 7311-7317
169. Davis, B. & Lazarus, N. R. (1974) *Biochem. Soc. Trans.* 2, 409-411
170. Lambert, M., Camus, J. & Christophe, J. (1973) *Biochem. Biophys. Res. Commun.* 52, 935-942
171. Tsien, R. W. (1977) *Adv. Cyclic Nucleotide Res.* 8, 363-420
172. Levene, P. A. & Hill, D. W. (1933) *J. Biol. Chem.* 101, 711-718
173. Kirchberger, M. A., Tada, M. & Katz, A. M. (1974) *J. Biol. Chem.* 249, 6166-6173
174. Tada, M., Kirchberger, M. A., Repke, D. I. & Katz, A. M. (1974) *J. Biol. Chem.* 249, 6714-6180
175. Tada, M., Kirchberger, M. A. & Katz, A. M. (1975) *J. Biol. Chem.* 250, 2640-2647
176. Kirchberger, M. A. & Tada, M. (1976) *J. Biol. Chem.* 251, 725-729
177. Tada, M., Kirchberger, M. A. & Li, H.-C. (1975) *J. Cyclic Nucleotide Res.* 1, 329-338
178. Kirchberger, M. A. & Raffo, A. (1977) *J. Cyclic Nucleotide Res.* 3, 45-53
179. Wray, H. L., Gray, R. R. & Olsson, R. A. (1973) *J. Biol. Chem.* 248, 1496-1498
180. Schwartz, A., Entman, M. L., Kaniike, K., Lane, L. K., Van Winkle, W. B. & Bornet, E. P. (1976) *Biochim. Biophys. Acta* 426, 57-72
181. Sulakhe, P. V. & Drummond, G. I. (1974) *Arch. Biochem. Biophys.* 161, 448-455
182. Hui, C.-W., Drummond, M. & Drummond, G. I. (1976) *Arch. Biochem. Biophys.* 173, 415-427
183. Sulakhe, P. V., Leung, N. L.-K. & St. Louis, P. J. (1976) *Can. J. Biochem.* 54, 438-445
184. Andrew, C. G., Roses, A. D., Almon, R. R. & Appel, S. H. (1973) *Science* 182, 927-929
185. Trevor, A. J., Rodnight, R. & Schwartz, A. (1965) *Biochem. J.* 95, 883-888

186. Weller, M. & Rodnight, R. (1971) *Biochem. J.* 124, 393-406
187. Jones, D. A. & Rodnight, R. (1971) *Biochem. J.* 121, 597-600
188. Huang, C.-J., Reisman, L., Ripoche, P. A. & Schwartz, I. L. (1978) *Fed. Proc.* 37, 343
189. Carnegie, P. R., Dunkley, A. W., Kemp, B. E. & Murray, A. W. (1974) *Nature(London)* 249, 147-150
190. Bretscher, M. S. (1971) *Nature New Biol.* 231, 229-232
191. Yu. J. & Steck, T. L. (1974) *Fed. Proc.* 33, 1532
192. Guthrow, C. E., Rasmussen, H., Brunswick, D. J. & Coopermen, B. S. (1973) *Proc. Natl. Acad. Sci. U.S.A.* 70, 3344-3346
193. Weiss, I. W., Morgan, K. & Phang, J. M. (1972) *J. Biol. Chem.* 247, 760-764
194. Schwartz, I. L., Schlatz, L. J., Kinne-Saffron, E. & Kinne, R. (1974) *Proc. Natl. Acad. Sci. U.S.A.* 71, 2595-2599
195. Rasmussen, H. (1970) *Science* 170, 404-412
196. Montague, W. & Howell, S. L. (1975) *Adv. Cyclic Nucleotide Res.* 6, 201-243
197. Prince, W. T., Berridge, M. J. & Rasmussen, H. (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69, 553-557
198. Dumont, J. E., Willems, C., Van Sande, J. & Neve, P. (1971) *Ann. N. Y. Acad. Sci.* 185, 291-316
199. Mühlrad, A., Bálint, M. & Biró, N. A. (1964) *Acta Physiol. Acad. Sci. Hung.* 25, 339-350
200. Perrie, W. T., Smillie, L. B. & Perry, S. V. (1972) *Cold Spring Harbor Symp. Quant. Biol.* 37, 17-18
201. Perrie, W. T., Smillie, L. B. & Perry, S. V. (1973) *Biochem. J.* 135, 151-164
202. Frearson, N. & Perry, S. V. (1975) *Biochem. J.* 151, 99-107
203. Frearson, N., Focant, B. W. W. & Perry, S. V. (1976) *FEBS Lett.* 63, 27-32
204. Frearson, N., Solaro, R. J. & Perry, S. V. (1976) *Nature (London)* 264, 801-802
205. Gorecka, A., Aksoy, M. O. & Hartshorne, D. J. (1976) *Biochem. Biophys. Res. Commun.* 71, 325-331
206. Sobieszek, A. (1977) *Eur. J. Biochem.* 73, 477-483

207. Small, J. V. & Sobieszek, A. (1977) Eur. J. Biochem. 76, 521-530
208. Adelstein, R. S. (1978) Trends Biochem. Sci. 3, 27-30
209. Adelstein, R. S., Conti, M. A. & Anderson, W., Jr. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3115-3119
210. Adelstein, R. S. & Conti, M. A. (1975) Nature(London) 256, 597-599
211. Scordilis, S. P., Anderson, J. L., Pollack, R. & Adelstein, R. S. (1977) J. Cell Biol. 74, 940-949
212. Scordilis, S. P. & Adelstein, R. S. (1977) Nature(London) 268, 558-560
213. Chacko, S., Conti, M. A. & Adelstein, R. S. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 129-133
214. Haslam, R. J. & Lynham, J. A. (1977) Biochem. Biophys. Res. Commun. 77, 714-722
215. Stull, J. T. & High, C. W. (1977) Biochem. Biophys. Res. Commun. 77, 1078-1083
216. McPherson, J., Fenner, C., Smith, A., Mason, D. T. & Wikman-Coffelt, J. (1974) FEBS Lett. 47, 149-154
217. Muhlrاد, A. & Oplatka, A. (1977) FEBS Lett. 77, 37-40
218. Pollard, T. D. & Korn, E. D. (1973) J. Biol. Chem. 248, 4691-4697
219. Maruta, H. & Korn, E. D. (1977) J. Biol. Chem. 252, 8329-8332
220. Pires, E., Perry, S. V. & Thomas, M. A. W. (1974) FEBS Lett. 41, 292-296
221. Daniel, J. L. & Adelstein, R. S. (1976) Biochemistry 15, 2370-2377
222. Morgan, M., Perry, S. V. & Ottaway, J. (1976) Biochem. J. 157, 687-697
223. Bárány, K. & Bárány, M. (1977) J. Biol. Chem. 252, 4752-4754
224. Bailey, C. & Villar-Palasi, C. (1971) Fed. Proc. 30, 1147
225. Stull, J. T., Brostrom, C. O. & Krebs, E. G. (1972) J. Biol. Chem. 247, 5272-5274
226. England, P. J., Stull, J. T. & Krebs, E. G. (1972) J. Biol. Chem. 247, 5275-5277

227. Huang, T. S., Bylund, D. B., Stull, J. T. & Krebs, E. G. (1974) FEBS Lett. 42, 249-252
228. England, P. J., Stull, J. T., Huang, T. S. & Krebs, E. G. (1973) Metabolic Interconversions of Enzymes 1973 (Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds.), pp. 175-184, Spring-Verlag, New York
229. Moir, A. J. G., Wilkinson, J. M. & Perry, S. V. (1974) FEBS Lett. 42, 253-256
230. Cole, H. A. & Perry, S. V. (1975) Biochem. J. 149, 525-533
231. Moir, A. J. G. & Perry, S. V. (1977) Biochem. J. 167, 333-343
232. England, P. J. (1975) FEBS Lett. 50, 57-60
233. Ray, K. P. & England, P. J. (1976) FEBS Lett. 70, 11-16
234. England, P. J. (1976) Biochem. J. 160, 295-304
235. Stull, J. T. & Buss, J. E. (1977) J. Biol. Chem. 252, 851-857
236. Blumenthal, D. K., Stull, J. T. & Gill, G. N. (1978) J. Biol. Chem. 253, 334-336
237. Lincoln, T. M. & Corbin, J. D. (1978) J. Biol. Chem. 253, 337-339
238. Perry, S. V. & Cole, H. A. (1974) Biochem. J. 141, 733-743
239. Ribolow, H., Bárány, K., Steinschneider, A. & Bárány, M. (1977) Arch. Biochem. Biophys. 179, 81-85
240. Perry, S. V. & Cole, H. A. (1973) Biochem. J. 131, 425-428
241. Moir, A. J. G., Cole, H. A. & Perry, S. V. (1977) Biochem. J. 161, 371-382
242. Pratje, E. & Heilmeyer, L. M. G., Jr. (1972) FEBS Lett. 27, 89-93
243. Ribolow, H. & Bárány, M. (1977) Arch. Biochem. Biophys. 179, 718-720
244. Bárány, M., Bárány, K., Gaetjens, E. & Horvath, B. Z. (1974) in Exploratory Concepts in Muscular Dystrophy II (Mihorac, A. T., ed.), 451-462, Excerpta Medica, Amsterdam
245. Weeds, A. G. & Lowey, S. (1971) J. Mol. Biol. 61, 701-725
246. Greaser, M. L. & Gergely, J. (1971) J. Biol. Chem. 246, 4226-4233

