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Substrate directed control of rabbit liver protein phosphatases

by

© MICHAEL OKECHUKWU MONANU

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To my family.

Abstract

The modulation of four molecular forms of rabbit liver phosphorylase *a* phosphatase, the 'catalytic' subunit (35 000 M_r) phosphatase and phosphatases-1, $2A_1$ and $2A_2$, by some physiologically important compounds were studied using rabbit skeletal muscle and liver ^{32}P-labelled phosphorylase *a* as substrates. Glucose and caffeine showed independent and additive activations. The caffeine effect was seen at μM to mM concentrations and glucose was activating at concentrations below the normal blood glucose level.

The nucleotides ATP and AMP, at their presumed physiological concentrations in the liver, were strong inhibitors. Inhibition by these nucleotides and other inhibitors tested showed varied response to the presence of glucose and caffeine, depending on the phosphatase form. Thus, significant relief of ATP inhibition was afforded by glucose and caffeine acting independently for the 35 000 M_r phosphatase, whereas relief of inhibition for phosphatases-1, $2A_1$ and $2A_2$ required a combination of glucose, caffeine and Mg^{2+}. Phosphatase-1 differed from phosphatases-$2A_1$ and $2A_2$ in its response to either AMP or AMP plus ATP inhibition as the combined presence of glucose, caffeine and Mg^{2+} could not completely overcome the inhibition.

The modes of effector actions on the four phosphatase forms were distinguished by using the natural substrate

(phosphorylase *a*) and two alternative substrates (histone and a tetradecapeptide consisting of residues 5 to 18 of rabbit skeletal muscle phosphorylase *a*). Glucose, caffeine, AMP, ADP, P_i and glucose 1-P showed substrate-directed effects for all the phosphatase forms. ATP exerted both substrate and enzyme-directed effects for the 35 000 M_r phosphatase and phosphatases-1 and $2A_2$ but only a substrate-directed effect for phosphatase-$2A_1$, suggesting that the γ-subunit of the type-2 phosphatases may prevent ATP binding. Mg^{2+} showed substrate-directed effect for phosphatase-1, $2A_1$ and $2A_2$, and an additional enzyme-directed effect for the 35 000 M_r phosphatase form.

The phosphorylase *a* inhibition of glycogen synthase activation was investigated using gel-filtered rabbit liver extracts, particularly with respect to the glucose-caffeine effect on the phosphorylase *a* phosphatase activity. The onset of glycogen synthase activation was preceded by the characteristic latency observed with rat liver filtrates. Glucose (20 mM) or caffeine (1 mM), decreased the lag period while acting independently, with a further decrease occurring in their combined presence. AMP (0.3 mM), inhibited strongly the glycogen synthase activation, but the independent and additive actions by glucose and caffeine afforded relief of this inhibition. Similar patterns were seen for inhibitions by Mg.ATP and exogenous phosphorylase *a*. Using purified proteins, the dephosphorylation or activation of glycogen synthase *b* by protein phosphatase-1 was

significantly inhibited by phosphorylase *a* whereas protein phosphatases 2A$_1$ and 2A$_2$ and the M_r 35 000 phosphatase activities on glycogen synthase *b* were not appreciably affected.

The results in these studies are consistent with the view that the phosphorylase *a* level modulates the activation of glycogen synthase activation in liver extracts. Furthermore the additive effects seen for glucose and caffeine, with respect to the inhibitory conditions observed in these studies, support the contention that glucose alone may not be the sole physiological regulator of glucose homeostasis through its action on phosphorylase *a* in the liver. Hence, the inhibitory site on phosphorylase *a* which binds nucleosides and purine derivatives such as caffeine may be of physiological significance.

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Table of Contents

List of Tables

Table | Page

List of Figures

Figures Page

List of Abbreviations

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
AMP	adenosine 5'-monophosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 5'-monophosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetate
EGTA	ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
G-1-P	α-D-glucose-1-phosphate
G-6-P	α-D-glucose-6-phosphate
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid
MES	2[N-morpholino]ethane sulfonic acid
$Mg(OAc)_2$	magnesium acetate
P_i	inorganic phosphate
PMSF	phenylmethyl sulfonyl fluoride
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
TCA	trichloro acetic acid
UDPG	uridine-5'-diphosphoglucose
wt	weight
v	volume
U	units
K_m	apparent Michaelis constant

V_{max}	initial velocity at infinite substrate concentration
M_r	relative molecular mass
K_i	dissociation constant for enzyme-inhibitor interaction

I. Introduction

Phosphorylase *a* phosphatase (EC 3.1.3.17) catalyzes the conversion of phosphorylase *a* into phosphorylase *b*, a reaction that has been defined as a hydrolytic removal of the phosphate on a serine, 14 residues from the N-terminus (1,2), and is simply represented as:

$$\text{Phosphorylase } a + n\ H_2O \xrightarrow{\text{enzyme}} n/2\ \text{Phosphorylase } b + n\ P_i$$

Phosphorylase *a* from rabbit skeletal muscle is tetrameric (3) while that from rabbit liver (4) and some muscle tissues is dimeric (see ref. 5); thus *n* in the above equation is 4 and 2, respectively, for tetrameric and dimeric phosphorylase *a*. Further, there are indications that the phosphatase reaction may occur via the ordered uni-bi kinetic mechanism common to hydrolytic enzymes (see ref. 5).

Partial purifications of the rabbit skeletal muscle (6) and liver(2) phosphorylase *a* phosphatase were reported following the initial description of the enzymatic activity in 1943 by Cori and Green (7). However, further detailed biochemical investigation of the dephosphorylation event and its effect on cellular regulation was hampered by the apparent presence of multiple molecular forms of protein phosphatases with broad substrate specificities in a wide variety of mammalian tissues (reviewed in refs. 8 and 9).

This notwithstanding, knowledge of protein phosphatases has increased dramatically in the last decade following the

purification to homogeneity of a low molecular weight protein phosphatase (M_r 35 000) from a wide variety of tissues including rabbit liver (10,11), skeletal muscle (12), and heart (13), bovine and canine heart (14,15), and liver cell nuclei (16). The various purification schemes employed dissociation steps including ethanol precipitation of crude preparations at room temperature (10,13), freezing and thawing in the presence of 2-mercaptoethanol (17, 18), or storage at 4°C for 1 to 2 days (11). Of these, the ethanol precipitation has gained wide use, possibly due to ease of purification of relatively pure enzyme.

These dissociation steps suggested the existence of higher molecular weight protein phosphatases which may contain regulatory subunits (10,19), thus the 5- to 10-fold activation caused by the ethanol treatment(10). Concerted efforts by numerous investigators have led to partial and homogeneous preparations of high molecular weight forms of protein phosphatase from a variety of tissues including rabbit and rat skeletal muscle and liver (reviewed in refs. 8 and 9).

A. Classification and substrate specificity

Extensive studies carried out recently by Cohen and associates demonstrated that 'most of the cytoplasmic protein phosphatases reported in the literature can be explained by just four enzymes, termed protein phosphatases-1, 2A, 2B and 2C, which appear to account for

all the phosphatase activity in mammalian tissues towards phosphorylated enzymes involved in the regulation of carbohydrate, lipid and protein metabolism' (20) (see Table I.1). These phosphatases have been classified into types-1 and 2, and could be distinguished by their substrate specificity and sensitivity to inhibitor proteins, ATP, and Mn^{2+} (21,22). Thus, type-1 phosphatases (Phosphatase-1) are strongly inhibited by two heat-stable protein inhibitors-1 and 2, and preferentially dephosphorylate the β-sububit of phosphorylase *b* kinase. Type-2 phosphatases (Phosphatases-2A, 2B and 2C) dephosphorylate preferentially the α-subunit of phosphorylase *b* kinase and are insensitive to inhibitors-1 and 2. Characterization studies indicate that the catalytic entities of protein phosphatases-1 and 2 are distinct gene products (21,23) and both are present in the 35 000 M_r phosphatase preparations (21).

With phosphorylase *a* as substrate, both types can also be differentiated by the sensitivity of type-1 phosphatases to inhibition by heparin and protamine sulfate while type-2 phosphatases are unaffected to any appreciable extent by these compounds (24,25). Furthermore, the type-1 phosphatases are not activated to the same extent as phosphatase-2A by spermine, and there has been some discussion about the possible physiological regulation of phosphatases by this compound (26, and see Section C.).

In rabbit liver, protein phosphatase-1 accounts for 20-50% of the phosphorylase *a* and glycogen synthase *b*

Table I.1. Some characteristics of protein phosphatases (PrP) in mammalian tissues.[a]

	Protein phosphatase			
	PrP-1	PrP-2A	PrP-2B	PrP-2C
Distribution	protein-glycogen complex	cytosol	cytosol	cytosol
Specificity for phosphorylase *b* kinase	β-subunit	α-subunit	α-subunit	α-subunit
Phosphorylase *a* phosphatase activity	high	high	very low	very low
Substrate specificity	broad	broad	narrow	broad
Sensitivity to inhibitors-1 and 2	yes	no	no	no
Regulators	inhibitors-1 and 2	not known	Ca^{++}-calmodulin	Mg^{++}
Approximate M_r	137 KDa[b]	$2A_1$ 185 KDa[c] $2A_2$ 160 KDa[c] $2A_0$ 181 KDa[b]	75 KDa[b]	45 KDa[c]
Subunit structure	G:C	$2A_1$ $\alpha_2\beta\gamma$ $2A_2$ $\alpha\beta_2$ $2A_0$ $\alpha_2\beta\gamma'$	A:[redacted]	monomeric

[a]compiled from references 20,33,41,42 and 43. [b]rabbit skeletal muscle. [c]rat liver. M_r's of subunits are: α-35 KDa; β-69 KDa; γ-58 KDa; γ'-54 KDa; G-103 KDa; C-37 KDa; A-61 KDa; B-15 KDa. KDa is kilodaltons.

phosphatase activities, and for 75% of the phosphorylase *a* kinase (β-subunit) phosphatase activity. Phosphatase-2A (three forms designated $2A_0$, $2A_1$ and $2A_2$ have been identified in mammalian tissues) accounts for about 60% of phosphorylase *a* and glycogen synthase *b* (phosphorylated at various sites) phosphatase activities, and for 25% of the phosphorylase *b* kinase (α-subunit) phosphatase activity (27). Protein phosphatases-1 and 2A have broad substrate specificity, acting on proteins involved in glycogen metabolism (28), glycolysis (29), fatty acid synthesis, cholesterol synthesis, and protein synthesis (30). They also are the major phosphatase activity in the rabbit skeletal muscle directed towards the regulatory protein, inhibitor-2 (31). Phosphatases-$2A_1$ and 2C are also involved in the regulation of muscle contraction (32).

Protein phosphatase-2C (a Mg^{2+}-dependent enzyme) also has a broad substrate specificity but can be distinguished from phosphatase-2A by its extremely low phosphorylase *a* and histone H1 phosphatase activities (20). Protein phosphatase-2C is similar to the enzyme termed 'smooth muscle phosphatase-II' which is active against the phosphorylated form of smooth muscle myosin light chain (M_r 20 000) (32). Protein phosphatase-2B (a Ca^{2+}-calmodulin-dependent enzyme) is the most specific, having activity against only the α-subunit of phosphorylase *b* kinase, inhibitor-1, and myosin light chain at significant rates amongst a large number of substrates tested, and is also specifically inhibited by

trifluoperazine (20). It is possibly identical to Calcineurin or CaM-BP_{80} (33,34,35), a major calmodulin-binding protein in neural tissue.

B. Distribution and subunit composition

Protein phosphatase-1 is the major phosphatase activity associated with the protein-glycogen complex in both skeletal muscle and liver of rabbit while the type-2 phosphatases are located exclusively in the cytosol (28,36; see Table I.1). Tamura and Tsuiki reported that protein phosphatase activity in rat liver extracts can be resolved into three fractions, namely IA, IB and II, with approximate M_r's of 69 000, 300 000, and 160 000, respectively, on gel filtration (37,38). Protein phosphatases-IA, IB and II correspond to protein phosphatases-2C, $2A_1$ and $2A_2$, respectively (28).[1]

SDS-PAGE revealed that homogeneous preparation of rat liver phosphatase-$2A_1$ contains three types of subunits, namely α, β and γ of M_r 35 000, 69 000, and 58 000, respectively (39). Densitometric tracing suggest a subunit composition of $\alpha_2\beta\gamma$ for phosphatase-$2A_1$ while gel filtration indicates an approximate M_r of 260 000. However, an M_r of 185 000 was obtained from sucrose density centrifugation indicating that phosphatase-$2A_1$ is asymmetric. The α subunit is catalytically active, and is found in the 35 000 M_r

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[1]The nomenclature used for the protein phosphatases in this thesis are those from Cohen's laboratory and are presently widely used by investigators in this field of research.

protein phosphatase prepared from liver extracts through ethanol precipitation at room temperature (39,40). Phosphatase-$2A_2$ contains only the α and β subunits and has a subunit composition of $\alpha\beta_2$ (40). Similar subunit structures and composition have been reached for phosphatases-2A, and $2A_2$ from rabbit skeletal muscle while phosphatase-$2A_0$ from this tissue has a smaller γ subunit (M_r 54 000) with a subunit composition of $\alpha_2\beta\gamma$ (41). There are indications that phosphatase-$2A_2$ is generated from phosphatase-2A, by loss of the γ subunit during purification (28,39,40).

Protein phosphatase-2B from rabbit skeletal muscle is composed of two subunits with approximate M_r's of 60 000 and 15 000 [an M_r of 19 200 was obtained from amino acid sequencing of this latter subunit (42)], and the rabbit skeletal muscle and bovine brain enzymes are structurally very similar (33,34). Protein phosphatase-2C from rat liver is a monomeric enzyme (M_r 45 000) (43) and has identical structural and catalytic properties with the turkey gizzard enzyme (32). Protein phosphatase-1 from rabbit skeletal muscle has a molecular mass of 137 000 and contains two subunits- G (M_r 103 000) and C (M_r 37 000) [same as the type-1 catalytic subunit present in the M_r 35 000 phosphatase preparations] in a 1:1 molar ratio (44).

C. Protein effectors

Inhibitors-1 and 2

The initial assertion on the possible existence of protein inhibitors of protein phosphatases was made by Lee and co-workers (10,19) who observed the presence of heat-stable, ethanol- and trypsin-labile proteins in extracts from rabbit liver that were inhibitors of phosphorylase *a* phosphatase from the same tissue. The finding was extended by Huang and Glinsmann in their purification of two different phosphorylase *a* phosphatase inhibitor proteins, termed inhibitors-1 and 2, from rabbit skeletal muscle (45).

Inhibitor-1, a 26 000 M_r protein, is active only after phosphorylation on a specific threonine, 35 residues from the N-terminus (46,47), by cAMP-dependent protein kinase (45). The phosphorylation of rabbit skeletal muscle inhibitor-1 increases in response to adrenalin (48), and decreases in response to insulin (49). These observations suggest a physiological role for this inhibitor protein in the hormonal control of glycogen metabolism.

Inhibitor-2 is a 33 000 M_r protein (same as the M_r 31 000 subunit of the Mg.ATP-dependent protein phosphatase; see **Section D**) that interacts with phosphatase-1 in two distinct ways. At low concentrations (K_d = 0.1 nM), it interacts in a 1:1 molar ratio with the catalytic subunit of protein phosphatase-1 to produce an enzyme species indistinguishable from the Mg.ATP-dependent protein phosphatase

(50,51, and see **Section D.**). Activation of this species requires incubation with Mg.ATP and a protein kinase termed factor F_A (52), identified as glycogen synthase kinase-3 (53), and results from phosphorylation of the inhibitor-2 on a threonine residue (50,51). At higher concentrations (K_d = 5 nM), inhibitor-2 inactivates protein phosphatase-1 by a second mechanism which appears to result from direct binding of the inhibitor protein to a distinct site on the enzyme, termed site-2 (50).

Both forms of inhibitor proteins have been reported to occur in other tissues and are specific inhibitors for protein phosphatase-1 as they do not affect type-2 phosphatases (21,22).

Activators

Phosphorylase *a* phosphatase, isolated from swine adrenal cortex, was shown to be activated by a heat-stable protein from the same tissue (54). This activator was subsequently identified as histone H1 (55), and later observations led to the discovery of 'latent' phosphorylase *a* phosphatases in rabbit skeletal muscle (56) and bovine aorta smooth muscle (57) that were markedly stimulated by histone H1 and other highly basic polypeptides like protamine and polylysine. These polypeptides were shown in a recent study to cause appreciable increases in the phosphorylase *a* phosphatase activity of protein phosphatase-2A in rat liver cytosol (25).

While the dephosphorylation of phosphorylase *a* by protein phosphatase-1 present in the rat liver cytosol was inhibited by protamine and polylysine, the enzyme's activity on other substrates such as glycogen synthase, acetyl-CoA carboxylase and pyruvate kinase, was stimulated. However, protamine and polylysine do not exist in mammalian cells and histone H1 is present in the nucleus, and these facts shed some doubts on the possible physiological significance of the effects of these compounds on protein phosphatase activities (58).

In another study using homogeneous enzymes from rabbit skeletal muscle and partially purified preparations from rat liver, spermine increased the activity of protein phosphatase-2A towards eight of nine substrates tested (26). The most impressive activation was obtained with glycogen synthase phosphorylated at site-3 (8- to 15-fold). Spermine, however, inhibited the dephosphorylation of phosphorylase *a* by protein phosphatase-2A. A similar pattern was observed for protein phosphatase-1. The effects of spermine were suggested to be the result of its interaction with the protein phosphatases and their substrates, and an attractive hypothesis was made that the spermine activation of protein phosphatase-1 and/or 2A may underlie the relatively specific dephosphorylation of site-3 of glycogen synthase by insulin (26,58).