247. Wang, K., Ash, J. F. & Singer, S. J. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4483-4486
248. Wang, K. & Singer, S. J. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2021-2025
249. Wang, K. (1977) Biochemistry 16, 1857-1865
250. Shizuta, Y., Shizuta, H., Gallo, M., Davies, P., Pastan, I. & Lewis, M. S. (1976) J. Biol. Chem. 251, 6562-6567
251. Davies, P., Shizuta, Y., Olden, K., Gallo, M. & Pastan, I. (1977) Biochem. Biophys. Res. Commun. 74, 300-307
252. Wallach, D., Davies, P. J. A. & Pastan, I. (1978) J. Biol. Chem. 253, 3328-3335
253. Goodman, D. B. P., Rasmussen, H., DiBella, F. & Guthrow, C. E., Jr. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 652-659
254. Soifer, D. (1975) J. Neurochem. 24, 21-33
255. Soifer, D., Laszlo, A., Mack, K., Scotto, J. & Siconolfi, L. (1975) Ann. N. Y. Acad. Sci. 253, 598-610
256. Eipper, B. A. (1974) J. Biol. Chem. 249, 1398-1406
257. Eipper, B. A. (1974) J. Biol. Chem. 249, 1407-1416
258. Lagnado, J., Tan, L. P. & Reddington, M. (1975) Ann. N. Y. Acad. Sci. 253, 577-597
259. Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L. & Greengard, P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 177-181
260. Nunez, J., Rappaport, L. & Leterrier, J. F. (1974) NATO Adv. Study Inst. Ser. A-9, 407-434
261. Rappaport, L., Leterrier, J. F. & Nunez, J. (1975) Ann. N. Y. Acad. Sci. 253, 611-629
262. Sheterline, P. (1976) Biochem. Soc. Trans. 4, 789-791
263. Piras, R. & Piras, M. M. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 1161-1165
264. Piras, M. M. & Piras, R. (1974) Eur. J. Biochem. 47, 443-452
265. Sandoval, I. V. & Cuatrecascas, P. (1976) Nature(London) 262, 511-514
266. Langan, T. A. (1971) Ann. N. Y. Acad. Sci. 185, 166-180

267. Langan, T. A. (1976) Fed. Proc. 35, 1623
268. Dixon, G. H., Candido, E. P. M., Honda, B. M., Louie, A. J., MacLeod, A. R. & Sung, M. T. (1974) in The Structure and Function of Chromatin, Ciba Foundation Symp. 28, 229-258, Elsevier, Amsterdam
269. Shlyapnikov, S. V., Arutyunyan, A. A., Kurochkin, S. N., Memelova, L. V., Nesterova, M. V., Sashchenko, L. P. & Severin, E. S. (1975) FEBS Lett. 53, 316-319
270. Louie, A. J., Candido, E. P. M. & Dixon, G. H. (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 803-819
271. Farago, A., Romhanyi, T., Antoni, F., Takats, A. & Fabian, F. (1975) Nature(London) 254, 88
272. Hashimoto, E., Takeda, M. & Nishizuka, Y. (1975) Biochem. Biophys. Res. Commun. 66, 547-555
273. Ruiz-Carrillo, A., Wamgh, L. J. & Allfrey, V. G. (1975) Science 190, 117-128
274. Gurley, L. R., Walters, R. A. & Tobey, R. A. (1975) J. Biol. Chem. 250, 3936-3944
275. Sung, M. T. (1977) Biochemistry 16, 286-290
276. Ingle, C. J. & Dixon, G. H. (1967) Proc. Natl. Acad. Sci. U.S.A. 58, 1011-1018
277. Sung, M. T. & Dixon, G. H. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 1616-1623
278. Sung, M. T., Harford, J., Bundman, M. & Vidalakas, G. (1977) Biochemistry 16, 279-285
279. Kleinsmith, L. J., Allfrey, V. G. & Mirsky, A. E. (1966) Proc. Natl. Acad. Sci. U.S.A. 55, 1182-1189
280. Ord, M. G. & Stocken, L. A. (1966) Biochem. J. 98, 888-897
281. Garrard, W. T., Kidd, G. H. & Bonner, J. (1976) Biochem. Biophys. Res. Commun. 70, 1219-1224
282. Langan, T. A. (1973) in Protein Phosphorylation in Control Mechanism (Huijing, F. & Lee, E. Y. C., eds.), 287-292, Academic Press, New York
283. Langan, T. A. (1973) Adv. Cyclic Nucleotide Res. 3, 99-153
284. Delange, R. J. & Smith, E. L. (1971) Ann. Rev. Biochem. 40, 279-314

285. Allfrey, V. G., Faulkner, R. & Mirsky, A. E. (1964) Proc. Natl. Acad. Sci. U.S.A. 51, 786-794
286. Paik, W. K. & Kim, S. (1971) Science 174, 114-119
287. Marzluff, W. F., Jr. & McCarty, K. S. (1970) J. Biol. Chem. 245, 5635-5642
288. Ord, M. G. & Stocken, L. A. (1974) in The Structure and Function of Chromatin, Ciba Foundation Symp. 28, 259-267, Elsevier, Amsterdam
289. Otake, H., Miwa, M., Fujimura, S. & Sugimura, T. (1969) J. Biochem. (Tokyo) 65, 145-146
290. Johnson, E. M. (1977) Adv. Cyclic Nucleotide Res. 8, 267-309
291. Allfrey, V. G., Inoue, A., Karn, J., Johnson, E. M. & Vidali, G. (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 785-801
292. Stein, G. S., Spelsberg, T. C. & Kleinsmith, L. J. (1974) Science 183, 817-824
293. Teng, C. S., Teng, C. T. & Allfrey, V. G. (1971) J. Biol. Chem. 246, 3597-3609
294. Kostraba, N. C., Montagna, R. A. & Wang, T. Y. (1975) J. Biol. Chem. 250, 1548-1555
295. Allfrey, V. G., Johnson, E. M., Karn, J. & Vidali, G. (1973) in Protein Phosphorylation in Control Mechanism (Huijing, F. & Lee, E. Y. C., eds.) 217-249, Academic Press, New York
296. Olson, M. O. J., Orrick, L. R., Jones, C. & Busch, H. (1974) J. Biol. Chem. 249, 2823-2827
297. Kang, Y. J., Olson, M. O. J. & Busch, H. (1974) J. Biol. Chem. 249, 5580-5585
298. Grummt, I. (1974) FEBS Lett. 39, 125-127
299. Kawashima, K. & Izawa, M. (1977) Biochem. Biophys. Res. Commun. 74, 265-272
300. Paul, J. & Gilmour, R. S. (1974) in The Structure and Function of Chromatin, Ciba Foundation Symp. 28, 181-198
301. Ahmed, K. & Ishida, H. (1971) Mol. Pharmacol. 7, 323-327
302. Trajkovic, D., Ribarac-Stepic, N. & Kanazir, D. (1974) Arch. Int. Physiol. Biochim. 82, 211-216