D. Mg.ATP-dependent protein phosphatase

Merlevede and Riley first reported this enzyme activity in adrenal cortex (59). It has been identified subsequently in a wide variety of tissues including dog liver, rat and rabbit liver, heart, and skeletal muscle (reviewed in ref. 60), and pig brain (61). The enzyme is present in an inactive form consisting of a catalytic protein (F_C) and a modulator subunit (M), a unique protein previously characterized as phosphatase inhibitor-2 (62). The inactive F_C.M complex requires preincubation with Mg.ATP and an activating protein termed F_A (52), identified as glycogen synthase kinase-3 (53), to exhibit activity.

A mechanism of activation of this enzyme (F_C.M) has been proposed by Jurgensen *et al.* (62) in which the transient phosphorylation of the modulator subunit by the kinase is the driving force for the transition of the inactive catalytic subunit (F_C) into its active conformation. A more recent study using adenosine 5'-(γ-thio)triphosphate concluded that the modulator protein does not prevent the conformational change in the F_C subunit but partially inhibits the expression of the phosphorylase *a* phosphatase activity (64).

The relationship between the 'native' Mg.ATP-dependent protein phosphatase and protein phosphatase-1 isolated from rabbit skeletal muscle has been studied in great detail (65,66,67,68). The Mg.ATP-dependent protein phosphatase consists of two major proteins of approximate M_r's of 37 000

and 31 000, in a 1:1 molar ratio. The 37 000 M_r subunit co-migrates with the catalytic subunit of protein phosphatase-1 from rabbit skeletal muscle while the 31 000 M_r subunit comigrates with authentic inhibitor-2 from the same tissue. The C subunit of protein phosphatase-1 (see **Section B**) is identical in all respects to the 37 000 M_r type-1 phosphatase catalytic subunit, while its G subunit interacts with inhibitor-2 to form an inactive species indistinguishable from the native Mg.ATP-dependent protein phosphatase.

E. Objective for studies in this thesis

It has long been known that glucose, caffeine, or nicotinamide accelerated the inactivation (dephosphorylation) of phosphorylase *a* in rat liver extracts (69,70). Extensive investigation of the glucose effect led to the hypothesis by Hers, Stalmans and their associates, that phosphorylase *a* acts as the glucose receptor of the liver cell (71,72, reviewed in ref. 73). Thus, by reducing the level of phosphorylase *a* (an inhibitor of glycogen synthase phosphatase) through its activation of phosphorylase *a* phosphatase, glucose action results in the activation of glycogen synthase leading to deposition of glycogen under hyperglycemic condition.

Some studies (12,74,75,76) employing isolated enzymes from rabbit skeletal muscle have been carried out towards understanding at the molecular level, the effects of ligands

on phosphorylase *a* phosphatase. However, under physiological conditions, there is no free glucose in mammalian muscle (77); hence the effect of glucose in this tissue would be of little practical significance.

Numerous studies over the years by several investigators, particularly Madsen and his associates, employing X-ray crystallography and enzyme kinetics, have revealed a number of major ligand binding sites on rabbit skeletal muscle phosphorylase *a* in its T-conformation (78,79). In the course of these studies, a nucleoside inhibitor site specific for nucleosides as well as purine derivatives such as caffeine, which are synergistic with glucose in inhibiting phosphorylases from muscle and liver, was defined (80,81). Intact liver cells show synergism between glucose and caffeine in the rate of inactivation (dephosphorylation) of phosphorylase *a* and the activation of glycogen synthase (82). However, the molecular basis for this effect had not been proven.

The projects undertaken in this study entail the purification of phosphorylase *a* and phosphorylase *a* phosphatase(s) from rabbit liver to study the effects of glucose and caffeine, separately and together, on the rate of the phosphatase reaction. Other effectors such as nucleotides and metabolic intermediates were to be tested for their modulation of the system (see **Chapter II**). Also, studies were to be done to distinguish between substrate and/or enzyme-directed effects of the different ligands that

modulate the phosphorylase *a* phosphatase activities (see Chapter III). Further, the inhibitory effect of phosphorylase *a* on the glycogen synthase phosphatase activity, the molecular essence of the Hers' hypothesis, was to be investigated in line with the ligand effects seen with phosphorylase *a* phosphatase (see Chapters IV and V).

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II. Additive activation of rabbit liver phosphorylase a phosphatases by glucose and caffeine[1]

A. Introduction

Holmes and Mansour (1) observed that incubation of rat diaphragm muscle in glucose caused a marked decrease in the activity of phosphorylase *a*. In other studies, Hers, Stalmans and their associates showed that glucose, caffeine or nicotinamide, and *in vivo* administration of glucocorticoids activated phosphorylase *a* phosphatase activity in rat liver filtrates (2,3). Their results led to the hypothesis that phosphorylase *a* acts as the glucose receptor of the liver cell (4, reviewed in refs. 5 and 6).

Determination of the molecular structure and major ligand binding sites on rabbit skeletal muscle phosphorylase *a* allowed Madsen and his associates to suggest a molecular basis for the Hers' hypothesis (7,8; see **Introduction to Chapter III**). In addition, a new inhibitor site on phosphorylase, specific for nucleosides and purine derivatives such as caffeine, which are synergistic with glucose in inhibiting phosphorylases from rabbit skeletal muscle and liver, was defined (9,10). Although the natural effector for this site remained undefined, caffeine is effective with glucose in accelerating the phosphorylase *a* phosphatase activity in intact rat hepatocytes (11), or

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[1]A version of this chapter has been published. Monanu, M. O., and Madsen, N. B. (1985) *Can. J. Biochem. Cell Biol.* **63**, 115-121.

in vitro (12; see **Results**).

Published reports in recent years indicated the existence of multiple molecular forms of protein phosphatases from both the skeletal muscle and liver of rabbit, which are capable of acting on a large number of substrates including phosphorylase *a* (13, reviewed in refs. 14 and 15). As is presently known, protein phosphatases-1 and 2A ($2A_0$, $2A_1$ and $2A_2$) account for virtually all the phosphorylase *a* phosphatase activity in dilute extracts from a wide variety of mammalian tissues including rabbit skeletal muscle and liver (16,17).

A number of studies, aimed at elucidating at the molecular level the ligand effects on phosphorylase *a* phosphatase activity, have been done using phosphorylase *a* phosphatase purified from rabbit skeletal muscle [possibly protein phosphatase-1 (16)] (18,19,20,21). However, there is no free glucose in this tissue under physiological conditions (22), hence the effect of this physiologically important ligand would be of little practical significance. Some other studies on the characterization and regulation of hepatic phosphorylase *a* phosphatase activity [possibly a mixture of the catalytic entities of protein phosphatases-1 and 2A (23,24)] (25,26,27) have used muscle phosphorylase *a* as substrate, often with the phosphatase activators, Mg^{2+} and caffeine or theophylline, present in the assay system. There is therefore the need to investigate the ligand action on the more recently defined holoforms of hepatic

phosphorylase *a* phosphatases with substrate from the same tissue, in the absence of activators in the basal assay.

Furthermore, in light of the synergism reported for the glucose-caffeine effect on phosphorylase *a* (9,10), studies of the hepatic proteins would provide better insight on the role of glucose as a physiological regulator of glucose homeostasis through its action on phosphorylase *a*.

B. Materials and Methods

Materials

Frozen rabbit livers were obtained from Pel-Freez Biologicals; [γ-^{32}P]-ATP, specific radioactivity 2900 Ci/mmol, was from New England Nuclear; Sephadex G-25 (fine and coarse grades), DEAE-Sephadex A-50, and AMP-Sepharose (N-linked) were from Pharmacia; aminohexyl-agarose, aminobutyl-agarose, aminohexyl-Sepharose 4B, ATP and AMP (99-100% pure), caffeine, human saliva α-amylase (type IX-A), rabbit liver glycogen (type III), and imidazole chloride were from Sigma; Dowex 1-Cl was from Bio Rad. The rabbit liver glycogen was purifiec Dowex 1-Cl and assayed as described by Ashwell (28) and the concentration expressed as the molar equivalent of its glucose residues; the imidazole chloride was recrystallized from acetone before use. DE 52 was obtained from Whatman; immersible CX-30 ultrafiltration units were from Millipore Corporation; Zeineh Instant dialyzer was from Biomed Instruments,

California, and D-glucose was from Fisher Scientific. New Zealand white rabbits were obtained through the Health Sciences animal unit of the University of Alberta and were maintained on normal diet. All other chemicals used were of the highest grades commercially available.

Purification of rabbit liver phosphorylase b

Although reports have been published on the purification of this protein (29,30,31), these procedures employed isolation of glycogen particulate fraction, a cumbersome process that results in low yields of pure protein. The project described in this thesis entails the use of liver phosphorylase *a* as a substrate for the phosphatase rather than as a catalyst, hence the need for large amounts of purified protein. This required the development of a more 'convenient' and suitable purification scheme that would overcome the handicaps of earlier methods.

Step1 [Acid precipitation]:

All steps were done at 4°C except where otherwise noted. About 350 gm frozen liver was broken into small pieces and homogenized in 3 volumes of cold buffer [20 mM imidazole-HCl (pH 7.0), 5 mM EDTA, 1 mM DTT, 0.25 M sucrose, 5 mM benzamidine, and 1 mM PMSF] in a Waring blender for 1 min at medium speed. The pH of the homogenate was adjusted to 5.8 with 1 N acetic acid (usually 16 ml). The homogenate was then centrifuged at 10 000 g for 40 min to remove cell debris and precipitated proteins. Following passage of the

supernatant through cheese cloth the pH was adjusted to 7.0 with 2 M Tris base.

Step 2 [$(NH_4)_2SO_4$ precipitation]:

The supernatant was brought to 70% saturation with solid $(NH_4)_2SO_4$ and the precipitate collected, after storing for 10-20 min, by centrifugation at 10 000 g for 20 min. 2 M Tris base was added to maintain pH during salt addition.

Step 3 [Polyethylene glycol (PEG 8000) precipitation]:

The pellet from step 2 was dissolved in buffer A [50 mM imidazole-HCl (pH 7.4), 5 mM EDTA, 0.5 mM DTT] up to a final volume two-and-a-half times the starting liver material. PEG was added from a 50%(wt/v) stock PEG in buffer A to 10%(v/v) final concentration. After continuous stirring for 20 min, the precipitate was collected by centrifugation at 10 000 g for 20 min and resuspended in buffer A containing [redacted] mM benzamidine using a Potter-Elvehjem homogenizer. The protein solution was incubated at 30°C for 1 hr to ensure complete conversion to phosphorylase *b* by endogenous phosphatase, and then dialyzed against 20 volumes of buffer A for 16-18 hr.

Step 4 [Aminohexyl-agarose chromatography]:

The dialyzate from step 3 was diluted to 300 ml with dialysis buffer and applied to a 2.5x30 cm aminohexyl agarose column equilibrated with buffer A. The column was washed with the same buffer until the absorbance at 280 nm was close to baseline. Bound protein was eluted with a linear gradient made from 500 ml buffer A with and without 0.7 M NaCl. Active fractions were pooled, concentrated by

70% $(NH_4)_2SO_4$ saturation and salt removed by dialysis against buffer B [25 mM imidazole-HCl (pH 7.4), 5 mM EDTA, 1 mM DTT].

Step 5 [DE 52 chromatography]:

The dialyzate from three successive preparations (up to step 4) was applied at 80 ml/hr to a 2.5x35 cm DE 52 column equilibrated with buffer B. After washing with two bed volumes of buffer, bound protein was eluted with a linear gradient made from 600 ml buffer B with and without 0.5 M NaCl. Active fractions were pooled, concentrated by 60% $(NH_4)_2SO_4$ saturation, and desalted by overnight dialysis against buffer A.

Step 6 [AMP-Sepharose 4B chromatography]:

The protein solution obtained from two successive preparations (up to step 5) was applied at 10 ml/hr to a 1.6x20 cm AMP-Sepharose 4B column equilibrated with buffer A. Following a wash with two bed volumes of buffer A, bound protein was eluted with buffer A containing 0.4 M NaCl. Active fractions were pooled, desalted by dialysis against buffer containing 0.5 mM EDTA, and concentrated using immersible CX-30 ultrafiltration units. The concentrated protein was stored at 4°C prior to use in the preparation of ^{32}P-labelled phosphorylase *a*. The enzyme preparation to this stage had specific activity of 20-26 U/mg as determined using 16 mM glucose 1-P, 1% rabbit liver glycogen, 5 mM AMP, 0.7 M Na_2SO_4, pH 6.5, at 30°C and exhibited a single band on SDS-PAGE. About 36 mg of pure

liver phosphorylase *b* was usually obtained from 1 kg of frozen liver within 10 days.

Purification of rabbit liver protein phosphatases

The 35 000 M_r phosphatase was prepared from frozen rabbit liver by ethanol precipitation at room temperature by the procedure developed by Brandt *et al.* (25), up to and including the first DEAE-Sephadex A-50 step. The specific activity of the phosphatase preparation used for most of the studies was about 2 000 U/mg using liver phosphorylase *a* at 40 μM as determined by the assay described in this report (note the absence of phosphatase activators like Mg^{2+} and caffeine or theophylline). The enzyme was stable for over 6 months when stored at -20°C in the presence of 60% glycerol.

Protein phosphatases-$2A_1$ and $2A_2$ were purified (up to the first DE 52 and aminohexyl-Sepharose 4B chromatographies) from fresh livers from mature New Zealand white rabbits following the procedure of Tamura and Tsuiki (32). Further purification had been shown to cause dissociation of these holophosphatases (23,32). The preparations of phosphatases-$2A_1$ and $2A_2$ had specific activities of 1.2-1.6 and 0.07-0.09 U/mg, respectively, using liver phosphorylase *a* at 40 μM with the assay in this report (see above). Both enzyme preparations were dialysed against buffer containing 50% glycerol and stored at -20°C, under which condition they retained activity for several weeks.

Protein phosphatase-1, the main protein phosphatase activity in the protein-glycogen complex (17), was prepared from fresh livers obtained from mature New Zealand white rabbits by a procedure adapted from the work of Ingebritsen *et al.* (23). All steps were done at 4°C unless otherwise indicated. Liver tissue was homogenized in 3 volumes of 4 mM EDTA, 0.25 M sucrose, 0.1%(v/v) 2-mercaptoethanol (pH 7.0) for 1 min at 13 000 rpm using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 6 000 g for 10 min and the supernatant passed through cheese cloth. The protein solution was then centrifuged at 100 000 rpm for 90 min. The clear glycogen pellet was resuspended by homogenization in buffer A [10 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 0.1%(v/v) 2-mercaptoethanol] containing 1 mM PMSF, 1 mM benzamidine, and 1 μg/ml of leupeptin and pepstatin.

The suspension was incubated at 30°C for about 40 min in the presence of 300 U/ml of human saliva α-amylase to digest the glycogen present. The solution was cooled to 4°C and applied to a 2.5x15 cm DE 52 column equilibrated with buffer A. After washing with two bed volumes of buffer A containing 0.05 M NaCl, a 400 ml linear gradient of 0.05-0.5 M NaCl in buffer A was developed. Active fractions, assayed with phosphorylase *a*, were pooled, concentrated using CX-30 ultrafiltration unit, and dialyzed against buffer containing 50% glycerol. The preparation was stored at -20°C and retained activity for several weeks.

With phosphorylase *a* as substrate, protamine sulfate inhibited the protein phosphatase-1 but not protein phosphatases-$2A_1$ and $2A_2$. This is the expected result for one of the criteria used to distinguish the types-1 and 2 protein phosphatases (33,34).

Protein concentration

Protein concentration of purified proteins was determined from absorbance measurements at 280 nm using the absorbance index $E^{1\%}_{1cm}$ of 13.2 for phosphorylase (35) and assuming an $E^{1\%}_{1cm}$ of 10 for the protein phosphatases.

Preparation of rabbit liver ^{32}P-labelled phosphorylase a

This was prepared from liver phosphorylase *b* using [γ-^{32}P]-ATP and rabbit skeletal muscle phosphorylase *b* kinase as described by Krebs *et al.* (36). The kinase was removed by passing the ^{32}P-labelled phosphorylase *a* preparation through a 2x5 cm aminobutyl-agarose column equilibrated with 50 mM imidazole-HCl (pH 7.4), 5 mM EDTA, 0.5 mM DTT, and 30 mM NaCl under which condition the kinase is retained (Dr. B. Osterlund, personal communication). Contaminating nucleotide was removed by extensive dialysis against buffer containing 50 mM glucose and 1% activated charcoal. The preparation was further purified by Sephadex G-25 (fine) chromatography. Those fractions with $A_{260/280}$ ratios of 0.57-0.58 (37) were pooled, concentrated using an immersible CX-30 ultrafiltration unit, and then used in the

phosphatase experiments. The specific activity of the ^{32}P-labelled phosphorylase *a* was 45-50 U/mg assayed with 16 mM glucose 1-P, 2% rabbit liver glycogen, pH 6.5, at 30°C.

Preparation of rabbit skeletal muscle phosphorylase a

This was prepared as described for the liver protein using muscle phosphorylase *b* and either ATP or [γ-^{32}P]-ATP. The phosphorylase *a* preparations were recrystallized three times before chromatography on Sephadex G-25 (fine) to remove contaminating nucleotide.