- 303. Hirschmann, P. N. & Itzhaki, S. (1976) Biochem. Soc. Trans. 4, 339-340
- 304. Capps, M. J. & Itzhaki, S. (1976) Biochem. Soc. Trans. 4, 816-817
- 305. Johnson, E. M. & Allfrey, V. G. (1972) Arch. Biochem. Biophys. 152, 786-794
- 306. Mamrack, M. D., Olson, M. & Busch, H. (1977) Biochem. Biophys. Res. Commun. 76, 150-157
- 307. Berezney, R. & Coffey, D. S. (1974) Biochem. Biophys. Res. Commun. 60, 1410-1417
- 308. Berezney, R. & Coffey, D. S. (1975) Science 189, 291-293
- 309. Berezney, R. & Coffey, D. S. (1976) Adv. Enzyme Regul. 14, 63-100
- 310. Allen, S. L., Berezney, R. & Coffey, D. S. (1977) Biochem. Biophys. Res. Commun. 75, 111-116
- 311. Kabat, D. (1970) Biochemistry 9, 4160-4175
- 312. Walton, G. M. & Gill, G. N. (1973) Biochemistry 12, 2064-2611
- 313. Traugh, J. A. & Porter, G. G. (1976) Biochemistry 15, 610-616
- 314. Roberts, S. & Ashby, C. D. (1978) J. Biol. Chem. 253, 288-296
- 315. Traugh, J. A., Mumby, M. & Traut, R. R. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 373-376
- 316. Schubart, U. K., Shapiro, S., Fleischer, N. & Rosen, O. M. (1977) J. Biol. Chem. 252, 92-101
- 317. Levin, D. H., Ranu, R. S., Ernst, V. & London I. M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3112-3116
- 318. Ranu, R. S. & London, I. M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4349-4353
- 319. Levin, D. & London, I. M. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1121-1125
- 320. Issinger, O.-G., Benne, R., Hershey, J. W. B. & Traut, R. R. (1976) J. Biol. Chem. 251, 6471-6474
- 321. Issinger, O.-G. (1977) Biochim. Biophys. Acta 477, 185-189
- 322. Gordon, J. (1971) Biochem. Biophys. Res. Commun. 44, 579-586

323. Fakunding, J. L., Traugh, J. A., Traut, R. R. & Hershey, J. W. B. (1972) *J. Biol. Chem.* 247, 6365-6367
324. Eil, C. & Wool, I. G. (1973) *J. Biol. Chem.* 248, 5130-5136
325. Hébert, J., Pierre, M. & Loeb, J. E. (1977) *Eur. J. Biochem.* 72, 167-174
326. Fantl, P. & Ward, H. A. (1962) *Biochim. Biophys. Acta* 64, 568-570
327. Bettelheim, F. R. (1956) *Biochim. Biophys. Acta* 19, 121-130
328. Osbahr, A. J., Colman, R. W., Laki, K. & Gladner, J. A. (1964) *Biochem. Biophys. Res. Commun.* 14, 555-558
329. Blombäck, B., Blombäck, M., Edman, P. & Hessel, B. (1966) *Biochim. Biophys. Acta* 115, 371-390
330. Blombäck, B., Blombäck, M. & Searle, J. (1963) *Biochim. Biophys. Acta* 74, 148-151
331. Witt, I. & Hasler, K. (1972) *Biochim. Biophys. Acta* 271, 357-362
332. Wolff, D. J. & Siegel, F. L. (1972) *Biochim. Biophys. Acta* 247, 4180-4185
333. Wolff, D. J., Huebner, J. A. & Siegel, F. L. (1972) *J. Neurochem.* 19, 2855-2862
334. Brooks, J. C. & Siegel, F. L. (1973) *J. Biol. Chem.* 248, 4189-4193
335. Brooks, J. C. & Siegel, F. L. (1973) *Biochem. Biophys. Res. Commun.* 55, 710-716
336. Wolff, D. J. & Brostrom, C. O. (1974) *Arch. Biochem. Biophys.* 163, 349-358
337. Bennick, A. (1975) *Biochem. J.* 145, 557-567
338. Bennick, A. (1976) *Biochem. J.* 155, 163-169
339. Bennick, A. (1977) *Biochem. J.* 163, 229-239
340. Bennick, A. (1977) *Biochem. J.* 163, 241-245
341. Donella, A., Pinna, L. A. & Moret, V. (1972) *FEBS Lett.* 26, 249-251
342. Greengard, O., Sentenac, A. & Mendelsohn, N. (1964) *Biochim. Biophys. Acta* 90, 406-407
343. Hurd, S. S., Teller, D. & Fischer, E. H. (1966) *Biochem. Biophys. Res. Commun.* 24, 79-84
344. Gratecos, D. & Fischer, E. H. (1974) *Biochem. Biophys.*

- Res. Commun. 58, 960-967
345. Meyer, F., Heilmeyer, L. M. G., Jr., Haschke, R. H. & Fischer, E. H. (1970) J. Biol. Chem. 245, 6642-6648
346. Hedrick, J. L. & Fischer, E. H. (1965) Biochemistry 7, 1337-1342
347. Cohen, P., Duewer, T. & Fischer, E. H. (1971) Biochemistry 10, 2683-2694
348. Cohen, P., Saari, J. C. & Fischer, E. H. (1973) Biochemistry 12, 5233-5241
349. Fischer, E. H. & Krebs, E. G. (1958) J. Biol. Chem. 231, 65-71
350. Green, A. A. & Cori, G. T. (1943) J. Biol. Chem. 151, 21-29
351. Cori, C. F. & Cori, G. T. (1945) J. Biol. Chem. 158, 341-345
352. Fischer, E. H., Graves, D. J., Crittenden, E. R. S. & Krebs, E. G. (1959) J. Biol. Chem. 234, 1698-1704
353. Hughes, R. C., Yunis, A. A., Krebs, E. G. & Fischer, E. H. (1962) J. Biol. Chem. 237, 40-43
354. Nolan, C., Novoa, W. B., Krebs, E. G. & Fischer, E. H. (1964) Biochemistry 3, 542-551
355. Wolf, D. P., Fischer, E. H. & Krebs, E. G. (1970) Biochemistry 9, 1923-1929
356. Fischer, E. H. & Krebs, E. G. (1966) Fed. Proc. 25, 1511-1520
357. Fischer, E. H., Heilmeyer, L. M. G., Jr. & Haschke, R. H. (1971) Curr. Top. Cell. Regul. 4, 211-251
358. Riley, W. D., DeLange, R. J., Bratvold, G. E. & Krebs, E. G. (1968) J. Biol. Chem. 243, 2209-2215
359. Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S. & Krebs, E. G. (1971) J. Biol. Chem. 246, 1968-1976
360. Hayakawa, T., Perkins, J. P. & Krebs, E. G. (1973) Biochemistry 12, 574-579
361. Cohen, P. (1973) Eur. J. Biochem. 34, 1-14
362. Cohen, P., Watson, D. C. & Dixon, G. H. (1975) Eur. J. Biochem. 51, 79-92

- 363. Yeaman, S. J. & Cohen, P. (1975) *Eur. J. Biochem.* 51, 93-104
- 364. Cohen, P. & Antoniw, J. F. (1973) *FEBS Lett.* 34, 43-47
- 365. Cohen, P., Antoniw, J. F., Davison, M. & Taylor, C. (1973) *Metabolic Interconversions of Enzymes* (Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds.) 33-42, Springer-Verlag, New York
- 366. Larner, J. & Sanger, F. (1965) *J. Mol. Biol.* 11, 491-500
- 367. Rosenkrans, A. M. & Larner, J. (1973) *Biochim. Biophys. Acta* 315, 317-332
- 368. Larner, J. & Villar-Palasi, C. (1971) *Curr. Top. Cell. Regul.* 3, 195-236
- 369. Itarte, E., Robinson, J. C. & Huang, K. P. (1977) *J. Biol. Chem.* 252, 1231-1234
- 370. Nimmo, H. G., Proud, C. G. & Cohen, P. (1976) *Eur. J. Biochem.* 68, 31-44
- 371. Proud, C. G., Rylatt, D. B., Yeaman, S. J. & Cohen, P. (1977) *FEBS Lett.* 80, 435-442
- 372. Segal, H. L. & Lin, D. C. (1973) *Metabolic Interconversions of Enzymes 1973* (Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds.) 75-87, Springer-Verlag, New York
- 373. Schlender, K. K. & Reimann, E. M. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 2197-2201
- 374. Schlender, K. K. (1973) *Biochim. Biophys. Acta* 297, 384-398
- 375. Soderling, T. R., Jett, M. F., Hutson, N. J. & Khatra, B. S. (1977) *J. Biol. Chem.* 252, 7517-7524
- 376. Hutson, N. J., Khatra, B. S. & Soderling, T. R. (1978) *J. Biol. Chem.* 253, 2540-2545
- 377. Huang, T.-S. & Krebs, E. G. (1977) *Biochem. Biophys. Res. Commun.* 75, 643-650
- 378. Cohen, P. (1976) *Trends Biochem. Sci.* 1, 38-40
- 379. Yeaman, S. J., Cohen, P., Watson, D. C. & Dixon, G. H. (1977) *Biochem. J.* 162, 411-421
- 380. Corbin, J. D., Reimann, E. M., Walsh, D. A. & Krebs, E. G. (1970) *J. Biol. Chem.* 245, 4849-4851