Phosphorylase a phosphatase assay

Phosphorylase *a* phosphatase activity was measured by the release of ^{32}P from ^{32}P-labelled phosphorylase *a* at 30°C by a modification of method I of Haschke *et al.* (38). The standard incubation mixture contained 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.5 mM EDTA, 0.1 mg BSA, ^{32}P-labelled phosphorylase *a*, and the phosphatase preparation in a total of 100 μl. For phosphatases-1, $2A_1$ and $2A_2$, the reaction mixture also contained 10%(v/v) glycerol in addition. Ligands and substrate, in a total of 50 μl, were preincubated for 10-15 min before starting the reaction by the addition of 50 μl of phosphatase (also preincubated). The enzyme was suitably diluted and the reaction was timed to allow for release of not more than 20% of the radioactive phosphate. Under these conditions the reaction was

proportional with time. The reaction was terminated by the addition of 350 µl of ice-cold 10%(v/v) TCA and 50 µl of ice-cold 25 mg/ml BSA was then added. After storage at 4°C for 10 min, all tubes were centrifuged at 12 000 rpm for 10 min in a Beckman microfuge 12. Aliquots of the clear supernatant were then counted for radioactivity with 5 ml of Aquasol scintillation fluid in a Beckman LS 6800. Blank values obtained when the enzyme was added after the stopping reagent were subtracted from all assays. Total available substrate was determined by digesting protein pellets from blank tubes with 500 µl 88% formic acid and aliquots equivalent to those in the tests were counted. Phosphatase activities were expressed in nanomoles of ^{32}P per minute per milligram of enzyme and relative activities were obtained by setting the control activity (no additions) at 100%.

C. Results

Effects of Mg^{2+}, glucose, and caffeine on K_m and V_{max}

Glucose and caffeine, acting alone or in combination, had variable activating effects reflected by increases in V_{max} for the different phosphatase forms used in this study. As shown in Table II.1, these increases were in most cases associated with small (for the 35 000 M_r phosphatase) to appreciable (for phosphatases-$2A_1$ and $2A_2$) increases in K_m. The K_m of the 35 000 M_r phosphatase for muscle phosphorylase *a* was about 4 µM in the absence of ligands;

Table II.1. Effects of Mg^{2+}, glucose and caffeine, in different combinations, on K_m and V_{max} of three rabbit liver phosphatase forms using rabbit liver and skeletal muscle ^{32}P-labelled phosphorylase *a*.

Phosphorylase *a* phosphatase activity was determined as described under Materials and Methods. Final ligand concentrations used were: (A) glucose (10 mM) and caffeine (1 mM); (B) glucose (10 mM), caffeine (1 mM) and $MgCl_2$ (5 mM). K_m and V_{max} values were obtained using the Wilkinson kinetics computer program and are expressed in micromolar concentrations and nanomoles ^{32}P per minute per milligram phosphatase, respectively. Errors quoted are the standard errors derived by the program and are for individual determinations of K_m and V_{max}, as described by Wilkinson (39).

Table II.1

Conditions	Phosphatase forms							
	35 000 M_r phosphatase				Phosphatase $2A_1$, using liver substrate		Phosphatase $2A_2$, using liver substrate	
	Using muscle substrate		Using liver substrate					
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
				Experiment A				
Control	4.3±0.29	91.8±2.4	54.6±5.7	2281±123	17.1±4.5	0.21±0.03	12.6±3.5	1.68±0.3
Glucose	7.3±0.65	234.1±9.9	56.8±8.3	3589±279	8.5±2.1	0.12±0.01	35.6±12.6	6.08±1.7
Caffeine	6.6±0.65	264.0±11.7	89.8±8.1	5168±291	13.2±2.4	0.19±0.02	77.3±19.4	16.10±4.4
Glucose–caffeine	7.7±0.81	326.5±16.3	74.1±4.3	4876±165	25.8±4.3	0.39±0.04	123.7±33.4	33.5±11.6
				Experiment B				
Control					19.8±3.5	0.26±0.02	16.4±4.8	2.61±0.5
$MgCl_2$					30.5±5.2	0.74±0.07	25.7±6.8	7.21±1.2
$MgCl_2$–glucose					31.6±4.7	0.88±0.08	65.8±13.8	26.8±6.0
$MgCl_2$–caffeine					47.4±7.6	1.56±0.17	45.9±10.3	24.2±4.3
$MgCl_2$–glucose–caffeine					35.4±2.2	1.62±0.06	47.8±6.2	29.5±3.2

about 1.7-fold increase in K_m observed with glucose and caffeine acting independently or together was associated with a fourfold increase in V_{max} for the ligands acting together and smaller increases for independent actions. With liver substrate, about 13-fold and 25-fold increases in K_m and V_{max}, respectively, over the value obtained with muscle substrate were observed for this phosphatase form. Thus, while showing a better activity with the "natural substrate", the liver 35 000 M_r phosphatase had a lower apparent affinity for the liver substrate when compared with the muscle substrate. Furthermore, there was no additive effect of glucose and caffeine when the liver substrate was employed.

The holophosphatases-$2A_1$ and $2A_2$ exhibited K_m's for the liver substrate which were fourfold lower than those obtained with the 35 000 M_r phosphatase (Table II.1). These findings might be explained by observing that the latter two phosphatase forms represent more closely the native enzyme, as the 35 000 M_r was generated by harsh treatment of crude liver preparations during purification. Mg^{2+} is known to strongly inhibit the 35 000 M_r phosphatase, hence its effect was not tested. However, Mg^{2+} had an activating effect on phosphatases-$2A_1$ and $2A_2$, in agreement with earlier findings (32). The independent and additive effects of glucose and caffeine on V_{max} for these phosphatase forms were more significant with Mg^{2+} present for phosphatase-$2A_1$, while there was a smaller increase in K_m in the presence of Mg^{2+} for

phosphatase-2A$_s$. Thus, the 1.9-fold increase in V_{max} for phosphatase-2A$_1$ in the presence of both glucose and caffeine increased to about fourfold with Mg^{2+} present, suggesting an almost complete dependence on Mg^{2+} by this phosphatase form. For phosphatase-2A$_2$, the 10-fold increase in K_m with glucose and caffeine present decreased to 3-fold with Mg^{2+}, while the 20-fold increase in V_{max} dropped to a 10-fold increase.

Relief of inhibition by glucose and caffeine

Glucose and caffeine, acti[redacted]alone and in combination, in most cases relieved the inh[redacted]on by the various inhibitors tested. For the 35 [redacted] phosphatase using muscle substrate, data in Fig. II.1 show that the inhibition by ATP (2.5 mM) was relieved by combined activations by glucose (1.5 mM) and caffeine (25 μM). These concentrations were used in the quoted data to show the sensitivity of the system, but the effects were repeatable at higher values. Inhibition by phosphorylase *b* (0.1 mM) (not shown) and inorganic phosphate (50 mM) was severe and relief was not afforded by the presence of both activators. Glucose 1-P inhibition was relieved by glucose and caffeine acting independently and together (Fig. II.1). AMP inhibition was observed in the micromolar range (not shown) and relief required a combination of glucose and caffeine at 15 and 1 mM, respectively (% relative activities of 8 in the presence of 30 μM AMP and 88 in the presence of a combination of 15 mM glucose and 1 mM caffeine).

Figure II.1. Effects of different combinations of ligands on the activity of 35 000 M_r phosphatase towards ^{32}P-labelled rabbit skeletal muscle phosphorylase *a*.

Phosphorylase *a* phosphatase activity of rabbit liver 35 000 M_r phosphatase was determined as described under Methods using rabbit skeletal muscle ^{32}P-labelled phosphorylase *a* in the presence of ligands. Final concentrations were: phosphorylase *a* (10 μM), ATP (2.5 mM), ADP (2.5 mM), glycogen (1%), glucose 1-P (G-1-P) (1 mM), P_i (50 mM), glucose (1.5 mM), and caffeine (25 μM). Results are expressed as relative activity with the control value set at 100%. Data in this and subsequent figures in this chapter are from duplicate incubation mixtures for a single set of experiments representative of several repeated trials. *t* statistics for two means was applied to determine the significance ($p<0.03$) of the differences from the control values.

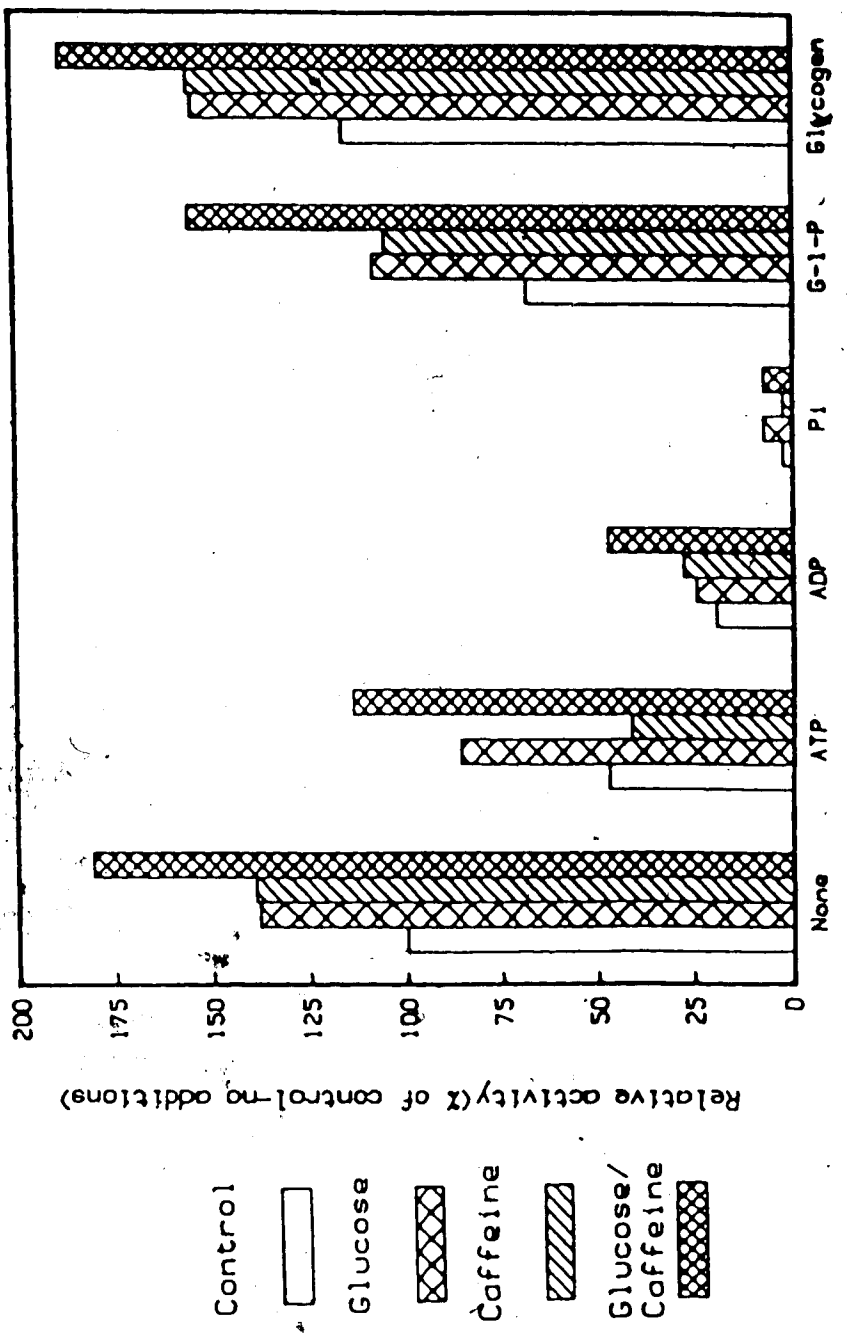
Control
Glucose
Caffeine
Glucose/
Caffeine
Relative activity (% of control-no additions)
200
175
150
125
100
75
50
25
0
None
ATP
ADP
Pi
G-1-P
Glycogen

FIGURE II.1

Using liver substrate, ATP inhibition at 0.2 mM could be overcome by glucose (25 mM) and caffeine (1 mM), acting alone and in combination (data not shown). These concentration of glucose and caffeine were used in this and subsequent experiments because they had been used previously to demonstrate synergistic inhibition of liver phosphorylase *a* (10). AMP (0.3 mM) or ATP plus AMP required the presence of both activators for relief of inhibition. At the presumed physiological concentration in the liver cell of 7 mM for ATP (40), relief of inhibition was not observed (Fig. II.2). With excess of Mg^{2+}, some relief of inhibition by glucose and caffeine was seen but the activity was less than the control value, possibly owing to excess Mg^{2+}, which is inhibitory for this phosphatase form. Glucose 6-P and UDP-glucose inhibitions (Fig.II.2) could be overcome significantly by glucose and caffeine acting alone or in combination. The inhibition by glucose 6-P disagrees with the activating effect seen for the complete muscle system (18,20), probably owing to tissue and assay differences.

Phosphatase-$2A_2$ showed a different pattern with respect to relief of ATP and AMP inhibition by glucose and caffeine, as shown in Fig. II.3. The inhibition by ATP could not be overcome by glucose and caffeine acting alone or together in the absence of Mg^{2+}. Some relief of AMP inhibition was afforded by both ligands. Relief of combined inhibition by ATP and AMP required glucose and caffeine acting together and the activity was below the control value. The presence

Figure II.2. Effects of ligands in different combinations on the activity of 35 000 M_r phosphatase towards ^{32}P-labelled rabbit liver phosphorylase *a*.

Phosphorylase *a* phosphatase activity of rabbit liver 35 000 M_r phosphatase was determined as described under Methods using rabbit liver ^{32}P-labelled phosphorylase *a* in the presence of ligands. Final concentrations were: phosphorylase *a* (40 μM), ATP (7.5 mM), $MgCl_2$ (10 mM), glucose 6-P (G-6-P) (1 mM), UDP-glucose (UDPG) (1.86 mM), glucose (25 mM), and caffeine (1 mM). Results are expressed as relative activity with the control set at 100%.

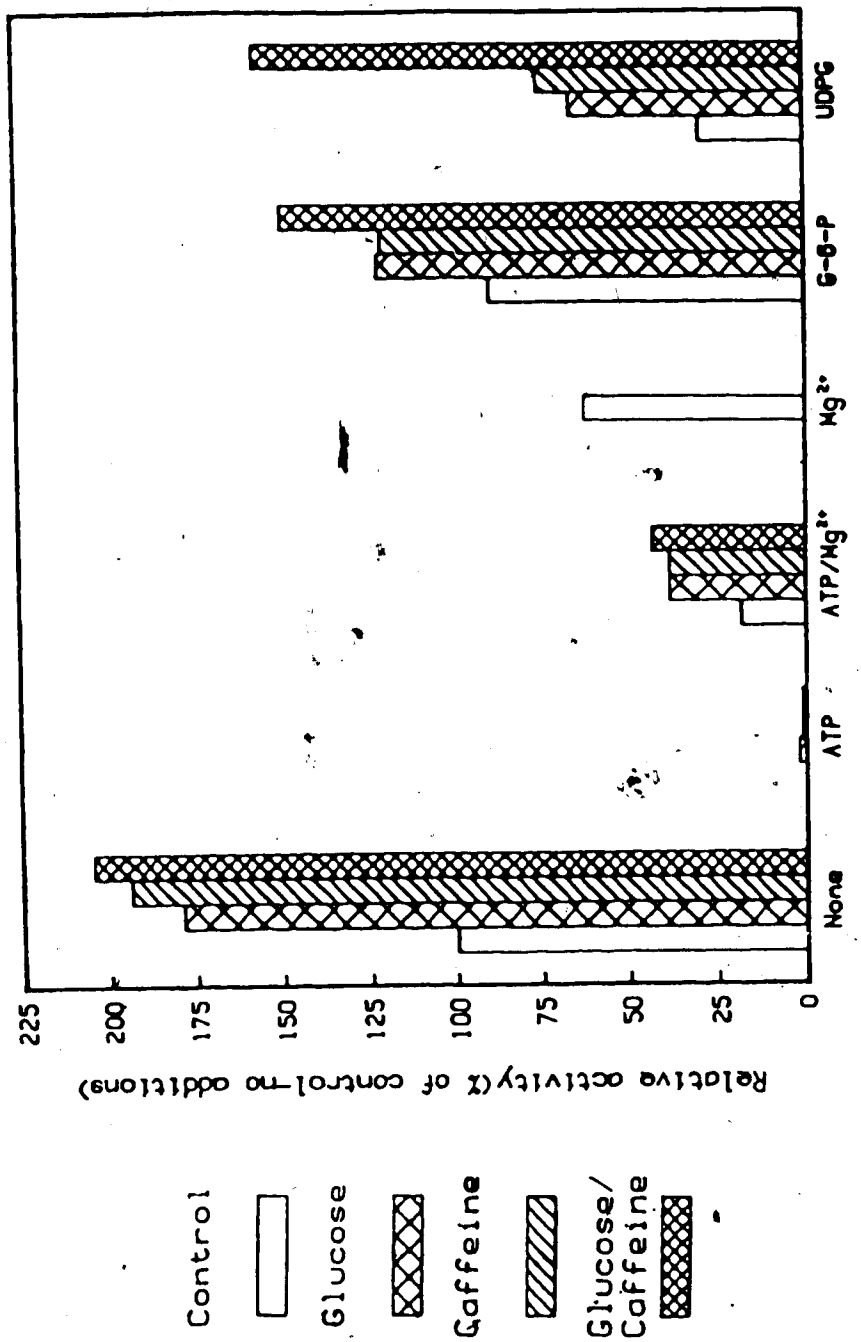
Relative activity (% of control-no additions)
225
200
175
150
125
100
75
50
25
0
None
ATP
ATP/Mg2+
Mg2+
G-6-P
UDPG
Control
Glucose
Caffeine
Glucose/
Caffeine

FIGURE II.2

Figure II.3. Effects of Mg^{2+}, ATP, AMP, glucose, and caffeine, in different combinations, on the activity of phosphatase-$2A_2$ towards ^{32}P-labelled rabbit liver phosphorylase *a*.