381. Huttunen, J. K. & Steinberg, D. (1971) *Biochim. Biophys. Acta* 239, 411-427
382. Khoo, J. C., Fong, W. W. & Steinberg, D. (1972) *Biochem. Biophys. Res. Commun.* 49, 407-413
383. Khoo, J. C., Steinberg, D., Huang, J. J. & Vagelos, P. R. (1976) *J. Biol. Chem.* 251, 2882-2890
384. Khoo, J. C., Sperry, P. J., Gill, G. N. & Steinberg, D. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 4843-4847
385. Severson, D. L., Khoo, J. C. & Steinberg, D. (1977) *J. Biol. Chem.* 252, 1484-1489
386. Khoo, J. C., Steinberg, D. & Lee, E. Y. C. (1978) *Biochem. Biophys. Res. Commun.* 80, 418-423
387. Carlson, C. A. & Kim, K.-H. (1973) *J. Biol. Chem.* 248, 378-380
388. Carlson, C. A. & Kim, K.-H. (1974) *Arch. Biochem. Biophys.* 164, 478-489
389. Carlson, C. A. & Kim, K.-H. (1974) *Arch. Biochem. Biophys.* 164, 490-501
390. Lee, K.-H. & Kim, K.-H. (1977) *J. Biol. Chem.* 252, 1748-1751
391. Inoue, H. & Lowenstein, J. M. (1972) *J. Biol. Chem.* 247, 4825-4832
392. Farstad, M. (1967) *Biochim. Biophys. Acta* 146, 272-283
393. Farstad, M. (1968) *Acta Physiol. Scand.* 74, 568-576
394. Aas, M. (1970) *Biochim. Biophys. Acta* 202, 250-258
395. Ljungström, O., Hjelmquist, G. & Engström, L. (1974) *Biochim. Biophys. Acta* 358, 289-298
396. Hjelmquist, G., Andersson, J., Edlund, B. & Engström, L. (1974) *Biochem. Biophys. Res. Commun.* 61, 509-513
397. Titanji, V. P. K., Zetterqvist, O. & Engström, L. (1976) *Biochim. Biophys. Acta* 422, 98-108
398. Berglund, L., Ljungström, O. & Engström, L. (1977) *J. Biol. Chem.* 252, 613-619
399. Feliu, J. E., Hue, L. & Hers, H.-G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 2762-2766
400. Ekman, P., Dahlqvist, U., Humble, E. & Engström, L. (1976) *Biochim. Biophys. Acta* 429, 374-382

401. Mapes, J. P. & Harris, R. A. (1976) *J. Biol. Chem.* 251, 6189-6196
402. Riou, J. P., Claus, T. H. & Pilkis, S. J. (1978) *J. Biol. Chem.* 253, 656-659
403. Linn, T. C., Pettit, F. H. & Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* 62, 234-241
404. Linn, T. C., Pettit, F. H., Hucho, F. & Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* 64, 227-234
405. Roche, T. E. & Reed, L. J. (1972) *Biochem. Biophys. Res. Commun.* 48, 840-846
406. Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* 151, 328-342
407. Barrera, C. R., Namihira, G., Hamilton, L., Munk, P., Eley, M. H., Linn, T. C. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* 148, 343-358
408. Pettit, F. H., Roche, T. E. & Reed, L. J. (1972) *Biochem. Biophys. Res. Commun.* 49, 563-571
409. Davis, P. F., Pettit, F. H. & Reed, L. J. (1977) *Biochem. Biophys. Res. Commun.* 75, 541-549
410. Butler, J. R., Pettit, F. H., Davis, P. F. & Reed, L. J. (1977) *Biochem. Biophys. Res. Commun.* 74, 1667-1674
411. Reed, L. J., Pettit, F. H., Roche, T. E., Butterworth, P. J., Barrera, C. R. & Tsai, C. S. (1973) *Metabolic Interconversions of Enzymes 1973* (Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds.) 99-106, Springer-Verlag, New York
412. Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* 125, 115-127
413. Cooper, R. H., Denton, R. M., Pask, H. T., Randle, P. J., Severson, D. L. & Whitehouse, S. (1973) *Metabolic Interconversion of Enzymes 1973* (Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds.) 107-116, Springer-Verlag, New York
414. Severson, D. L., Denton, R. M., Pask, H. T. & Randle, P. J. (1974) *Biochem. J.* 140, 225-237

- 415. Stansbie, D., Denton, R. M., Bridges, B. J., Pask, H. T. & Randle, P. J. (1976) *Biochem. J.* 154, 225-236
- 416. Walsh, D. A., Cooper, R. H., Denton, R. M., Bridges, B. J., & Randle, P. J. (1976) *Biochem. J.* 157, 41-67
- 417. Coore, H. G. (1974) *NATO Adv. Study Inst. Ser.* A-9, 533-548
- 418. Mukherjee, C. & Jungas, R. L. (1975) *Biochem. J.* 148, 229-235
- 419. Wieland, O. & Jagow-Westermann, B. V. (1969) *FEBS Lett.* 3, 271-274
- 420. Wieland, O. & Siess, E. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 65, 947-954
- 421. Siess, E. A. & Wieland, O. H. (1976) *FEBS Lett.* 65, 163-168
- 422. Leiter, A. B., Weinberg, M., Isohashi, F., Utter, M. F. & Linn, T. (1978) *J. Biol. Chem.* 253, 2716-2723
- 423. Lornitzo, F. A., Qureshi, A. A. & Porter, J. W. (1975) *J. Biol. Chem.* 250, 4520-4529
- 424. Qureshi, A. A., Kim, M., Lornitzo, F. A., Jenik, R. A. & Porter, J. W. (1975) *Biochem. Biophys. Res. Commun.* 64, 836-844
- 425. Qureshi, A. A., Jenik, R. A., Kim, M., Lornitzo, F. A. & Porter, J. W. (1975) *Biochem. Biophys. Res. Commun.* 66, 344-351
- 426. Brand, I. A. & Söling, H. D. (1975) *FEBS Lett.* 57, 163-168
- 427. Brand, I. A., Müller, M. K., Unger, C. & Söling, H. D. (1976) *FEBS Lett.* 68, 271-274
- 428. Hofer, H. W. & Fürst, M. (1976) *FEBS Lett.* 62, 118-122
- 429. Taunton, O. D., Stifel, F. B., Greene, H. L. & Herman, R. H. (1974) *J. Biol. Chem.* 249, 7228-7239
- 430. Mendicino, J., Beaudreau, C. & Bhattacharyya, R. N. (1966) *Arch. Biochem. Biophys.* 116, 436-445
- 431. Caron, M. G., Goldstein, S., Savard, K. & Marsh, J. M. (1975) *J. Biol. Chem.* 250, 5137-5143
- 432. Trzeciak, W. H. & Boyd, G. S. (1974) *Eur. J. Biol.* 46, 201-207

433. Pittman, R. C., Khoo, J. C. & Steinberg, D. (1975) J. Biol. Chem. 250, 4505-4511
434. Beckett, G. J. & Boyd, G. S. (1975) Biochem. Soc. Trans. 3, 892-894
435. Naghshineh, S., Treadwell, C. R., Gallo, L. & Vahouny, G. V. (1974) Biochem. Biophys. Res. Commun. 61, 1076-1082
436. Wallat, S. & Kunau, W.-H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 949-960
437. Vahouny, G. V., Naghshineh, S., Gallo, L. & Treadwell, C. R. (1977) Fed. Proc. 36, 390
438. Milstien, S., Abita, J.-P., Chang, N. & Kaufman, S. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1591-1593
439. Abita, J. P., Milstien, S., Chang, N. & Kaufman, S. (1976) J. Biol. Chem. 251, 5310-5314
440. Jedlicki, E., Kaufman, S. & Milstien, S. (1977) J. Biol. Chem. 252, 7711-7714
441. Harris, J. E., Baldesarini, R. J., Morgenroth, V. H., III, & Roth, R. H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 789-793
442. Ames, M. M., Lerner, P. & Lovenberg, W. (1978) J. Biol. Chem. 253, 27-31
443. Lovenberg, W., Bruckwick, E. A. & Hanbauer, I. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2955-2958
444. Lloyd, T. & Kaufman, S. (1975) Biochem. Biophys. Res. Commun. 66, 907-913
445. Hoeldtke, R. & Kaufman, S. (1977) J. Biol. Chem. 252, 3160-3169.
446. Lee, K.-L. & Nickol, J. M. (1974) J. Biol. Chem. 249, 6024-6026
447. Chuah, C.-C. & Oliver, I. T. (1972) Biochemistry 11, 2547-2553
448. Narumi, S. & Miyamoto, E. (1974) Biochim. Biophys. Acta 350, 215-224
449. Martelo, O. J. (1973) in Protein Phosphorylation in Control Mechanism (Huijing, F. & Lee, E. Y. C., eds.) 199-216, Academic Press, New York
450. Hirsch, J. & Martelo, O. J. (1976) J. Biol. Chem. 251, 5408-5413

- 451. Hirsch, J. & Martelo, O. J. (1978) *Biochem. J.* 169, 355-359
- 452. Jungmann, R. A., Hiestand, P. C. & Schweppe, J. S. (1974) *J. Biol. Chem.* 249, 5444-5451
- 453. Kranias, E. G., Schweppe, J. S. & Jungmann, R. A. (1977) *J. Biol. Chem.* 252, 6750-6758
- 454. Dahmus, M. E. (1976) *Biochemistry* 15, 1821-1829
- 455. Buhler, J. M., Iborra, F., Sentenac, A. & Fromageot, P. (1976) *FEBS Lett.* 71, 37-41
- 456. Bell, G. I., Valenzuela, P. & Rutter, W. J. (1977) *J. Biol. Chem.* 252, 3082-3091
- 457. Vakhitova, E. A., Amirkhanyan, M. M. & Stepanov, V. M. (1970) *Biokhimiya* 35, 1164-1169
- 458. Kraut, J. (1971) in *The Enzyme*, 3rd ed. (Boyer, P. D., ed.) 3, 165-183, Academic Press, New York
- 459. Bot, G. & Dosa, I. (1971) *Acta Biochim. Biophys. Acad. Sci. Hung.* 6, 73-87
- 460. Cori, G. T. & Green, A. A. (1943) *J. Biol. Chem.* 151, 31-38
- 461. Cori, G. T. & Cori, C. F. (1945) *J. Biol. Chem.* 158, 321-332
- 462. Fischer, E. H. & Krebs, E. G. (1955) *J. Biol. Chem.* 216, 121-132
- 463. Rall, T. W., Wosilait, W. D. & Sutherland, E. W. (1956) *Biochim. Biophys. Acta* 20, 69-76
- 464. Sutherland, E. W. & Wosilait, W. D. (1955) *Nature (London)* 175, 169-170
- 465. Wosilait, W. D. & Sutherland, E. W. (1956) *J. Biol. Chem.* 218, 469-481
- 466. Rall, T. W., Sutherland, E. W. & Wosilait, W. D. (1956) *J. Biol. Chem.* 218, 483-495
- 467. Fischer, E. H., Graves, D. J., Crittenden, E. R. S. & Krebs, E. G. (1959) *J. Biol. Chem.* 234, 1698-1704
- 468. Delange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A. & Krebs, E. G. (1968) *J. Biol. Chem.* 243, 2200-2208
- 469. Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkeler, F. L. & Walsh, D. A. & Krebs, E. G. (1970) *J. Biol. Chem.* 245, 6317-6328

- 470. Villar-Palasi, C. & Larner, J. (1961) Arch. Biochem. Biophys. 94, 436-442
- 471. Rosell-Perez, M., Villar-Palasi, C. & Larner, J. (1962) Biochemistry 1, 763-768
- 472. Smith, C. H., Brown, N. D. & Larner, J. (1971) Biochim. Biophys. Acta 242, 81-88
- 473. Sooranna, S. R. & Saggerson, E. D. (1976) FEBS Lett. 64, 36-39
- 474. Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) Biochem. Biophys. Res. Commun. 65, 575-582
- 475. Koritz, S. B., Bhargava, G. & Schwantz, E. (1977) Ann. N. Y. Acad. Sci. 297, 329-335
- 476. Stadtman, E. R. & Chock, P. B. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2761-2765
- 477. Chock, P. B. & Stadtman, E. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2766-2770
- 478. Roach, P. J. (1977) Trends Biochem. Sci. 2, 87-90
- 479. Sung, M. T. & Freedlender, E. F. (1978) Biochemistry 17, 1884-1890
- 480. Datta, A., De Haro, C. & Ochoa, S. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1148-1152
- 481. Beg, Z. H., Allmann, D. W. & Gibson, D. M. (1973) Biochem. Biophys. Res. Commun. 54, 1362-1369
- 482. Nordstrom, J. L., Rodwell, V. W. & Mitschelen, J. J. (1977) J. Biol. Chem. 252, 8924-8934
- 483. Ingebritsen, T. S., Lee, H.-S., Parker, R. A. & Gibson, D. M. (1978) Biochem. Biophys. Res. Commun. 81, 1268-1277
- 484. Smith, D. L., Bruegger, B. B., Halpern, R. M. & Smith, R. A. (1973) Nature(London) 246, 103-104
- 485. Walsh, D. A. & Krebs, E. G. (1971) in The Enzyme (Boyer, P. D., ed.) 8, 555-581, Academic Press, New York
- 486. Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1975) Adv. Cyclic Nucleotide Res. 5, 241-251
- 487. Rosen, O. M., Rangel-Aldao, R. & Erlichman, J. (1977) Curr. Top. Cell. Regul. 12, 39-74
- 488. Rosen, O. M., Erlichman, J. & Rubin, C. S. (1975) Adv. Cyclic Nucleotide Res. 5, 253-263

489. Rosen, O. M., Rubin, C. S. & Erlichman, J. (1975) Adv. Enzyme Regul. 13, 173-185
490. Burnett, G. Kennedy, E. P. (1954) J. Biol. Chem. 211, 969-980
491. Sundararajan, T. A., Kumar, K. S. V. & Sarma, P. S. (1958) Biochim. Biophys. Acta 29, 449-450
492. Rabinowitz, M. & Lipmann, F. (1960) J. Biol. Chem. 235, 1043-1050
493. Pinna, L. A., Donella, A., Clari, G. & Moret, V. (1975) Biochim. Biophys. Acta 397, 519-528
494. Walsh, D. A., Perkins, J. P. & Krebs, E. G. (1968) J. Biol. Chem. 243, 3763-3765
495. Traugh, J. A., Ashby, C. D. & Walsh, D. A. (1974) Methods Enzymol. 38C, 290-299
496. Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H. & Krebs, E. G. (1971) J. Biol. Chem. 246, 1977-1985
497. Bloch, A. (1974) Biochem. Biophys. Res. Commun. 58, 652-659
498. Bloch, A., Dutschman, G. & Maue, R. (1974) Biochem. Biophys. Res. Commun. 59, 955-959
499. Bloch, A. (1975) Adv. Cyclic Nucleotide Res. 5, 331-338
500. Cech, S. Y. & Ignarro, L. J. (1977) Science 198, 1063-1065
501. Kuo, J. F., Brackett, N. L., Shoji, M. & Tse, J. (1978) J. Biol. Chem. 253, 2518-2521
502. Cheng, Y.-C. & Bloch, A. (1978) J. Biol. Chem. 253, 2522-2524
503. Kuo, J. F., Krueger, B. K., Sanes, J. R. & Greengard, P. (1970) Biochim. Biophys. Acta 212, 79-91
504. Kuo, J. F. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4073-4041
505. Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1974) Methods Enzymol. 38C, 299-308
506. Rubin, C. S., Erlichman, J. & Rosen, O. M. (1974) Methods Enzymol. 38C, 308-315

507. Miyamoto, E., Petzold, G. L., Kuo, J. F. & Greengard, P. (1973) J. Biol. Chem. 248, 179-189
508. Takai, Y., Nakaya, S., Inoue, M., Kishimoto, A., Nishiyama, K., Yamamura, H. & Nishizuka, Y. (1976) J. Biol. Chem. 251, 1481-1487
509. Ashby, C. D. & Walsh, D. A. (1972) J. Biol. Chem. 247, 6637-6642
510. Ashby, C. D. & Walsh, D. A. (1973) J. Biol. Chem. 248, 1255-1261
511. Donnelly, T. E., Jr., Kuo, J. F., Reyes, P. L., Liu, Y.-P. & Greengard, P. (1973) J. Biol. Chem. 248, 190-198
512. Donnelly, T. E., Jr., Kuo, J. F., Miyamoto, E. & Greengard, P. (1973) J. Biol. Chem. 248, 199-203
513. Kuo, W.-N. & Kuo, J. F. (1976) J. Biol. Chem. 251, 4283-4286
514. Corbin, J. D., Keely, S. L. & Park, C. R. (1975) J. Biol. Chem. 250, 218-225
515. Reimann, E. M., Walsh, D. A. & Krebs, E. G. (1971) J. Biol. Chem. 246, 1986-1995
516. Hoffmann, F., Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1975) J. Biol. Chem. 250, 7795-7801
517. Erlichman, J., Rosenfeld, R. & Rosen, O. M. (1974) J. Biol. Chem. 249, 5000-5003
518. Rosen, O. M. & Erlichman, J. (1975) J. Biol. Chem. 250, 7788-7794
519. Rangel-Aldao, R. & Rosen, O. M. (1976) J. Biol. Chem. 251, 3375-3380
520. Chou, C.-K., Alfano, J. & Rosen, O. M. (1977) J. Biol. Chem. 252, 2855-2859
521. Williams, R. E. (1976) Science 192, 473-474
522. Bylund, D. B. & Krebs, E. G. (1975) J. Biol. Chem. 250, 6355-6361
523. Kemp, B. E., Bylund, D. B., Huang, T.-S. & Krebs, E. G. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3448-3452
524. Kemp, B. E., Benjamini, E. & Krebs, E. G. (1975) Proc. Natl. Acad. Sci. U.S.A. 73, 1038-1042