Phosphorylase *a* phosphatase activity of purified rabbit liver phosphatase-$2A_2$ was determined as described under Methods using rabbit liver ^{32}P-labelled phosphorylase *a* in the presence of ligands. Final concentrations were: phosphorylase *a* (40 μM), ATP (7.2 mM), AMP (0.3 mM), glucose (25 mM), caffeine (1 mM), $MgCl_2$ (2.8 mM; 9 mM with ATP). Results are expressed as relative activity with the control set at 100%.

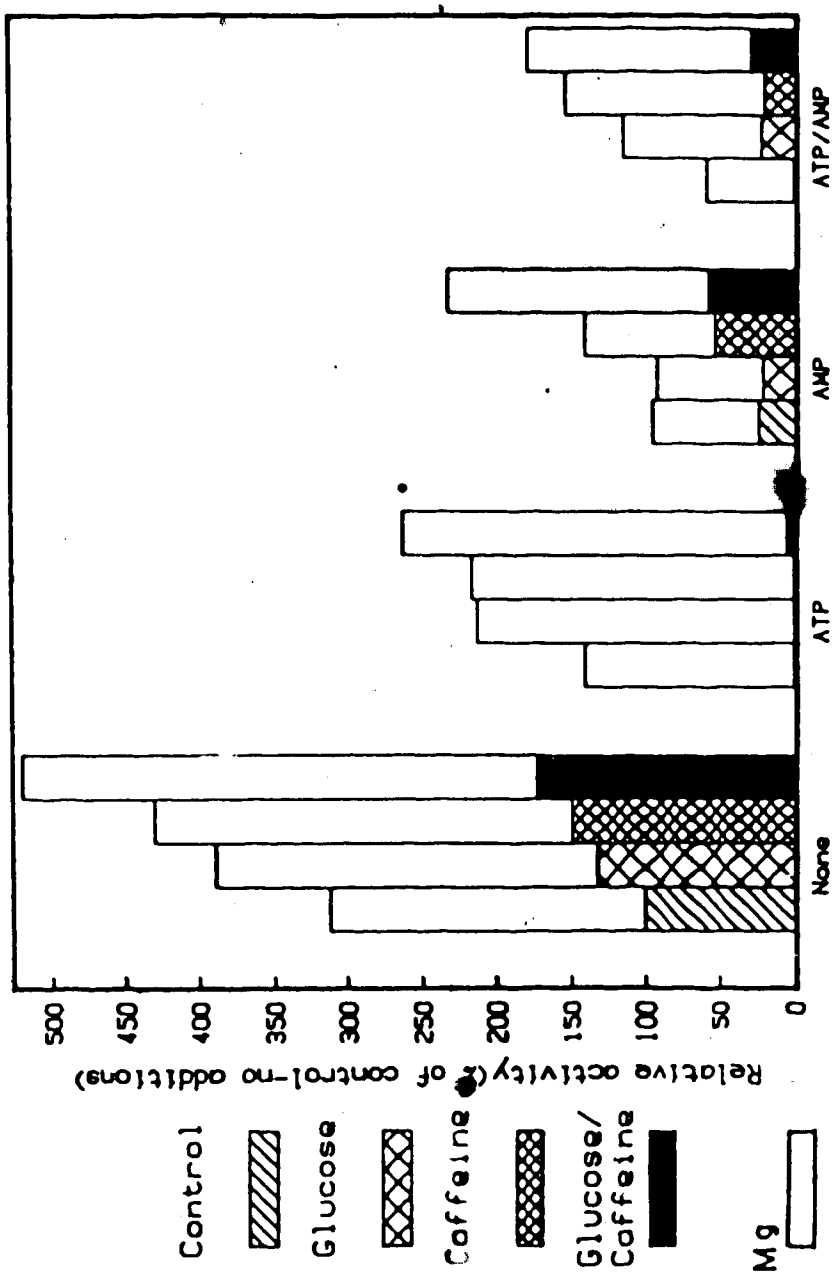
Relative activity (% of control-no additions)
500
450
400
350
300
250
200
150
100
50
0
None
ATP
AMP
ATP/AMP
Control
Glucose
Caffeine
Glucose/
Caffeine
Mg

FIGURE II.3

of Mg^{2+} significantly altered the response of phosphatase-$2A_2$ to ATP inhibition, resulting in relief of inhibition by Mg^{2+} acting alone, as well as with glucose and caffeine acting independently and together (Fig. II.3).

The inhibition of phosphatase-$2A_1$ by ATP could be relieved by glucose and caffeine acting alone and in combination (Fig. II.4). AMP inhibition was abolished by combined activations by glucose and caffeine and the combined inhibition by ATP and AMP was completely overcome only by the combination of glucose and caffeine. Mg^{2+} enhanced the independent and additive activities of glucose and caffeine, suggesting an augmenting role for Mg^{2+} in the relief of inhibition of this phosphatase.

Using muscle phosphorylase *a* as substrate, glucose and caffeine, acting alone and together, activated phosphatase-1 (Fig. II.5). Mg^{2+} also activated this phosphatase either alone or in combination with glucose or caffeine. Phosphatase-1 differed from phosphatases-$2A_1$ and $2A_2$ in its response to either AMP or AMP plus ATP inhibition as the combined presence of glucose, caffeine and Mg^{2+} could not completely overcome the inhibition. The pattern suggests a more potent inhibition of phosphatase-1 by AMP and could reflect important differences in enzymic properties with respect to ligand control.

Figure II.4. Effects of Mg^{2+}, ATP, AMP, glucose, and caffeine, in different combinations, on the activity of phosphatase-2A, towards ^{32}P-labelled rabbit liver phosphorylase *a*.

Phosphorylase *a* phosphatase activity of purified rabbit liver phosphatase-2A, was determined as described under Methods using rabbit liver ^{32}P-labelled phosphorylase *a* in the presence of ligands. Final concentrations were: phosphorylase *a* (40 μM), ATP (7.2 mM), AMP (0.3 mM), glucose (25 mM)), caffeine (1 mM), caffeine (1 mM), $MgCl_2$ (2.8 mM; 9 mM with ATP_4). Results are expressed as relative activity with the control set at 100%.

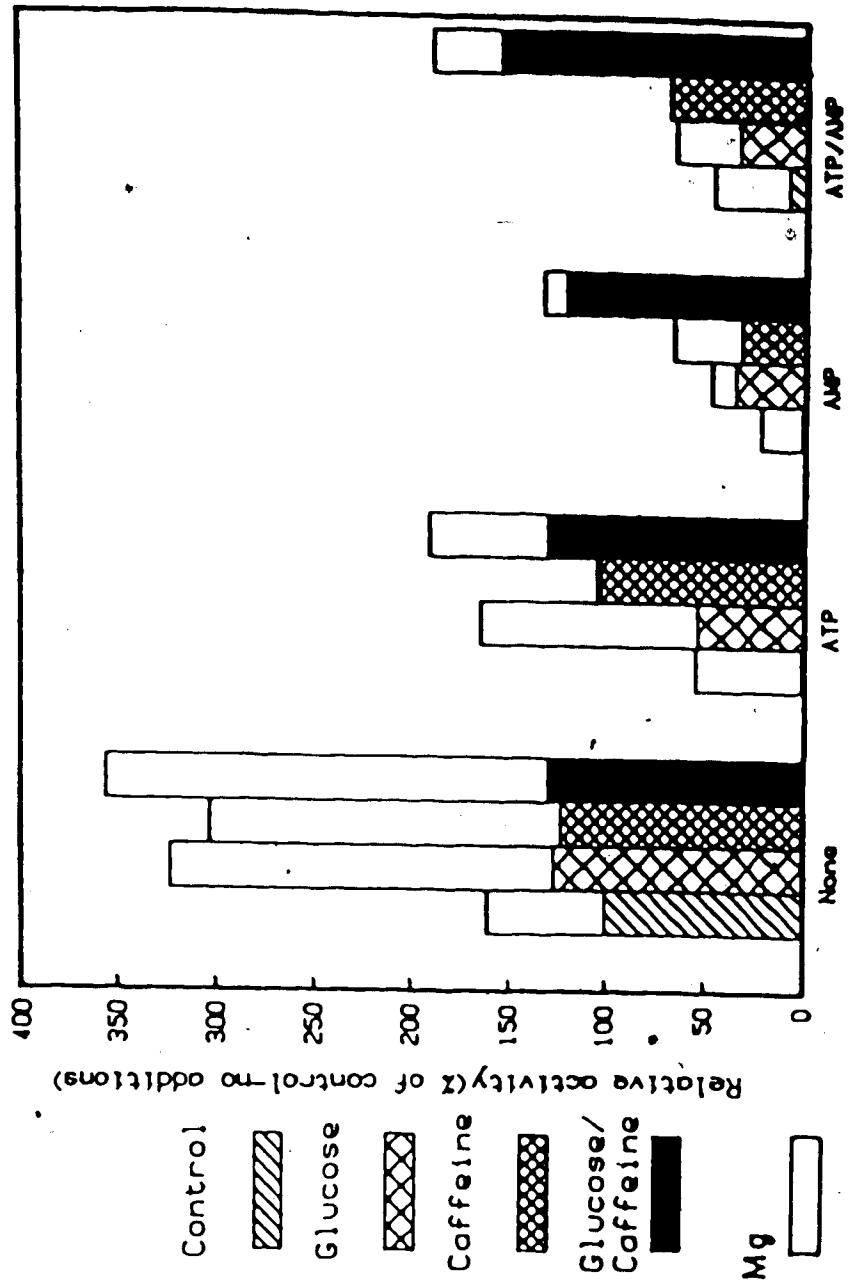
Relative activity(% of control-no additions)
400
350
300
250
200
150
100
50
0
None
ATP
AMP
ATP/AMP
Control
Glucose
Caffeine
Glucose/
Caffeine
Mg

FIGURE II.4

Figure II.5. Effects of Mg^{2+}, ATP, AMP, glucose, and caffeine, in different combinations, on the activity of phosphatase-1 towards ^{32}P-labelled rabbit skeletal muscle phosphorylase *a*.

Phosphorylase *a* phosphatase activity of purified rabbit liver phosphatase-1 was determined as described under Methods using rabbit skeletal muscle ^{32}P-labelled phosphorylase *a* in the presence of ligands. Final concentrations were: phosphorylase *a* (40 μM), ATP (7.2 mM), AMP (0.3 mM), glucose (25 mM), caffeine (1 mM), $MgCl_2$ (2.8 mM; 9 mM with ATP). Results are expressed as relative activity with control set at 100%.

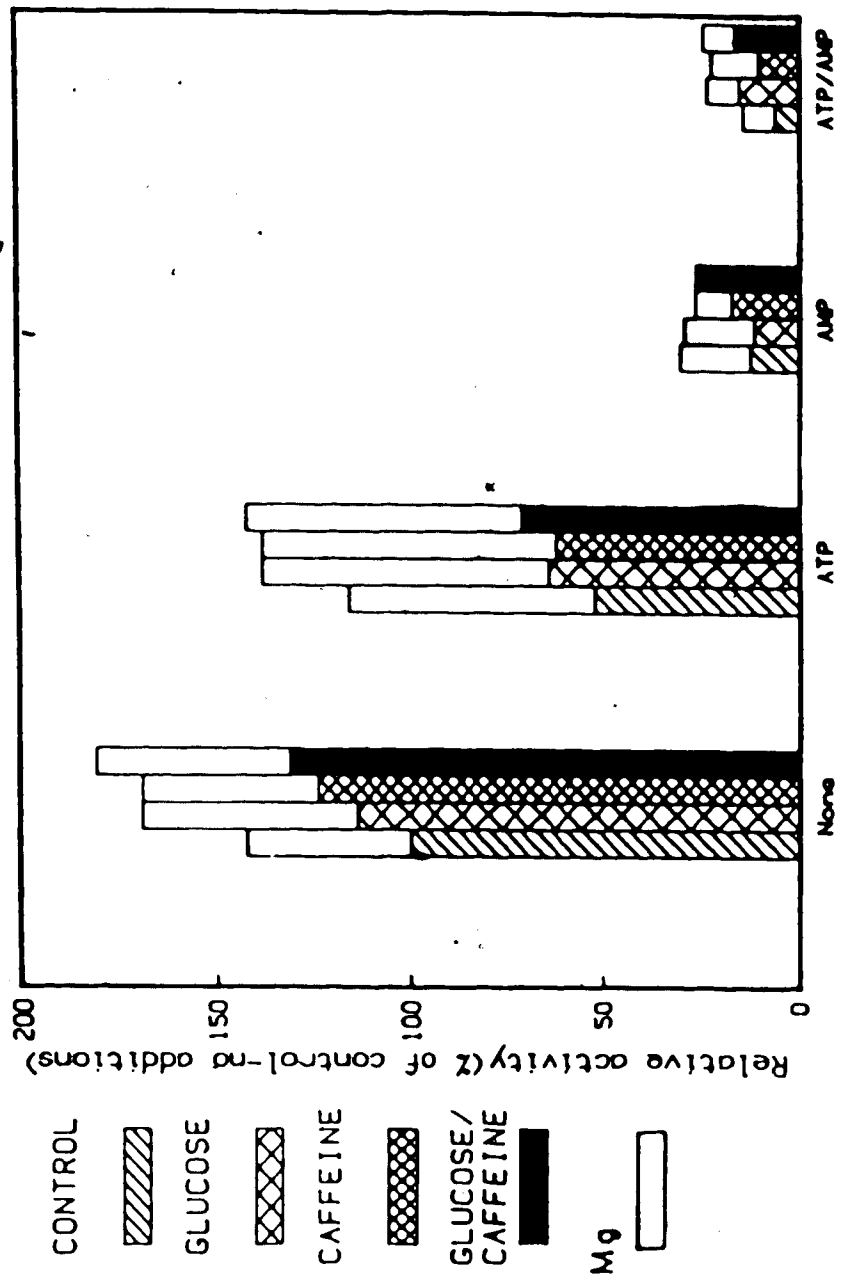
Relative activity (% of control-ng additions)
200
150
100
50
0
None
ATP
AMP
ATP/AMP
CONTROL
GLUCOSE
CAFFEINE
GLUCOSE/
CAFFEINE
Mg

FIGURE II.5

D. Discussion

The results obtained in studies in this chapter show that glucose and caffeine activated the four molecular forms of rabbit liver phosphorylase *a* phosphatase in an independent and additive manner. It is of significance that while glucose was able to overcome the inhibition by AMP or ATP of the 35 000 M_r protein phosphatase, this was not the case for the holoforms, especially when the two nucleotides were present together. In these situations a combination of glucose and caffeine was required. Furthermore, in all cases reported in the figures, the combined effect of glucose and caffeine, at the concentrations tested, was most potent in relieving the inhibition of the different phosphatase forms by the inhibitors tested.

Comparison of the phosphatase forms show an increasingly complex response to ligand control with increasing molecular size. The effect of Mg^{2+} changed from inhibitory for the 35 000 M_r form to stimulatory for phosphatases-1, $2A_1$ and $2A_2$. The 35 000 M_r form also showed different characteristics when acting on its natural substrate, liver phosphorylase *a* than on the substrate from muscle. In addition, the response to relief of inhibitions by various natural ligands such as ATP and AMP by glucose and caffeine, acting independently and in combination, would appear to be more pronounced with the larger phosphatase forms.

It is notable that while the concentration of caffeine required for significant effect was less than millimolar,

that for glucose was 25 mM, representing a 'hyperglycemic condition' which calls for storage of glucose as glycogen in the liver. This is achieved by inactivation of phosphorylase *a* leading to subsequent activation of glycogen synthase by phosphatase activity. Both ligands have been reported to stimulate glycogen synthase phosphatase activity in protein-glycogen suspensions from rat liver (41,42). Some synergism has also been reported for this effect (43) which has been suggested to be due to a direct interaction of these ligands with the phosphatase. In view of previously reported results from Madsen's laboratory (9,11) as well as those in this chapter, we believe that an indirect effect of glucose and caffeine, mediated through binding to phosphorylase *a*, may be exerted on the phosphorylase *a* phosphatase activity, thereby resulting in the activation of glycogen synthase.

While there is still disagreement over the identity of the phosphatase(s) acting on phosphorylase *a* and glycogen synthase *b* (reviewed in ref. 44), the finding that both reside in the same protein preparation is consistent with the observed physiological facts, as well as with the plausible molecular mechanism which provides a basis for the physiology (9,11). However, the existence of glycogen synthase phosphatase(s) which do not act on phosphorylase *a* does not negate the hypothesis advanced by Hers and associates and extended by us since the synthase phosphatase can be inhibited by phosphorylase *a*.

The additive effect by glucose and caffeine seen in this study supports the contention that the inhibitory site on phosphorylase *a*, which was probed with caffeine, may be of physiological significance. Consequently, allosteric modification of phosphorylase *a* remains an intriguing possibility for the control of phosphorylase *a* phosphatase activity *in vivo*.

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III. Distinction between substrate and/or enzyme-directed effects of modifiers of rabbit liver phosphorylase a phosphatases

A. Introduction

A number of physiologically important chemical effectors have been reported that affect the phosphorylase *a* phosphatase activity from both skeletal muscle and liver tissues (reviewed in ref. 1). These effectors could exert their actions by binding to the protein substrate, the phosphatase itself, or to both, resulting in alterations of protein structure that lead to the respective effects. The use of alternate substrates, in which the binding pockets for the ligands have been 'destroyed', allows distinction between ligands effects by any of the three possible modes.

Earlier studies on rabbit skeletal muscle phosphorylase *a* phosphatase (possibly protein phosphatase-1; see Discussion in ref. 2), utilizing phosphorylase *a* and the alternate substrate, a tetradecapeptide encompassing the phosphorylated site on rabbit skeletal muscle phosphorylase *a* (residues 5 to 18), established that AMP inhibition of the phosphatase occurred by its binding to phosphorylase *a* and was therefore substrate-directed (3,4). A separate study employing kinetic measurements supports this view (5). The AMP-phosphorylase *a* complex is a poor substrate for the phosphatase, reflected in a 50% increase in K_m and a V_{max} that is only 5% of the unliganded

substrate (4). Activation of phosphorylase *a* phosphatase by glucose resulted in a 3-fold increase in V_{max} with little change in K_m while there was no effect with the alternate substrate, indicating a substrate-directed effect for this ligand (6). Furthermore, substrate-directed effect has been suggested for activation by caffeine (3).

On the basis of crystallography studies of rabbit skeletal muscle phosphorylase *a*, Madsen and his associates suggested that AMP stabilizes the active R conformation of phosphorylase *a* to which the phosphatase can bind, but in which the serine phosphate is unavailable to its active site. Glucose stabilizes the inactive T conformation in which the serine phosphate is exposed to the phosphatase's active site and perturbation of the phosphatase binding site is insignificant (7,8). NMR results (9) and cross-linking studies (10) support these ideas. Caffeine is synergistic with glucose in promoting the T conformation of phosphorylase *a* (11), and may augment the activation of phosphorylase *a* phosphatase by glucose as was evident from studies done with intact hepatocytes (12) or *in vitro* (13; see **Chapter II** of this thesis). These findings on the effects of ligands on the alteration of the structure of the phosphatase's reaction site on phosphorylase *a* provided a molecular explanation for the observed effects of ligands on phosphatase activity.

Further studies on ligand interactions with phosphorylase *a* indicate that ATP binds to the [redacted]or

site and promotes the T conformation (14). This, by implication, suggested that this nucleotide would activate the phosphatase activity since glucose and caffeine promoted the T conformation and were phosphatase activators. However, results indicate that ATP inhibits the phosphorylase *a* phosphatase activity (5,13). Furthermore, despite numerous investigations, the modes by which ATP and Mg^{2+} affect the phosphatase reaction remain unclear.

Substrate-directed effects have been suggested for Mg^{2+} (3) and ATP (4), and binding sites on phosphorylase *a* for ATP and metal ions have been demonstrated (8,14,15). However, preincubation of extracts from tissues including rat liver (16) or purified protein preparations (17,18,19) with ATP has been shown to inactivate the phosphorylase *a* phosphatase activity present. This inactivation can be prevented or reversed by Mn^{2+} or Co^{2+} (19,20,21). Mg^{2+} has been variously reported to have no effect on the process (16), to protect the enzyme (19,20) or to reverse the inhibition (17,20). These findings suggest enzyme-directed effects for both ATP and Mg^{2+}. The reversal of ATP inhibition by Mg^{2+} has been generally thought to be due to its ability to form a complex with ATP in solution, but a recent report (22) proposed a model in which ATP, Mg^{2+}, Mn^{2+} and fluoride affect the phosphatase activity by binding to individual sites on the enzyme and not to one another.

The objective of the present study was to evaluate earlier findings on the modes of modifier actions on

phosphorylase *a* phosphatase activity, particularly the more recently defined holoforms of phosphorylase *a* phosphatase activity: phosphatases-1, $2A_1$ and $2A_2$, from rabbit liver. In view of the role of the hepatic system in the regulation of glycogen metabolism, it would be of physiological importance to understand ligand control of phosphorylase phosphatase activity in this tissue.

B. Materials and Methods

Materials

Sephadex G-25 (medium grade) was obtained from Pharmacia; calf thymus histone (type II-A) and the catalytic subunit of bovine heart cAMP-dependent protein kinase were from Sigma. Ag 1-X8 resin was from Bio Rad. The tetradecapeptide (residues 5 to 18 of rabbit skeletal muscle phosphorylase *a*) was provided by R. Parker and R. Hodges (University of Alberta), and was synthesized using the general procedures of Erickson and Merrifield (23). The peptide preparation was 90-95% pure as judged by HPLC and amino acid composition analyses. Sources of other materials used in this chapter have been reported in the preceding chapter.

Preparation of ^{32}P-labelled histone

This was prepared from calf thymus histone (type II-A) using the catalytic subunit of cAMP-dependent protein kinase as described by Killilea *et al.* (24). Incubation mixtures

(50 ml) at pH 7.0 contained 10 mg/ml histone, 75 mM KH_2PO_4, 4 mM MgCl, 1 mM DTT, 0:2 mM EGTA, 0.4 mM [γ-^{32}P]-ATP, and 3000 U of the protein kinase.[1] After 4 hr incubation at 30°C, the reaction was terminated by the addition of TCA to a final concentration of 2% (v/v). Isolation of the ^{32}P-labelled histone followed the procedure by Meisler and Langan (25). An incorporation of 18-25 nmole ^{32}P/mg protein was usually obtained. Protein concentration was determined from absorbance measurements at 260 nm and 280 nm using the method reported by Layne (26).

Preparation of ^{32}P-labelled tetradecapeptide

This substrate was prepared from the crude peptide using the catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]-ATP under the general conditions of Beavo *et al.* (27) as reported by Tessmer *et al.* (28). The crude peptide was first dissolved to 10 mg/ml in 25 mM MES (pH 6.9), 4 mM $Mg(OAc)_2$, and 0.25 mM EGTA, and centrifuged at 12 000 rpm for 5 min to remove insoluble material. [γ-^{32}P]-ATP was added to the clear supernatant to 0.2 mM and the phosphorylation initiated with 1 000 U (see above definition of U) of the kinase per ml of incubation mixture. After 3.5 hr incubation at 30°C, the mixture was put through a 7 ml Ag 1-X8 column equilibrated in 30% acetic acid (22). About 15 ml of the effluent was collected and the acetic

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[1] 1 U transfers 1.0 picomole of phosphate from [γ-^{32}P]-ATP to hydrolyzed and partially dephosphorylated casein (Sigma Prod. no. C-4765) per minute at pH 6.5 at 30°C.

acid removed by rotary evaporation at 35-40°C. The residue was dissolved in water, centrifuged at 12 000 rpm for 5 min, and the supernatant was lyophilized. The lyophilizate was dissolved in 0.1% NH_4HCO_3 and chromatographed on a 0.9x44 cm Sephadex G-25 (medium) column at 15 ml/hr. A single peak of radioactivity eluting at the void volume of the column was pooled and lyophilized thrice with repeated dissolution of the residue in water. The peptide concentration of the final solution was determined from amino acid analysis of an acid digest and incorporation of ^{32}P was between 0.7-0.9 mole ^{32}P/mole peptide (M_r 1600).

Phosphorylase a phosphatase assay

Phosphorylase *a* phosphatase activity was determined as described in the preceding chapter.

Histone phosphatase assay

This was measured by release of ^{32}P from ^{32}P-labelled histone at 30°C by a modification of the assay by Silberman *et al.* (29). Reaction mixtures contained 40 mM imidazole-HCl(pH 7.2), 0.5 mM DTT, 0.2 M NaCl, 1 mg/ml BSA, 50-80 μM ^{32}P-labelled histone (based on incorporated ^{32}P), and the phosphatase in a total of 100 μl. For phosphatases-1, $2A_1$ and $2A_2$, the reaction mixture also contained 10%(v/v) glycerol. Initiation and timing of the reaction was as for phosphorylase *a* phosphatase assay (see **Chapter II**). Termination of the reaction was done by the addition of

40 µl of a solution of 40 mM H_2SO_4 and 80 mM silicotungstic acid containing 0.5 mM inorganic phosphate. 160 µl of ice-cold 0.5 mg/ml BSA was then added, and after storage at 4°C for 10 min, the tubes were centrifuged at 12 000 rpm for 10 min in a Beckman microfuge 12. Aliquots of the clear supernatants were counted for radioactivity with 5 ml Aquasol scintillation fluid in a Beckman LS 6800.

Tetradecapeptide phosphatase assay

Phosphatase activity on ^{32}P-labelled tetradecapeptide was determined at 30°C in a standard incubation mixture containing 50 mM imidazole-HCl(pH 7.4), 0.5 mM EDTA, 0.5 mM DTT, 150 µM ^{32}P-labelled tetradecapeptide, and the phosphatase in a total of 100 µl. The reaction mixture for phosphatases-1, $2A_1$ and $2A_2$ contained 10%(v/v) glycerol in addition. Initiation and timing of the reaction was as for phosphorylase *a* phosphatase assay (see **Chapter II**). Release of ^{32}P was measured by a modification of the extraction procedure reported by Shacter-Noiman and Chock (22). The reaction was terminated by the addition of 200 µl of 20 mM silicotungstic acid in 0.02 N H_2SO_4. Following the sequential addition of 900 µl of water-saturated isobutanol:benzene(1:1) mixture and 100 µl of 5% ammonium molybdate in 4 N H_2SO_4, the reaction tubes were vortexed for 15 sec. The tubes were then centrifuged at 12 000 rpm for 5 min in a Beckman microfuge 12 and the radioactivity in 400 µl of the organic phase was determined with 5 ml Aquasol

scintillation fluid in a Beckman LS 6800.

In all enzyme assays, blank values obtained when the enzyme was added after the stopping reagent were subtracted from the assay values. Total available substrate (radioactivity) was determined from radioactivity in aliquots of the stock substrates. Phosphatase activities were expressed in μmoles per min per milligram of enzyme and relative activities were obtained by setting the control values (no addition) at 100%.

C. Results

The activity of phosphatase-2A$_1$ against rabbit skeletal muscle ^{32}P-labelled phosphorylase *a* was stimulated by glucose (25 mM), caffeine (1 mM), and Mg^{2+} (1 mM), as shown in Fig. III.1. This activating effect was not observed when the histone and tetradecapeptide phosphatase activities of this phosphatase form were examined in the presence of these ligands. AMP (0.3 mM), ATP (7.5 mM), ADP (2.5 mM), P_i (1 mM) and glucose 1-P (1 mM), each caused appreciable inhibitions of phosphorylase *a* phosphatase activity of phosphatase-2A$_1$, with the presence of Mg^{2+} suppressing significantly the ATP inhibition. With ^{32}P-labelled histone as substrate, a small inhibition by P_i was seen while inhibitions by AMP, ADP, and glucose 1-P were not seen. ATP activated histone dephosphorylation here, an effect that could arise from charged interactions between this nucleotide and the highly charged protein substrate. The tetradecapeptide phosphatase

Figure III.1. Effects of ligands on rabbit liver phosphatase-2A, activity towards three ^{32}P-labelled substrate forms.

Phosphatase-2A, activity was determined as described under Methods using [1] rabbit skeletal muscle ^{32}P-labelled phosphorylase *a* (40 μM), [2] ^{32}P-labelled histone (80 μM), [3] ^{32}P-labelled tetradecapeptide (150 μM), in the presence of ligands. Conditions were: (A) Control - no additions, (B) 25 mM glucose, (C) 1 mM caffeine, (D) 0.3 mM AMP, (E) 2.5 mM ADP, (F) 1 mM P_i (G) 1 mM glucose 1-P, (H) 7.5 mM ATP, (I) 1.5 mM $MgCl_2$, (J) 9 mM $MgCl_2$ + 7.5 mM ATP. Control activities were: [1] 1.16 nmoles ^{32}P/min/mg, [2] 1.32 μM/min/mg, [3] 32.7 μM/min/mg. Results are expressed as relative activity with the control value set at 100%. Data in this and subsequent figures in this chapter are from duplicate incubation mixtures for a single set of experiments representative of several repeated trials. *t* statistics was applied to determine the significance ($p \leq 0.03$) of the differences from the control values.

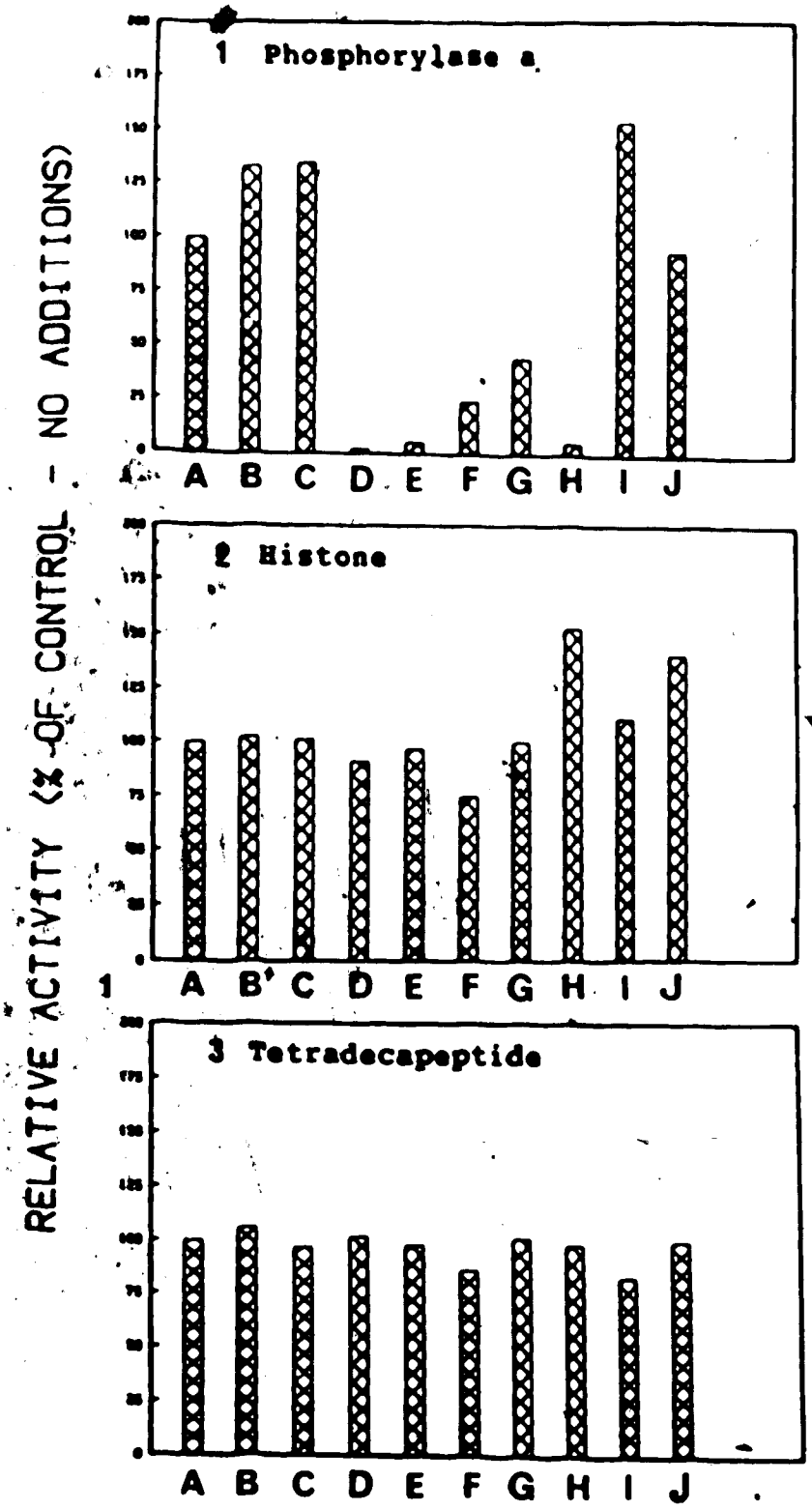
RELATIVE ACTIVITY (% OF CONTROL - NO ADDITIONS)
1 Phosphorylase a
2 Histone
3 Tetradecapeptide
A B C D E F G H I J
A B' C D E F G H I J
A B C D E F G H I J

FIGURE III.1

activity of phosphatase-$2A_1$ was unaffected to any significant extent by AMP, ADP, ATP and glucose 1-P. However, P_i and Mg^{2+} were slightly inhibitory of this activity (Fig. III.1).

Glucose, caffeine and Mg^{2+}, activated the phosphorylase *a* phosphatase activity of phosphatase-$2A_2$ (Fig. III.2). The activations by glucose and caffeine were not seen with histone and tetradecapeptide as substrates. AMP, ADP, ATP, P_i and glucose 1-P, inhibited the phosphorylase *a* phosphatase activity. ATP and Mg^{2+} caused some activation of the histone phosphatase activity while AMP, ADP and glucose 1-P had negligible effects. Some significant inhibition of this activity by P_i was seen. With tetradecapeptide substrate, ATP was inhibitory with little change seen in the additional presence of Mg^{2+}. This contrasts with the pattern for phosphatase-$2A_1$ (see above). The other ligands, AMP, ADP, P_i and glucose 1-P, had little or no effects as seen from comparison with the effects of these ligands on the phosphorylase *a* phosphatase activity of this phosphatase form (Fig. III.2).

For phosphatase-1, glucose, caffeine and Mg^{2+} stimulated its phosphorylase *a* phosphatase activity (Fig. III.3). The other ligands tested were inhibitory and some relief of ATP inhibition occurred in the presence of Mg^{2+}. The histone phosphatase activity of phosphatase-1[4] was unaffected by glucose, caffeine and P_i, while some slight inhibition was caused by AMP and Mg^{2+}. ADP, glucose 1-P

Figure III.2. Effects of ligands on rabbit liver phosphatase-$2A_2$ activity towards three ^{32}P-labelled substrate forms.

Phosphatase-$2A_2$ activity was determined as described under Methods using [1] rabbit skeletal muscle ^{32}P-labelled phosphorylase *a* (40 μM), [2] ^{32}P-labelled histone (80 μM), [3] ^{32}P-labelled tetradecapeptide (150 μM), in the presence of ligands. Conditions were: (A) Control - no additions, (B) 25 mM glucose, (C) 1 mM caffeine, (D) 0.3 mM AMP, (E) 2.5 mM ADP, (F) 1 mM P_i (G) 1 mM glucose 1-P, (H) 7.5 mM ATP, (I) 1.5 mM $MgCl_2$, (J) 9 mM $MgCl_2$ + 7.5 mM ATP. Control activities were: [1] 0.15 nmoles ^{32}P/min/mg, [2] 2.41 μM/min/mg, [3] 2.03 μM/min/mg. Results are expressed as relative activity with the control value set at 100%.