525. Kemp, B. E., Graves, D. J., Benjamini, E. & Krebs, E. G. (1977) *J. Biol. Chem.* 252, 4888-4894
526. Daile, P., Carnegie, P. R. & Young, J. D. (1975) *Nature (London)* 257, 416-418
527. Lincoln, T. M. & Corbin, J. D. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 3239-3243
528. Lincoln, T. M., Dills, W. L. & Corbin, J. D. (1977) 252, 4296-4257
529. Corbin, J. D. & Keely, S. L. (1977) *J. Biol. Chem.* 252, 910-918
530. Walter, U., Uno, I., Liu, A. Y.-C. & Greengard, P. (1977) *J. Biol. Chem.* 252, 6588-6590
531. Flockerzi, V., Speichermann, N. & Hofmann, F. (1978) *J. Biol. Chem.* 253, 3395-3399
532. Walinder, O. (1973) *Biochim. Biophys. Acta* 293, 140-149
533. Goldstein, J. L. & Hasty, M. A. (1973) *J. Biol. Chem.* 248, 6300-6307
534. Baggio, B., Pinna, L. A., Moret, V. & Siliprandi, N. (1970) *Biochim. Biophys. Acta* 207, 515-517
535. Baggio, B. & Moret, V. (1971) *Biochim. Biophys. Acta* 250, 346-350
536. Ruddon, R. W. & Anderson, S. L. (1972) *Biochem. Biophys. Res. Commun.* 46, 1499-1508
537. Tuazon, P. T. & Traugh, J. A. (1978) *J. Biol. Chem.* 253, 1746-1748
538. Traugh, J. A. & Traut, R. R. (1974) *J. Biol. Chem.* 249, 1207-1212
539. Kumar, R. & Tao, M. (1975) *Biochim. Biophys. Acta* 410, 87-98
540. Bingham, E. W. & Farrell, H. M., Jr. (1974) *J. Biol. Chem.* 249, 3647-3651
541. Gibson, K., Tichonicky, L. & Kruh, J. (1974) *Biochimie* 56, 1417-1423
542. Thorngurg, W. & Lindell, T. J. (1977) *J. Biol. Chem.* 252, 6660-6665
543. Takeda, M., Yamamura, H. & Ohga, Y. (1971) *Biochem. Biophys. Res. Commun.* 42, 103-110

544. Blum, H. E., Pocinwong, S. & Fischer, E. H. (1974) *Proc. Natl. Acad. Sci. U.S.A.* 71, 2198-2202
545. Rall, T. W., Sutherland, E. W. & Wosilait, W. D. (1956) *J. Biol. Chem.* 218, 483-495
546. Krebs, E. G. & Fischer, E. H. (1956) *Biochim. Biophys. Acta* 20, 150-157
547. Brostrom, C. O., Hunkeler, F. L. & Krebs, E. G. (1971) *J. Biol. Chem.* 246, 1961-1967
548. Pires, E. M. V. & Perry, S. V. (1977) *Biochem. J.* 167, 137-146
549. Dabrowska, R., Sherry, J. M. F., Aramatorio, D. K. & Hartshorne, D. J. (1978) *Biochemistry* 17, 253-258
550. Waisman, D. M., Singh, T. J. & Wang, J. H. (1978) *J. Biol. Chem.* 253, 3387-3390
551. Graves, D. J., Hayakawa, T., Horvitz, R. A., Beckman, E. & Krebs, E. G. (1973) *Biochemistry* 12, 580-585
552. Kishimoto, A., Takai, Y. & Nishizuka, Y. (1977) *J. Biol. Chem.* 252, 7449-7452
553. Krebs, E. G. (1973) in *Endocrinology, Proceeding of the 4th International Congress* (Scow, R. O., ed.) 17-29, Excerpta Medica, Amsterdam
554. Lebleu, B., Sen, G. C., Shaila, S., Cabrer, B. & Lengyel, P. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 3107-3111
555. Forn, J. & Greengard, P. (1976) *Arch. Biochem. Biophys.* 176, 721-733
556. Roques, M., Tirard, A. & Degroot, L. J. (1977) *Endocrinol.* 100, 967-973
557. Kaerlein, M. & Horak, I. (1976) *Nature(London)* 259, 150-151
558. Hirsch, J. D. & Martelo, O. J. (1976) *Biochem. Biophys. Res. Commun.* 71, 926-932
559. Nuttall, F. Q., Gannon, M. C., Corbett, V. A. & Wheeler, M. P. (1976) *J. Biol. Chem.* 251, 6724-6729
560. Lawrence, J. C. & Lerner, J. (1978) *J. Biol. Chem.* 253, 2104-2113
561. Vokaer, A., Iacobelli, S. & Kram, R. (1974) *Proc. Acad. Sci. U.S.A.* 71, 4482-4486

- 562. Neurath, H. & Walsh, K. A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3825-3832
- 563. Nakai, C. & Thomas, J. A. (1973) Biochem. Biophys. Res. Commun. 52, 530-536
- 564. Nakai, C. & Thomas, J. A. (1974) J. Biol. Chem. 249, 6459-6467
- 565. Thomas, J. A. & Nakai, C. (1973) J. Biol. Chem. 248, 2208-2213
- 566. Siess, E. A. & Wieland, O. H. (1972) Eur. J. Biochem. 26, 96-105
- 567. Imaoka, T., Imazu, M., Ishida, N. & Takeda, M. (1978) Biochim. Biophys. Acta 523, 109-120
- 568. Li, H.-C. (1975) FEBS Lett. 55, 134-137
- 569. Li, H.-C. & Hsiao, K.-J. (1977) Eur. J. Biochem. 77, 383-391
- 570. Li, H.-C. & Hsiao, K.-J. (1977) Arch. Biochem. Biophys. 179, 147-156
- 571. Kato, K. & Bishop, J. S. (1972) J. Biol. Chem. 247, 7420-7429
- 572. Kato, K. & Sato, S. (1974) Biochim. Biophys. Acta 358, 299-307
- 573. Kato, K., Kobayashi, M. & Sato, S. (1974) Biochim. Biophys. Acta 371, 89-101
- 574. Kobayashi, M. & Kato, K. (1977) J. Biochem. (Tokyo) 81, 93-97
- 575. Kato, K., Kobayashi, M. & Sato, S. (1975) J. Biochem. (Tokyo) 77, 811-815
- 576. Zieve, F. J. & Glinemann, W. H. (1973) Biochem. Biophys. Res. Commun. 50, 872-878
- 577. Nakai, C. & Glinemann, W. H. (1977) Mol. Cell. Biochem. 15, 141-143
- 578. Huang, F. L. & Glinemann, W. H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3004-3008
- 579. Huang, F. L. & Glinemann, W. H. (1976) FEBS Lett. 62, 326-329
- 580. Huang, F. L. & Glinemann, W. H. (1976) Eur. J. Biochem. 70, 419-426

- 581. Martensen, T. M., Brotherton, J. E. & Graves, D. J. (1973) J. Biol. Chem. 248, 8329-8336
- 582. Martensen, T. M., Brotherton, J. E. & Graves, D. J. (1973) J. Biol. Chem. 248, 8323-8328
- 583. Parrish, R. F. & Graves, D. J. (1976) Biochem. Biophys. Res. Commun. 70, 1290-1296
- 584. Graves, D. J., Martensen, T. M., Tu, J.-I. & Tessmer, G. M. (1973) Metabolic Interconversions of Enzymes 1973 (Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds.) 53-61, Springer-Verlag, New York
- 585. De Barsey, T., Stalmans, W., Laloux, M., De Wulf, H. & Hers, H.-G. (1972) Biochem. Biophys. Res. Commun. 46, 183-190
- 586. Antoniw, J. F. & Cohen, P. (1976) Eur. J. Biochem. 68, 45-54
- 587. Antoniw, J. F., Nimmo, H. G., Yeaman, S. J. & Conen, P. (1977) Biochem. J. 162, 423-433
- 588. Cohen, P., Nimmo, G. A. & Antoniw, J. F. (1977) Biochem. J. 162, 435-444
- 589. Cohen, P., Rylatt, D. B. & Nimmo, G. A. (1977) FEBS Lett. 76, 182-186
- 590. Riley, W. D., DeLange, R. J., Bratvold, G. E. & Krebs, E. G. (1968) J. Biol. Chem. 243, 2209-2215
- 591. Fischer, E. H., Hurd, S. S., Novoa, W. B. & Krebs, E. G. (1963) Biochem. J. 89, 39p-40p
- 592. Hurd, S. S., Novoa, W. B., Hickenbottom, J. P. & Fischer, E. H. (1966) Methods Enzymol. 8, 546-550
- 593. Haschke, R. H., Heilmeyer, L. M. G., Meyer, F. & Fischer, E. H. (1970) J. Biol. Chem. 245, 6657-6663
- 594. Gratecos, D., Detwiler, T. & Fischer, E. H. (1973) Metabolic Interconversions of Enzymes 1973 (Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds.) 43-52, Springer-Verlag, New York
- 595. Gratecos, D., Detwiler, T. C., Hurd, S. & Fischer, E. H. (1977) Biochemistry 16, 4812-4817
- 596. Detwiler, T. C., Gratecos, D. & Fischer, E. H. (1977) Biochemistry 16, 4818-4823

597. Varsanyi, M. & Bot, G. (1973) *Acta Biochim. Biophys. Acad. Sci. Hung.* 8, 23-31
598. Bot, G. & Dosa, I. (1971) *Acta Biochim. Biophys. Acad. Sci. Hung.* 6, 73-87
599. Bot, G., Varsanyi, M. & Gergely, P. (1975) *FEBS Lett.* 50, 351-354
600. Villar-Palasi, C. (1969) *Ann. N. Y. Acad. Sci.* 166, 719-730
601. Gilboe, D. P. & Nuttall, F. Q. (1972) *Biochem. Biophys. Res. Commun.* 48, 898-906
602. Ray, K. P. & England, P. J. (1976) *Biochem. J.* 157, 369-380
603. Bargoni, N., Fossa, T. & Sisini, A. (1963) *Enzymologia* 26, 65-74
604. Torres, H. & Chelala, C. A. (1970) *Biochim. Biophys. Acta* 198, 495-503
605. Chelala, C. A. & Torres, H. (1970) *Biochim. Biophys. Acta* 198, 504-513
606. Chelala, C. A. & Torres, H. N. (1969) *Biochim. Biophys. Acta* 178, 423-426
607. Wasner, H. K. (1975) *FEBS Lett.* 57, 60-63
608. Brandt, H., Capulong, Z. L. & Lee, E. Y. C. (1975) *J. Biol. Chem.* 250, 8038-8044
609. Brandt, H., Lee, E. Y. C. & Killilea, S. D. (1975) *Biochem. Biophys. Res. Commun.* 63, 950-956
610. Lee, E. Y. C., Brandt, H., Capulong, Z. L. & Killilea, S. D. (1975) *Adv. Enzyme Regul.* 14, 467-490
611. Killilea, S. D., Brandt, H., Lee, E. Y. C. & Whelan, W. . J. (1976) *J. Biol. Chem.* 251, 2363-2368
612. Killilea, S. D., Brandt, H., Lee, E. Y. C. & Whelan, W. J. (1974) *Fed. Proc.* 33, 1431
613. Brandt, H., Killilea, S. D. & Lee, E. Y. C. (1974) *Biochem. Biophys. Res. Commun.* 61, 548-554
614. Lee, E. Y. C., Mellgren, R. L., Killilea, S. D. & Aylward, J. H. (1977) in *Regulation of Carbohydrate Metabolism: Proceedings of 11th FEBS meeting, Copenhagen*
615. Kobayashi, M., Kato, K. & Sato, S. (1975) *Biochim. Biophys. Acta* 377, 343-355

616. Khandelwal, R. L., Vandenheede, J. R. & Krebs, E. G. (1976) J. Biol. Chem. 251, 4850-4858
617. Khandelwal, R. L. (1977) Biochim. Biophys. Acta 485, 379-390
618. Khandelwal, R. L. & Zinman, S. M. (1978) J. Biol. Chem. 253, 560-565
619. Holmes, P. A. & Mansour, T. E. (1968) Biochim. Biophys. Acta 156, 275-284
620. Abe, N. & Tsuiki, S. (1974) Biochim. Biophys. Acta 350, 383-391
621. Kikuchi, K., Tamura, S., Hiraga, A. & Tsuiki, S. (1977) Biochem. Biophys. Res. Commun. 75, 29-37
622. Meisler, M. H. & Langan, T. A. (1969) J. Biol. Chem. 244, 4961-4968
623. Bailey, J. M. & Whelan, W. J. (1972) Biochem. Biophys. Res. Commun. 46, 191-197
624. Moret, V., Pinna, L. A. & Magni, G. (1970) Ital. J. Biochem. 19, 204-210
625. Magni, G., Caulini, G. & Moret, V. (1971) Biochim. Biophys. Acta 242, 123-128
626. Pinna, L. A., Donella, A., Clari, G. & Moret, V. (1976) Biochem. Biophys. Res. Commun. 70, 1308-1315
627. Clari, G., Donella, A., Pinna, L. A. & Moret, V. (1975) Arch. Biochem. Biophys. 166, 318-322
628. Gilboe, D. P. & Nuttall, F. Q. (1973) Biochem. Biophys. Res. Commun. 53, 164-171
629. Gilboe, D. P. & Nuttall, F. Q. (1974) Biochim. Biophys. Acta 338, 57-67
630. Gilboe, D. P. & Nuttall, F. Q. (1976) Physiol. Chem. Physics 8, 405-415
631. Tan, A. W. H. & Nuttall, F. Q. (1976) Biochim. Biophys. Acta 445, 118-130
632. Tan, A. W. H. & Nuttall, F. Q. (1978) Biochim. Biophys. Acta 522, 139-150
633. Titanji, V. P. K. (1977) Biochim. Biophys. Acta 481, 140-151
634. Titanji, V. P. K., Zetterqvist, O. & Ragnarsson, U.

- (1977) FEBS Lett. 78, 86-90
635. Merlevede, W., Goris, J. & De Brandt, C. (1969) Eur. J. Biol. 11, 499-502
636. Goris, J., Pijnenborg-Vercruysse, L. & Merlevede, W. (1972) Biochim. Biophys. Acta 268, 158-165
637. Kalala, L. R., Goris, J. & Merlevede, W. (1973) FEBS Lett. 32, 284-288
638. Merlevede, W., Defreyn, G., Goris, J., Kalala, L. R. & Rosemont, J. (1976) Arch. Int. Physiol. Biochim. 84, 359-423
639. Goris, J. & Merlevede, W. (1974) FEBS Lett. 48, 184-187
640. Goris, J., Defreyn, G. & Merlevede, W. (1977) Biochimie 59, 171-178
641. Defreyn, G., Goris, J. & Merlevede, W. (1976) Arch. Int. Physiol. Biochim. 84, 595-596
642. De Wulf, H., Stalmans, W. & Hers, H.-G. (1970) Eur. J. Biochem. 15, 1-8
643. Stalmans, W., De Wulf, H., Lederer, B. & Hers, H.-G. (1970) Eur. J. Biochem. 15, 9-12
644. Stalmans, W., De Wulf, H. & Hers, H.-G. (1971) Eur. J. Biochem. 18, 582-587
645. Hers, H.-G., Stalmans, W., De Wulf, H., Laloux, M. & Hue, L. (1973) Metabolic Interconversions of Enzymes 1973 (Fischer, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R., eds.) 89-98, Springer-Verlag, New York
646. Paigen, K. (1958) J. Biol. Chem. 233, 388-394
647. Paigen, K. & Griffiths, S. K. (1959) J. Biol. Chem. 234, 299-303
648. Ullman, B. & Perlman, R. L. (1975) Biochim. Biophys. Acta 403, 393-411
649. Merlevede, W. & Riley, G. A. (1966) J. Biol. Chem. 241, 3517-3524
650. Kalala, L. R., Goris, J. & Merlevede, W. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 575-581
651. Rosemont, J. L. (1976) Arch. Int. Physiol. Biochim. 84, 184-185