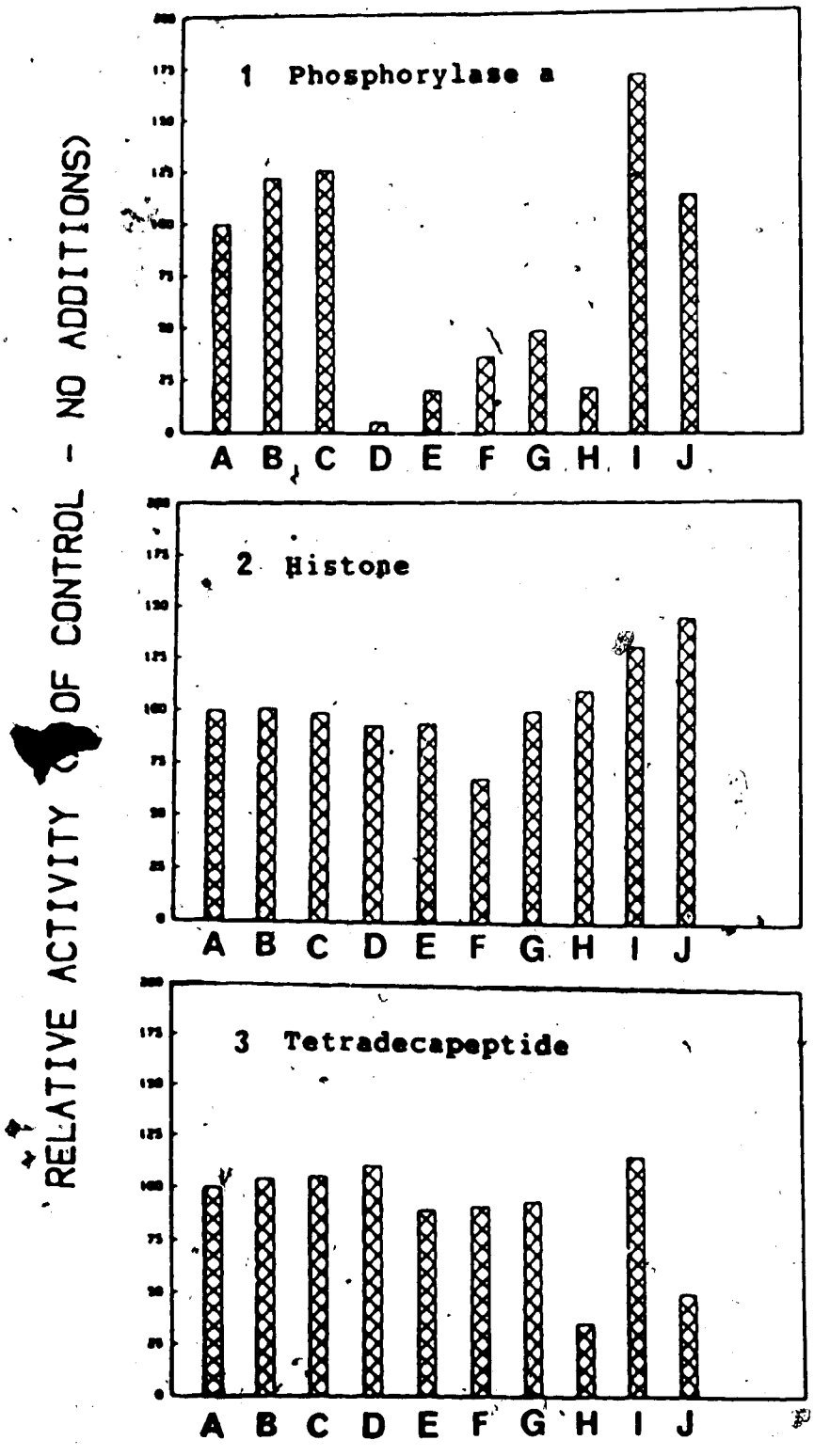
RELATIVE ACTIVITY OF CONTROL - NO ADDITIONS)
1 Phosphorylase a
2 Histone
3 Tetradecapeptide
A B C D E F G H I J

FIGURE III.2

Figure III.3. Effects of ligands on rabbit liver phosphatase-1 activity towards three ^{32}P-labelled substrate forms.

Phosphatase-1 activity was determined as described under Methods using [1] rabbit skeletal muscle ^{32}P-labelled phosphorylase *a* (40 μM), [2] ^{32}P-labelled histone (80 μM), [3] ^{32}Plabelled tetradecapeptide (150 μM), in the presence of ligands. Conditions were: (A) Control → no additions, (B) 25 mM glucose, (C) 1 mM caffeine, (D) 0.3 mM AMP, (E) 2.5 mM ADP, (F) 1 mM P_i (G) 1 mM glucose 1-P, (H) 7.5 mM ATP, (I) 1.5 mM $MgCl_2$, (J) 9 mM $MgCl_2$ + 7.5 mM ATP. Control activities were: [1] 1.10 nmoles ^{32}P/min/mg, [2] 0.38 μM/min/mg, [3] 7.82 μM/min/mg. Results are expressed as relative activity with the control value set at 100%.

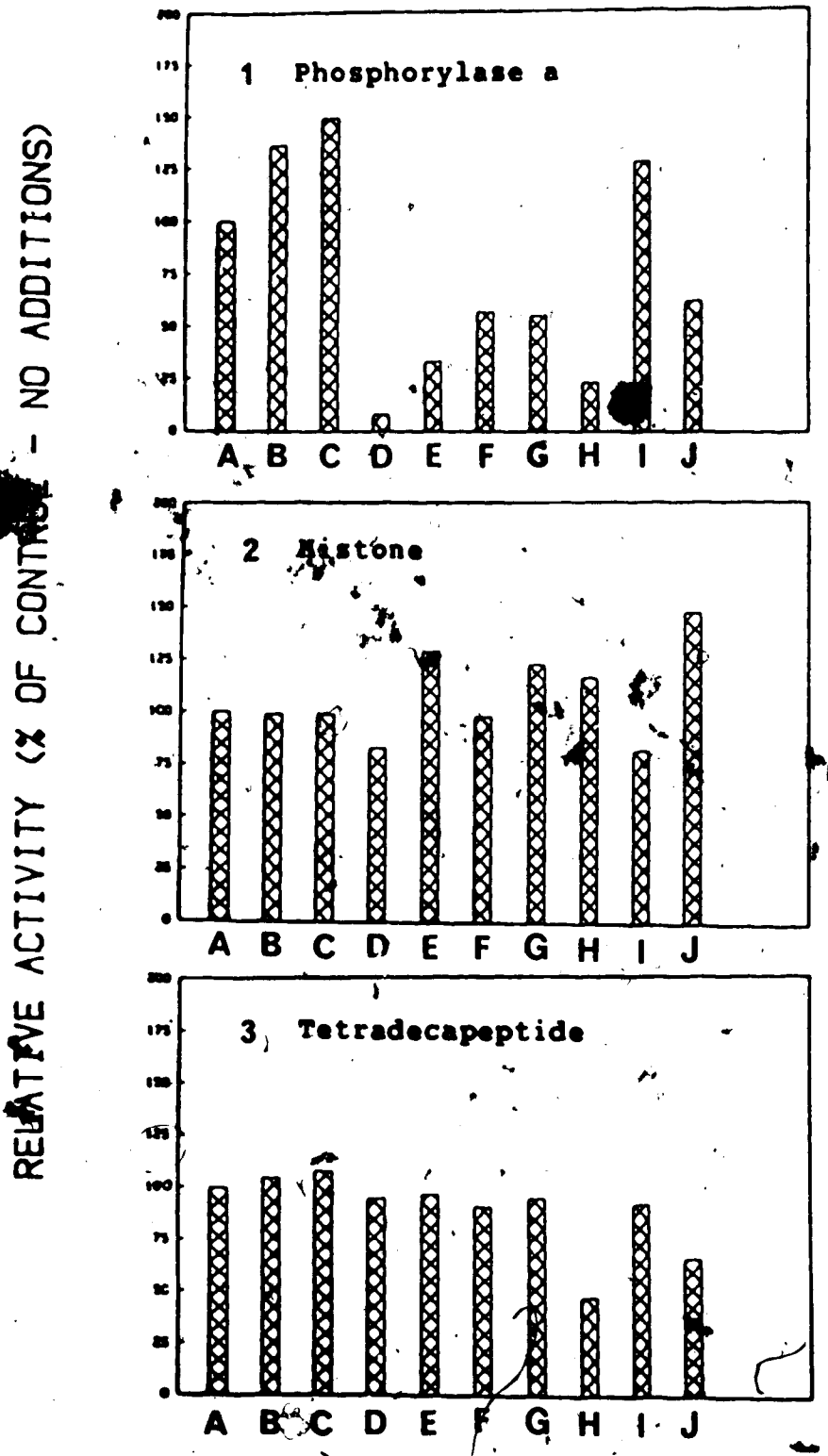
RELATIVE ACTIVITY (% OF CONTROL - NO ADDITIONS)
1 Phosphorylase a
2 Histone
3 Tetradecapeptide
A B C D E F G H I J

FIGURE III.3

Figure III.4. Effects of ligands on rabbit liver 35 000 M_r phosphatase activity towards three ^{32}P-labelled substrate forms.

The activity of 35 000 M_r phosphatase was determined as described under Methods using [1] rabbit skeletal muscle ^{32}P-labelled phosphorylase *a* (40 μM), [2] ^{32}P-labelled histone (80 μM), [3] ^{32}P-labelled tetradecapeptide (80 μM) in the presence of ligands. Conditions: (A) Control - no additions, (B) 25 mM glucose, (C) 10 mM caffeine, (D) 0.3 mM AMP, (E) 2.5 mM ADP, (F) 10 mM P_i, (G) 1 mM glucose 1-P, (H) 0.3 mM ATP, (I) 1.5 mM $MgCl_2$, (J) 9 mM $MgCl_2$ + 7.5 mM ATP. Control activities were: [1] 211.0 nmoles ^{32}P/min/mg, [2] 228.99 μM/min/mg, [3] 278.46 μM/min/mg. Results are expressed as relative activity with the control value set at 100%.

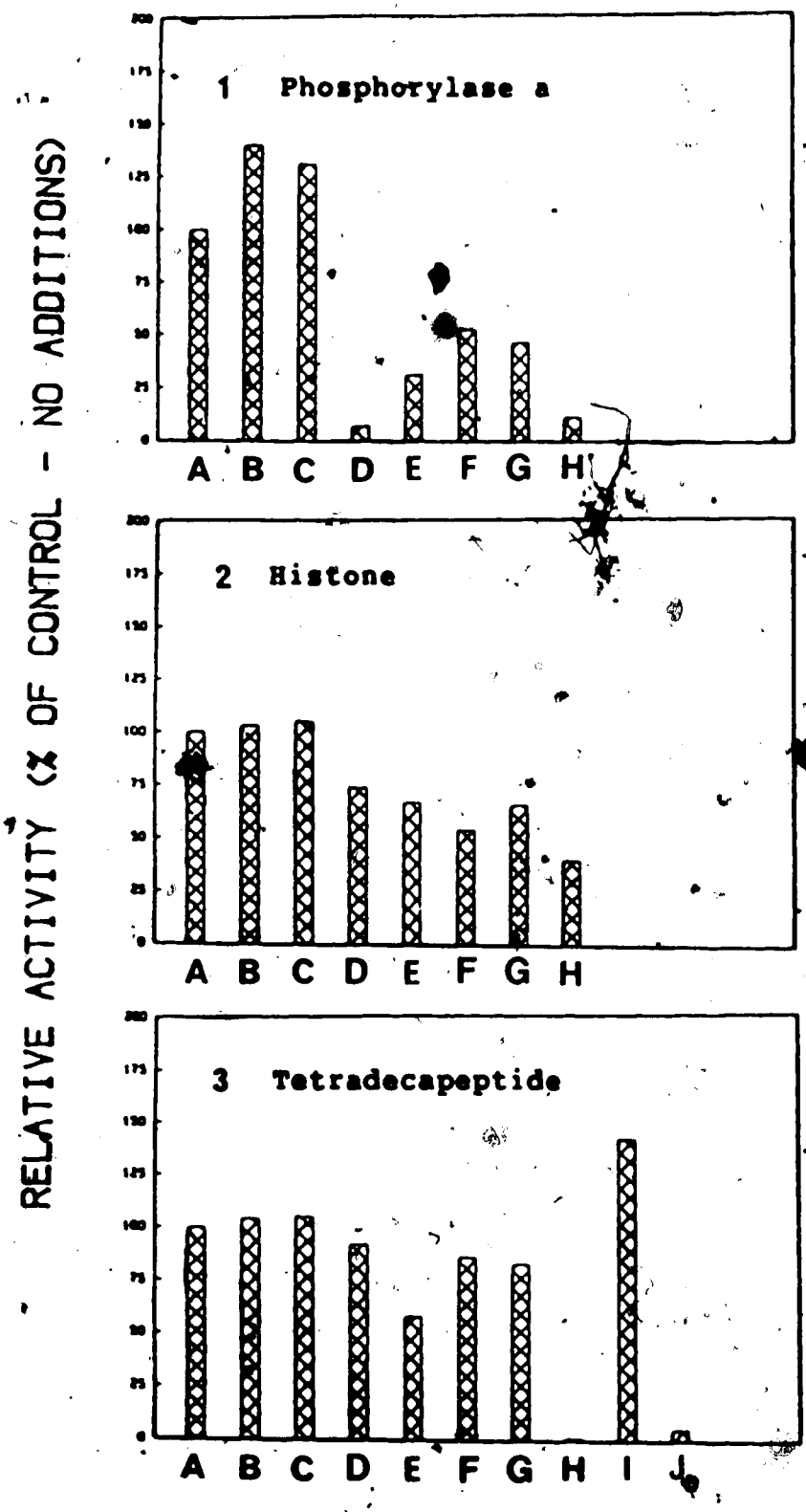
RELATIVE ACTIVITY (% OF CONTROL - NO ADDITIONS)
1 Phosphorylase a
A B C D E F G H
2 Histone
A B C D E F G H
3 Tetradecapeptide
A B C D E F G H I J

FIGURE III.4

and ATP were activating for this activity. With tetradecapeptide as substrate, all ligands except ATP had negligible effects. ATP inhibition remained even in the presence of Mg^{2+}, a pattern similar to that seen for the activity of phosphatase-$2A_2$ against this substrate.

As shown in Fig. III.4, glucose and caffeine activated the phosphorylase *a* phosphatase activity of the 35 000 M_r phosphatase form. AMP, ATP, ADP, P_i and glucose 1-P were inhibitory of this activity. With histone as substrate, the activation by glucose and caffeine was abolished while AMP, ADP, ATP, P_i and glucose 1-P remained inhibitory. The effect of Mg^{2+} was not tested with phosphorylase *a* and histone as substrates, but with tetradecapeptide, this ligand was activating, a surprising finding (see **Discussion**). Mg^{2+} had little effect on the ATP inhibition of the activity of this phosphatase on the tetradecapeptide. Glucose, caffeine and AMP did not affect this activity; ADP and ATP caused significant inhibition with smaller inhibitions seen for P_i and glucose 1-P.

D. Discussion

In this report, comparison of the activities relative to the control of four different molecular forms of rabbit liver phosphorylase *a* phosphatase, using the 'natural' (phosphorylase *a*) and two alternate substrates (histone and tetradecapeptide), allowed distinction of the modes of action by different physiologically important effectors of

the phosphatase activity. Glucose, caffeine and AMP did not affect the histone and tetradecapeptide phosphatase activity of the four phosphatase forms: the 35 000 M_r phosphatase, and phosphatases-1, $2A_1$ and $2A_2$, hence their effects are substrate-directed. By the same token, ADP, P_i and glucose 1-P exerted substrate-directed effects. Similar conclusions were reached in earlier studies on rabbit skeletal muscle phosphorylase *a* phosphatase (possibly protein phosphatase-1) (3,4,5,6).

ATP inhibited the phosphorylase *a* as well as the tetradecapeptide phosphatase activity of the 35 000 M_r phosphatase and phosphatases-1 and $2A_2$, and thus exhibited both substrate and enzyme-directed effects for these phosphatase forms. However, the ATP effect on phosphatase-$2A_1$ was substrate-directed as no effect of this ligand was seen with tetradecapeptide as substrate. There are indications that phosphatase-$2A_2$ is generated from phosphatase-$2A_1$ by loss of the γ-subunit during purification (30,31). The 'loss' of the enzyme-directed ATP effect for phosphatase-$2A_1$ in comparison to the effect seen for phosphatase-$2A_2$ suggests that the γ-subunit may prevent ATP binding, a speculation that needs further investigation.

The enzyme-directed inhibition by ATP was more potent for the 35 000 M_r phosphatase than for the other phosphatase forms studied. The sensitivity to ATP inhibition has been used as a criterion for distinguishing the type-2 phosphatase from phosphatase-1 (32). In the present study, ATP

inhibited, to a comparable extent, not only the type-2 phosphatase but also phosphatase-1 activity with the tetradecapeptide substrate, supporting a recent view that ATP inhibition is not a useful criterion for this purpose (32).