- 652. Maeno, H. & Greengard, P. (1972) J. Biol. Chem. 247, 3269-3277
- 653. Miyamoto, E. & Kakiuchi, S. (1975) Biochim. Biophys. Acta 384, 458-465
- 654. Weller, M. & Rodnight, R. (1971) Biochem. J. 124, 393-406
- 655. Rose, S. P. R. & Heald, P. J. (1960) Biochem. J. 77, 8p-9p
- 656. Rose, S. P. R. & Heald, P. J. (1961) Biochem. J. 81, 339-347
- 657. Rose, S. P. R. (1962) Nature(London) 194, 1280-1281
- 658. Yang, H.-Y., Costa, E., Majane, E. A. & Hong, J. (1977) J. Neurochem. 28, 1075-1080
- 659. Roberts, R. M. & Bazer, F. W. (1976) Biochem. Biophys. Res. Commun. 68, 450-455
- 660. Schlosnagle, D. C., Bazer, F. W., Tsibris, J. C. M. & Roberts, R. M. (1974) J. Biol. Chem. 249, 7574-7579
- 661. Davis, J. S. (1957) Proc. Soc. Exp. Biol. Med. 95, 247-249
- 662. Paietta, E. & Sands, H. (1978) Biochim. Biophys. Acta 523, 121-132
- 663. Graham, C. & Avruch, J. & Fairbanks, G. (1976) Biochem. Biophys. Res. Commun. 72, 701-708
- 664. Murray, A. W., Froscio, M. & Kemp, B. E. (1972) Biochem. J. 129, 995-1002
- 665. Traugh, J. A. & Sharp, S. B. (1977) J. Biol. Chem. 252, 3738-3744
- 666. Lightfoot, H. N., Mumby, M. & Traugh, J. A. (1975) Biochem. Biophys. Res. Commun. 66, 1141-1146
- 667. Mumby, M. & Traugh, J. A. (1977) Fed. Proc. 36, 778
- 668. Layne, P., Constantopoulos, A. Judge, J. F. X., Rauner, R. & Najjar, V. A. (1973) Biochem. Biophys. Res. Commun. 53, 800-805
- 669. Tsung, P. K., Sakamoto, T. & Weissmann, G. (1975) Biochem. J. 145, 437-448
- 670. Villar-Palasi, C. & Gazquez-Martinez, I. (1968) Biochim. Biophys. Acta 159, 479-489
- 671. Periasamy, M., Brunel, C., Blanchard, J.-M. & Jeanteur, P. (1977) Biochem. Biophys. Res. Commun. 79, 1077-1083

- 672. Olson, M. O. J. & Guetzow, K. (1976) Biochem. Biophys. Res. Commun. 70, 717-722
- 673. Tan, E. L., Levine, M. A., Yang, C. S. & Li, H.-C. (1976) Int. J. Biochem. 7, 21-26
- 674. Chou, M. W., Tan, E. L. & Yang, C. S. (1976) Fed. Proc. 35, 1410
- 675. Tang, F. Y. & Hoskins, D. D. (1975) Biochem. Biophys. Res. Commun. 62, 328-335
- 676. Azhar, S. & Menon, K. M. J. (1975) Eur. J. Biochem. 55, 487-495
- 677. Spaulding, S. W. & Burrow, G. N. (1975) Proc. Soc. Exp. Biol. Med. 150, 568-570
- 678. Miller, E., Fredholm, B., Miller, R. E., Steinberg, D., & Mayer, S. E. (1975) 2470-2480
- 679. Tellez De Inon, M. T. & Torres, H. N. (1973) Biochim. Biophys. Acta 297, 399-412
- 680. Albin, E. E. & Newburgh, R. W. (1975) Biochim. Biophys. Acta 377, 381-388
- 681. Sundararajan, T. A. & Sarma, P. S. (1954) Biochim. Biophys. Acta 13, 588-589
- 682. Sundararajan, T. A. & Sarma, P. S. (1957) Enzymologia 18, 234-240
- 683. Sundararajan, T. A. & Sarma, P. S. (1959) Biochem. J. 71, 537-544
- 684. Sundararajan, T. A. & Sarma, P. S. (1954) Biochem. J. 56, 125-130
- 685. Feinstein, R. N. & Volk, M. E. (1949) J. Biol. Chem. 177, 339-346
- 686. Norberg, B. (1950) Acta Chem. Scand. 4, 1206-1215
- 687. Hofman, T. (1958) Biochem. J. 69, 135-139
- 688. Hofman, T. (1958) Biochem. J. 69, 139-145
- 689. Revel, H. R. & Racker, E. (1960) Biochim. Biophys. Acta 43, 465-476
- 690. Revel, H. R. (1963) Methods Enzymol. 6, 211-214
- 691. Vreven, J., Lieberherr, M. & Vaes, G. (1973) Biochim. Biophys. Acta 293, 170-177

692. Harris, D. L. (1946) J. Biol. Chem. 165, 541-550
693. Harris, D. L., Mizock, B. J. & Pilakis, S. J. (1966) J. Biol. Chem. 241, 707-714
694. Nass, S. (1956) Trans. N. Y. Acad. Sci. 19, 118-129
695. Tsusaka, A. (1967) Acta Embryol. Morphol. Exp. 10, 44-53
696. Vittorelli, M. L. (1964) Experientia 20, 385-386
697. Foote, M. W. & Kind, C. A. (1953) Arch. Biochem. Biophys. 46, 254-256
698. Koehn, P. V. & Kind, C. A. (1965) Arch. Biochem. Biophys. 111, 614-618
699. Huang, C.-J., Ripoché, P. A., Li, H.-C., Wyssbord & Schwartz, I. L. (1977) Fed. Proc. 36, 631
700. Killilea, S. D., Brandt, H. & Lee, E. Y. C. (1976) Trends Biochem. Sci. 1, 30-33
701. Mellgren, R. L., Killilea, S. D., Aylward, J. H. & Lee, E. Y. C. (1978) Fed. Proc. 37, 1808
702. Killilea, S. D., Metieh, M. E. & Lee, E. Y. C. (1976) Fed. Proc. 35, 1410
703. Goris, J., Defreyn, G. & Merlevede, W. (1976) Arch. Int. Physiol. Biochim. 84, 626-627
704. Baba, T. & Tsuiki, S. (1974) Biochim. Biophys. Acta 370, 419-430
705. Picton, C., Brautigan, D. L., Builder, S. E. & Fischer, E. H. (1978) Fed. Proc. 37, 1809
706. Keller, P. J. & Cori, G. T. (1955) J. Biol. Chem. 214, 127-134
707. Mellgren, R. L., Slaughter, G. R. & Thomas, J. A. (1977) J. Biol. Chem. 252, 6082-6089
708. Huang, K.-P., Robinson, J. C. & Chou, J. Y. (1976) Fed. Proc. 35, 1410
709. Fischer, E. H. & Krebs, E. G. (1962) Methods Enzymol. 5, 369-373
710. Bárány, K. & Oppenheimer, H. (1967) Nature(London) 213, 626-627
711. Erlichman, J., Hirsch, A. H. & Rosen, O. M. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 731-735

- 712. Fiske, C. H. & Subbarow, Y. (1925) J. Biol. Chem. 66, 375-400
- 713. Martin, J. B. & Doty, D. M. (1949) Anal. Chem. 21, 965-967
- 714. Li, H.-C. & Felmly, D. A. (1973) Anal. Biochem. 52, 300-304
- 715. Shamoo, A. E., Gentile, D. E. & Brodsky, W. A. (1970) Biochim. Biophys. Acta 203, 484-494
- 716. Harigaya, S. & Schwartz, A. (1969) Circ. Res. 25, 781-794
- 717. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404-427
- 718. Laemmli, U. K. (1970) Nature(London) 227, 680-685
- 719. Siegel, L. M. & Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346-362
- 720. Schachman, H. K. (1959) in Ultracentrifugation in Biochemistry (Schachman, H. K., ed.) p. 239, Academic Press, New York
- 721. Martin, R. G. & Ames, B. N. (1961) J. Biol. Chem. 236, 1372-1379
- 722. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
- 723. Li, H.-C. & Hsiao, K.-J. (1976) Fed. Proc. 35, 1410
- 724. Khatra, B. S. & Soderling, T. R. (1977) Fed. Proc. 36, 527
- 725. Yan, S. C. B. & Graves, D. J. (1978) Fed. Proc. 37, 1425
- 726. Shizuta, Y., Khandelwal, R. L., Maller, J. L., Vandenheede, J. R. & Krebs, E. G. (1977) J. Biol. Chem. 252, 3408-3413