The tetradecapeptide phosphatase activities of phosphatase-1, $2A_1$ and $2A_2$ were not affected by Mg^{2+}, suggesting only substrate-directed effects for these phosphatase forms. Mg^{2+} is a strong inhibitor of the phosphorylase *a* phosphatase activity of the 35 000 M_r phosphatase (13,33) but activated the tetradecapeptide phosphatase activity of this phosphatase in the present study, indicating an enzyme-directed effect for this ligand. Perhaps the dual action of Mg^{2+} in binding to phosphorylase *a* (metal ion binding sites have been shown, see ref. 15) and the phosphatase results in inhibition of the phosphorylase *a* phosphatase activity, while the single binding to the phosphatase leads to activation of the tetradecapeptide phosphatase activity. However, significant interaction between the metal ion and the peptide substrate cannot be ruled out completely.

A number of studies indicate that Mg^{2+} protects phosphorylase *a* phosphatase from (19,20) or reverses (17,20) ATP inhibition of the activity. Direct interaction of either ligand with a 38 000 M_r phosphatase from bovine heart was reported recently (22). The results obtained in the present study show that the presence of Mg^{2+} could not abolish ATP inhibition of phosphatase-1 and $2A_2$ activities with the

tetradecapeptide, but this cation significantly overcame ATP inhibition of the phosphorylase *a* phosphatase activity of the phosphatase forms studied. Taken together, these findings suggest that Mg^{2+} reverses the ATP inhibition by a substrate-directed mechanism.

A major assumption that allows unambiguous interpretation of results in studies elucidating modes of modifier actions using alternate substrate is that the modifiers do not interact with the alternate substrate (34). Results obtained in this study using the alternate substrate, histone, indicated both substrate and enzyme-directed effects for such ligands including ATP, ADP, P_i, and Mg^{2+}. With the tetradecapeptide substrate, both modes of action were seen for only ATP and Mg^{2+}, indicating that interaction between the ligands and histone were sufficient to produce substrate-directed effects. For instance, the interaction between ATP and histone would explain the experimental observation that a mixture of the two yields a turbid solution which readily clears during the incubation periods for the experiments described in this chapter. This situation points to the caution needed when alternate substrate studies are being done.

Direct effects by glucose and caffeine have been reported for glycogen synthase phosphatase activity in rat liver protein-glycogen suspensions (35,36). The results obtained in this chapter established that both ligands affect the phosphorylase *a* phosphatase activity of the

phosphatase forms studied by substrate-directed mechanisms only. While the identity of the phosphatase(s) capable of acting on phosphorylase *a* and glycogen synthase *b* remain to be resolved, the view that the same enzyme, protein phosphatase-1, acts on both proteins in the protein-glycogen suspension is gaining increasing support as indicated by results in a recent report (37). Also, protein phosphatases-1 and 2A have been shown to account for virtually all the phosphorylase *a* and glycogen synthase *b* (sites 2 and 3 phosphorylated) phosphatase activities in dilute extracts from different mammalian tissues including rabbit skeletal muscle and liver (38,39,40). Thus, it seems unlikely that glucose and caffeine would have enzyme-directed effects with glycogen synthase *b* as substrate but not with phosphorylase *a*. The other reasonable explanation for the activation of the glycogen synthase phosphatase by glucose and caffeine is through the binding of the ligands to the synthase, and this has not been demonstrated.

It is interesting that despite the differences in molecular forms, the patterns of ligand action seen for the phosphatases were remarkably similar for the various ligands tested in this study. Substrate-directed effects were demonstrated for the ligands, thus indicating the importance of this form of control in the regulation of phosphorylase *a* phosphatase activity.

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IV. Phosphorylase a inhibition of glycogen synthase activation in gel-filtered rabbit liver extracts: effects of glucose and caffeine

A. Introduction

The inhibitory effect of phosphorylase *a* on the activation of glycogen synthase by dephosphorylation was reported by Hers, Stalmans and their associates, working with rat liver filtrates (1,2,3). Their results showed that the activation was preceded by a pronounced lag corresponding to the time needed to reduce phosphorylase *a* to a threshold level. This characteristic lag has been shown to occur in crude microsomal fractions (4), rat liver filtrates (5) and hepatocytes (6). It has also been reported to occur with isolated rat liver protein-glycogen suspensions (2), although some investigators disagree (7). The lag could not be detected with a homogeneous rabbit liver 35 000 M_r phosphatase (8) but phosphorylase *a* was stated to be inhibitory of the phosphatase activity on glycogen synthase in this and other reports (9,10).

The complexity of protein phosphatases and glycogen synthase, key proteins in the activation event, contibute to the difficulty in understanding the process at the molecular level. As is currently known, glycogen synthase (a tetramer) is phosphorylated on seven serine residues per subunit by at least five different protein kinases in both skeletal muscle and liver of rabbit (11) and similar tissues from rat

(12,13). The activity of the enzyme is known to be dependent on the states of phosphorylation of sites 1a, 2, and 3(a,b,c); sites 2 and 3(a,b,c) influencing the kinetic parameters to greater extent than site 1a, although the effects are additive with larger changes produced by phosphorylation of all five sites (11,14). On the other hand, phosphorylation of sites 1b and 5 does not seem to affect the enzyme activity (11,15). The responses of the different sites to hormone administration *in vivo* have been studied with respect to their phosphorylation states and the implication of the findings in the regulation of the phosphorylation (inactivation)/dephosphorylation (activation) of glycogen synthase *in vivo* has been a matter for current debate (16,17,18).

Although there is still no agreement on the identity of the phosphatase(s) acting on phosphorylase *a* and glycogen synthase *b* (7,19; reviewed in refs. 20,21), recent reports by Cohen and associates indicate that hepatic protein phosphatase-2A accounts for up to 60% of phosphorylase *a* phosphatase activity and 40-70% of the phosphatase activity with the various phosphorylated sites on glycogen synthase *b* (22). Furthermore, protein phosphatase-1 is the only phosphatase present in the protein-glycogen complex, and in the rabbit liver it accounted for 20-50% of the phosphorylase *a* phosphatase and glycogen synthase phosphatase, and for 75% of the phosphorylase *b* kinase (β-subunit) phosphatase activities (22). Other studies indicate that protein

phosphatase-1 and 2A account for virtually all the phosphorylase *a* and glycogen synthase *b* (sites-2 and 3 phosphorylated) phosphatase activities in dilute extracts from different mammalian tissues including rabbit skeletal muscle and liver (22,23,24). Also, protein phosphatase-1 and/or 2A have been implicated as the enzymes responsible for the activation of glycogen synthase in the earlier work by Hers and associates (22).

Glucose, a known stimulator of phosphorylase *a* phosphatase activity (25), has been shown to reduce the lag in glycogen synthase activation in rat liver filtrates (5), perfused rat liver and hepatocytes (26). In combination with caffeine, also an activator of phosphorylase *a* phosphatase (27), glucose further decreased the lag in glycogen synthase activation in rat hepatocytes (6). These effects have been considered to be indirect and mediated through the activation of phosphorylase *a* phosphatase activity by both ligands, thereby resulting in relief of phosphorylase *a* inhibition of the phosphatase activity required for glycogen synthase activation (28,29,30). However, there are reports suggesting direct effect of these ligands on the phosphatase activity involved (7,31,32).

In a recent report, glucose and caffeine were shown to activate three different molecular forms of rabbit liver phosphorylase *a* phosphatases (type-2 phosphatases) in an independent and additive manner (33 and Chapter II). The additive effect of both ligands was most potent in relieving

the inhibition by the nucleotide inhibitors, AMP and ATP. Similar results have been obtained for protein phosphatase-1 from rabbit liver using rabbit skeletal muscle substrate (Chapter II).

This chapter addresses the effect of glucose and caffeine on the lag in glycogen synthase activation in rabbit liver extracts, particularly with respect to the additive effect of both ligands on phosphorylase *a* phosphatase activity. The results obtained indicate that the characteristic latency in glycogen synthase activation also occurs with rabbit liver filtrate under the conditions used. Further, a combination of both ligands overcame the inhibition of the activation by AMP, ATP, and exogenous phosphorylase *a*.

B. Materials and Methods

Materials

UDP[^{14}C]-glucose was obtained from Amersham and HEPES was from Biochemical Corporation. Sources of other materials used have been reported in the preceding chapters.

Preparation of liver extracts

Liver extracts were prepared by the procedure reported by Strickland *et al*. (5) except that well-fed New Zealand white rabbits were used. Anaesthesia was accomplished by the intravenous injection of pentobarbital (100mg/kg body

weight), and blood was drained by heart puncture before obtaining the liver. The steps from anaesthesia to freezing the glass wool-filtered extract in liquid nitrogen were completed within 30 minutes.

Activation of glycogen synthase in liver filtrates

This was a slight modification of the method by Strickland *et al.* (5). Before experiments, stored liver extract was thawed at 4°C, and a 2.5 ml portion was chromatographed at 4°C on a 2x30 cm Sephadex G-25 (medium) column equilibrated with buffer A [50 mM HEPES (pH 7.1), 0.1 M NaCl]. The protein peak (approximately 4 ml) was collected and maintained at 4°C before use (within 5-10 min) in the activation experiments.

Activation, performed at 30°C, was initiated by mixing the filtrate (at 4°C) with an equal volume of buffer A (at 30°C) containing the various combinations of effectors at concentrations to give the desired final concentrations in the activation mixture.

For glycogen synthase activity assay, 50 µl aliquots of the activation mixture were withdrawn at intervals and added to 50 µl substrate mixtures containing 50 mM Tris-HCl(pH 7.6), 10 mM UDP[^{14}C]-glucose (0.4 µCi/mmole), 27 mM EDTA, 2.5 mM Na_2SO_4, 50 mM NaF, and 10%(wt/v) oyster glycogen. After 60 min incubation at 30°C, a 50 µl aliquot was spotted on Whatman #3 filter discs and processed as described by Thomas *et al.* (34).

Phosphorylase *a* activity was measured as described by Stalmans and Hers (35) by adding 50 µl aliquots of the activation mixture to 150 µl substrate mixtures at pH 6.1, formulated to give final concentrations of 50 mM glucose 1-P, 1% rabbit liver glycogen, and 2.5 mM EDTA. After adequate reaction time (60-90 min), the reaction was terminated by the addition of 50 µl ice-cold 10%(wt/v) trichloroacetic acid. The reaction tubes were then centrifuged at 12 000 rpm for 5 min in a Beckman microfuge 12. Inorganic phosphate in 200 µl of the clear supernatant was determined by the Fiske and Subbarow assay (36).

C. Results

Effects of glucose and caffeine on glycogen synthase activation

As shown in Fig. IV.1, glycogen synthase activation in rabbit liver filtrate in the absence of ligands was preceded by the characteristic lag seen with rat liver filtrates (5,37) or hepatocytes (6,26). Glucose (20 mM) or caffeine (1 mM), shortened the lag period, with a further decrease observed with both ligands present together. For comparison, the rates of glycogen synthase activation at 18 minutes incubation were 1.9-fold faster with glucose, 2-fold with caffeine, and 2.5-fold with both ligands present, than with the control. It is of significance that, in all incubations, the onset of glycogen synthase activation correlated with

Figure IV.1. The independent and additive effects of glucose and caffeine on glycogen synthase activation in rabbit liver filtrates.

Liver filtrates, obtained as described in Methods, were incubated in the presence of different combinations of ligands. Aliquots were withdrawn at indicated times and assayed for glycogen synthase (open symbols) or phosphorylase *a* (closed symbols) activities, as described in the text. Conditions of incubations were: Control - no additions (⊙—⊙), 20 mM glucose (△—△), 1 mM caffeine (□—□), and 20 mM glucose + 1 mM caffeine (◇—◇). Data in this and subsequent figures in this chapter are from duplicate incubation mixtures in experiments representative of several repeated trials. This and subsequent plots represent a combination of glycogen synthase and phosphorylase *a* activities measured in independent incubation mixtures under identical conditions.

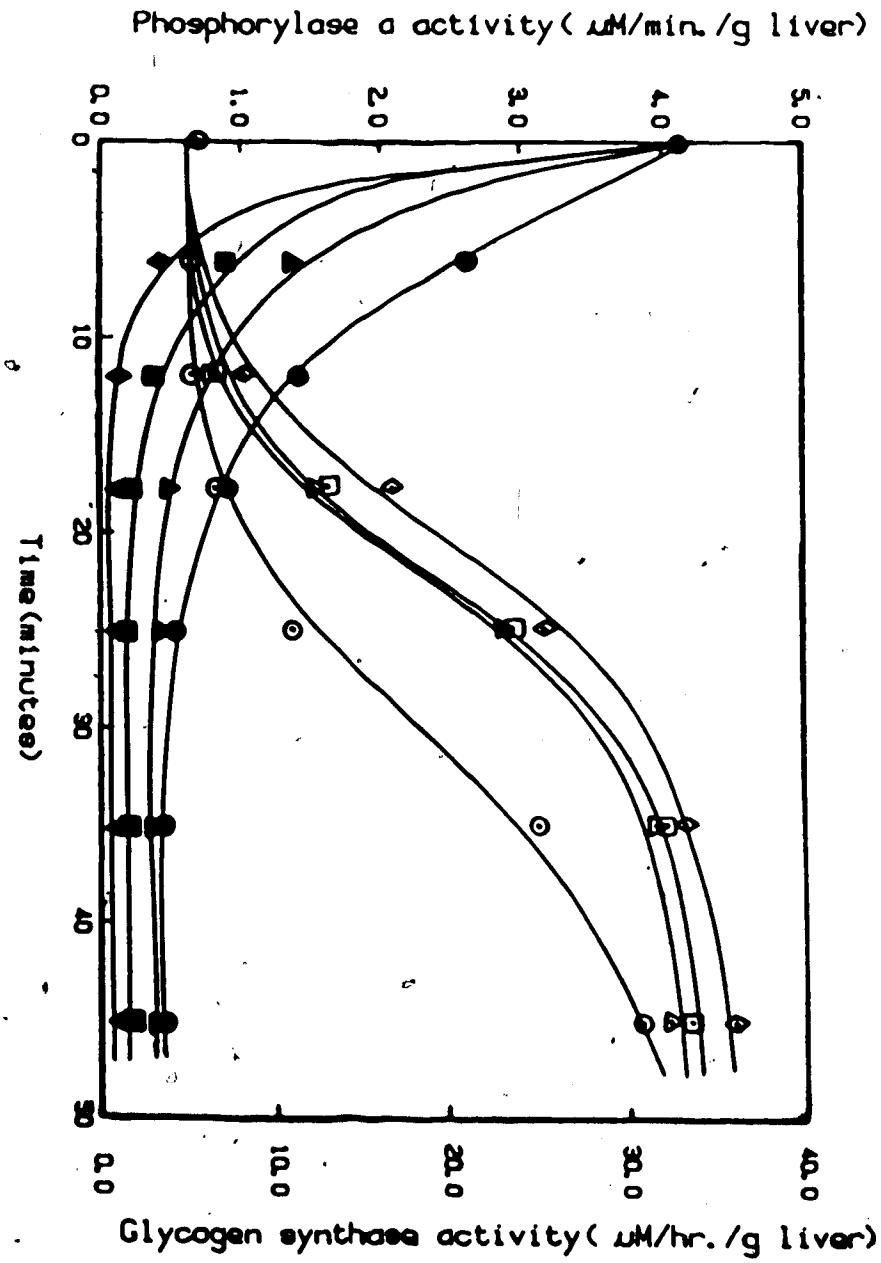

Phosphorylase a activity(μM/min./g liver)
0.0
1.0
2.0
3.0
4.0
5.0
0
10
20
30
40
50
Time(minutes)
0.0
10.0
20.0
30.0
40.0
Glycogen synthase activity(μM/hr./g liver)

FIGURE IV.1

the disappearance of phosphorylase *a*, and did not start until the level of phosphorylase *a* was below 1 μM/min/g liver. Similar patterns were observed in the presence of 10 mM glucose and 0.5 mM caffeine (not shown), but the reported concentrations of both ligands in this study were used for comparison with earlier studies on ligand action on phosphorylase *a* phosphatases (33).

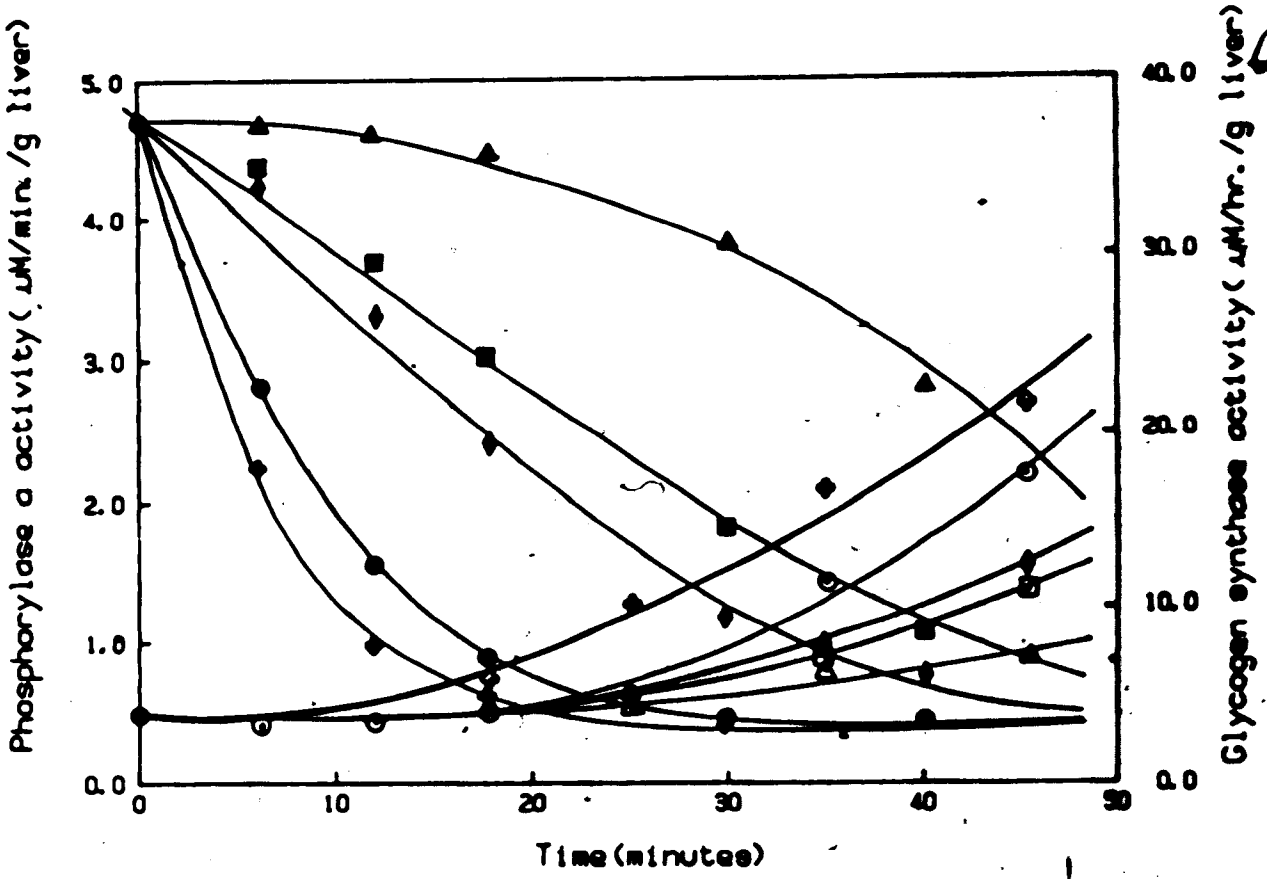
Effect of AMP on glycogen synthase activation

AMP (0.3 mM) significantly inhibited the onset of glycogen synthase activation (Fig. IV.2). This inhibitory action was reversed by the presence of 20 mM glucose or 1 mM caffeine, a combination of both ligands overcoming completely the AMP inhibition. At 30 minutes incubation, the rate of glycogen synthase activation was decreased to 25% of the control with AMP present. With 20 mM glucose present in addition, the rate improved to 50% ; in the presence of caffeine and AMP, the rate was about 40%; and in the combined presence of glucose, caffeine and AMP, there was a 1.3-fold increase in the rate over the control value. Again, glycogen synthase activation corresponded closely with the decrease in phosphorylase *a* activity. The drop in phosphorylase *a* activity in the presence of AMP towards the end of the sampling time could stem from two possible factors. While the AMP-phosphorylase *a* complex is a poor substrate for phosphorylase *a* phosphatase (see Chapter II), the phosphatase could still act on the complex but at a

Figure IV.2. Effects of glucose and caffeine, alone and together, on AMP inhibition of glycogen synthase activation in rabbit liver filtrates.

Liver filtrates, obtained as described under Methods, were incubated in the presence of different combinations of ligands. Aliquots were withdrawn at indicated times and assayed for glycogen synthase (open symbols) or phosphorylase *a* (closed symbols) activities, as described in the text. Conditions of incubation were: Control - no additions (○–○), 0.3 mM AMP (△–△), 0.3 mM AMP + 20 mM glucose (□–□), 0.3 mM AMP + 1 mM caffeine (◇–◇), 0.3 mM AMP + 20 mM glucose + 1 mM caffeine (◆–◆).

FIGURE IV.2


Phosphorylase a activity(μM/min./g liver)
Glycogen synthase activity(μM/hr./g liver)
Time (minutes)
0
10
20
30
40
50
0.0
1.0
2.0
3.0
4.0
5.0
10.0
20.0
30.0
40.0

low activity although this would not account for the accelerated rate at the end. Also a significant hydrolysis of AMP through deaminase activity would reduce the concentration of free nucleotide in the activation mixture. This pattern of phosphorylase *a* activity level with AMP present has been observed in previous studies (38).

Effect of Mg.ATP on glycogen synthase activation

Using filtered extract prepared as described under Methods, glycogen synthase activation was severely inhibited in the presence of 9 mM $MgCl_2$ plus 7.4 mM ATP, and the combined presence of 20 mM glucose and 1 mM caffeine could not overcome the inhibition (not shown). At the start of the activation experiment under these conditions, phosphorylase *a* activity started to drop, but levelled off at values above that for the control (no additions), hence partially explaining the shut-down of glycogen synthase activation. This could arise from either a kinase activity (residual Ca^{2+} could potentiate this) or a direct effect of Mg.ATP on the phosphatase activity involved.

To distinguish between these possibilities, the Mg.ATP effect was investigated in the presence of 1.0 mM EGTA. Fig. IV.3 shows that the inhibition of glycogen synthase activation remained, even with glucose and caffeine present together. A slight relief of this inhibition occurred with glucose and caffeine present together but the activation of the glycogen synthase levelled off at values below that of

Figure IV.3. Effects of glucose and caffeine, alone and together, on Mg.ATP inhibition of glycogen synthase activation in rabbit liver filtrates in the presence of 1.0 mM EGTA.

Liver filtrates, obtained as described under Methods, were incubated in the presence of different combinations of ligands. Aliquots were withdrawn at indicated intervals and assayed for glycogen synthase (open symbols) or phosphorylase *a* (closed symbols) activities, as described in the text. Conditions were: Control - no additions (⊙—⊙), Mg.ATP (△—△), Mg.ATP + glucose (□—□), Mg.ATP + caffeine (◇—◇), Mg.ATP + glucose + caffeine (◈—◈). All incubations contained 1.0 mM EGTA and final concentrations of ligands were: 9 mM $MgCl_2$, 7.4 mM ATP, 20 mM glucose, and 1 mM caffeine.

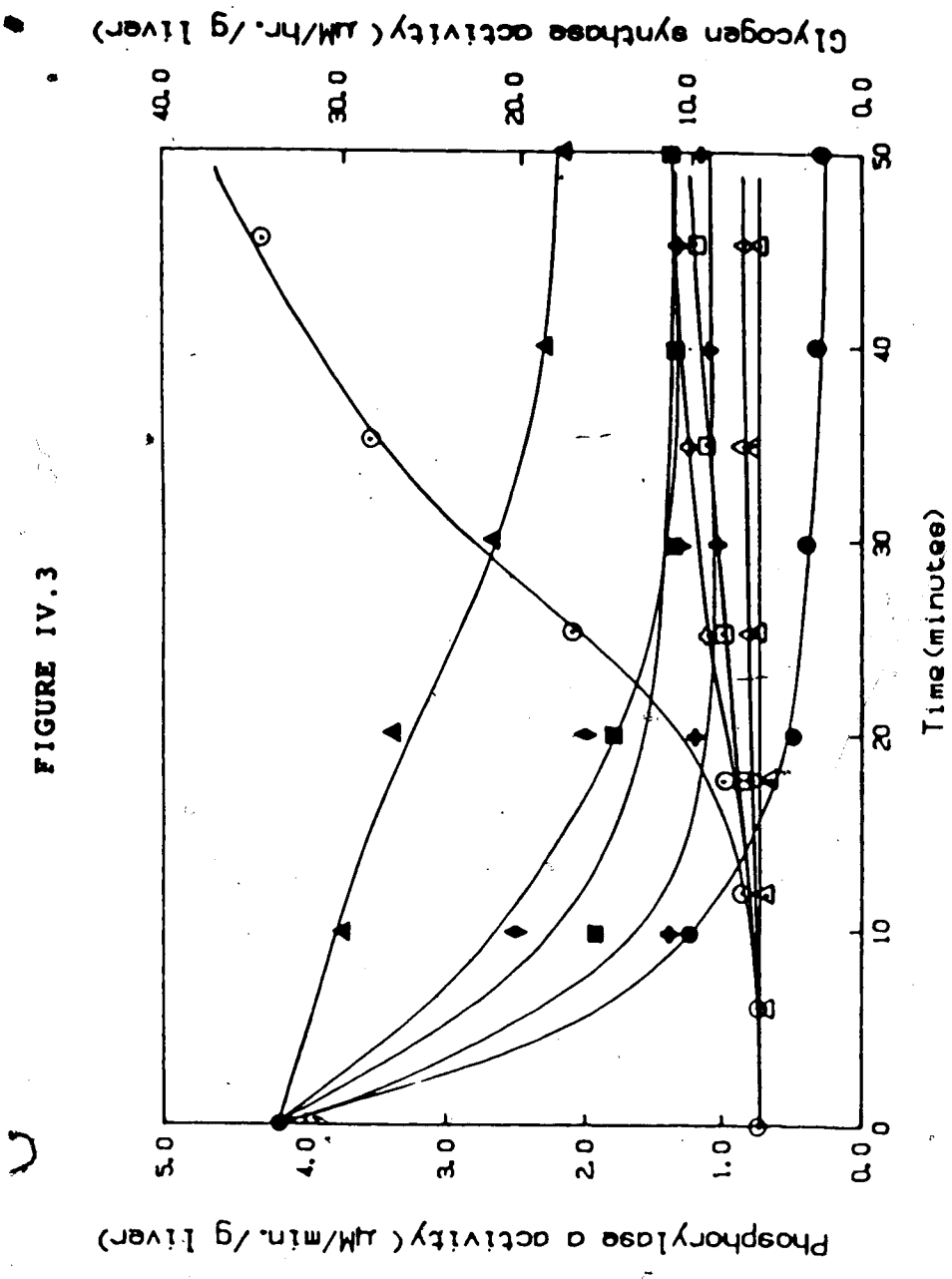

Glycogen synthase activity (μM/hr. /g liver)
40.0
30.0
20.0
10.0
0.0
Phosphorylase a activity (μM/min. /g liver)
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
Time (minutes)

FIGURE IV.3

the control. The phosphorylase *a* activity with glucose and caffeine present together decreased initially as expected but levelled off well above the value obtained for the control incubation. These findings support the view that a kinase activity was not responsible for the earlier observation that the combination of glucose and caffeine could not completely overcome the inhibition of glycogen synthase activation by 9 mM $MgCl_2$ plus 7.4 mM ATP. The drop in phosphorylase *a* activity in the presence of Mg.ATP towards the end of the sampling could be explained as for the case with AMP (see above discussion about Fig. IV.2).

At lower $MgCl_2$ and ATP concentrations (4 mM and 2.5 mM, respectively), glucose and caffeine, alone or together, relieved the inhibition of glycogen synthase activation (Fig. IV.4). In the presence of both glucose and caffeine, the onset of the activation occurred earlier than with the control, but levelled off at a lower value. This suggests that some form of modification of the phosphatase activity involved in the activation event may be taking place. Preincubation of liver extracts with ATP has been shown to lead to inactivation of protein phosphatases in the extracts (39). As with earlier experiments, glycogen synthase activation correlated with decreased phosphorylase *a* activity, occurring only when the activity of phosphorylase *a* was below 1 μM/min/g liver.

While the physiological concentration of ATP in the present study was taken to be about 7.4 mM (40), some

Figure IV.4. Effects of glucose and caffeine, alone and together, on Mg.ATP inhibition of glycogen synthase activation in rabbit liver filtrates in the presence of 1.0 mM EGTA.

Liver filtrates, obtained as described under Methods, were incubated in the presence of different combinations of ligands. Aliquots were withdrawn at indicated times and assayed for glycogen synthase (open symbols) or phosphorylase *a* (closed symbols) activities, as described in the text. Conditions were: Control - no additions (⊙—⊙), Mg.ATP (△—△), Mg.ATP + glucose (□—□), Mg.ATP + caffeine (◇—◇), Mg.ATP + glucose + caffeine (◈—◈). Final concentrations of ligands were: 4 mM $MgCl_2$, 2.5 mM ATP, 20 mM glucose, and 1 mM caffeine. All incubations contained 1.0 mM EGTA.

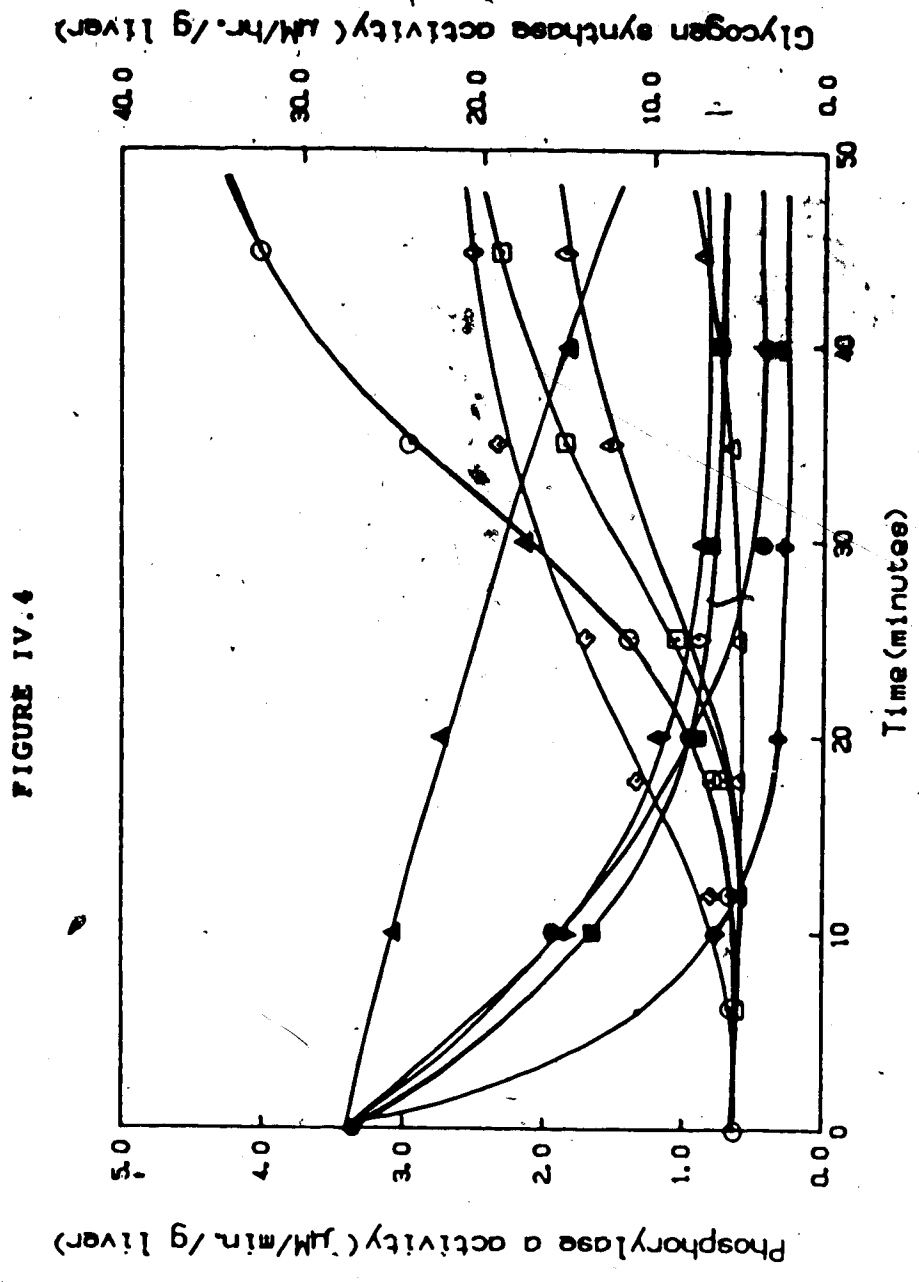

Glycogen synthase activity(μM/hr./g liver)
40.0
30.0
20.0
10.0
0.0
Phosphorylase a activity(μM/min./g liver)
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
Time(minutes)

FIGURE IV.4

investigators report a lower value of about 2.8 mM (41) and a free Mg^{2+} concentration of about 0.39 mM (42). It should be noted then that as a result of competition for ligand binding by the various cellular components, especially the interacting proteins in the rabbit liver filtrate, the effective concentrations of these ligands (ATP and Mg^{2+}) available to the components of the glycogen synthase activation process would be markedly lower than the total cellular concentrations and those used in Fig. IV.4. Consequently, as one approaches the concentration of Mg.ATP involved in the activation process, the ability of glucose and caffeine, alone and together, to overcome the inhibitory effects would become more significant.

Effect of added phosphorylase a on glycogen synthase activation

Results from earlier experiments (Figs. IV.1-4), suggested strongly the significance of phosphorylase *a* activity in the activation process. This was further investigated by adding rabbit skeletal muscle phosphorylase *a* to the activation mixture as indicated in Fig. IV.5. A significant delay in the onset of glycogen synthase activation was observed following the addition of 0.5 μg phosphorylase *a* per ml activation mixture, in agreement with other reports (5,43). In the presence of 20 mM glucose or 1 mM caffeine, the effect of added phosphorylase *a* was reversed, and a combination of both ligands overcame the inhibition of

Figure IV.5. Effect of added phosphorylase *a* on glycogen synthase activation in rabbit liver filtrates in the presence of glucose and caffeine.

Liver filtrates, obtained as described under Methods, were incubated in the presence of different combinations of ligands. About 10 seconds before zero time sampling, 4 µl of buffer (to control) or stock muscle phosphorylase *a* solution (to others) was added. Aliquots of the activation mixture were withdrawn at indicated times and assayed for glycogen synthase (open symbols) or phosphorylase *a* (closed symbols) activities, as described in the text. Conditions of incubations were: Control-no additions (⊙-⊙), phosphorylase *a* (△-△), phosphorylase *a* + glucose (□-□), phosphorylase *a* + caffeine (◇-◇), phosphorylase *a* + glucose + caffeine (◈-◈). The amount of added phosphorylase *a* was 0.5 µg/ml incubation mixture and final concentrations of glucose and caffeine were 20 mM and 1 mM, respectively.

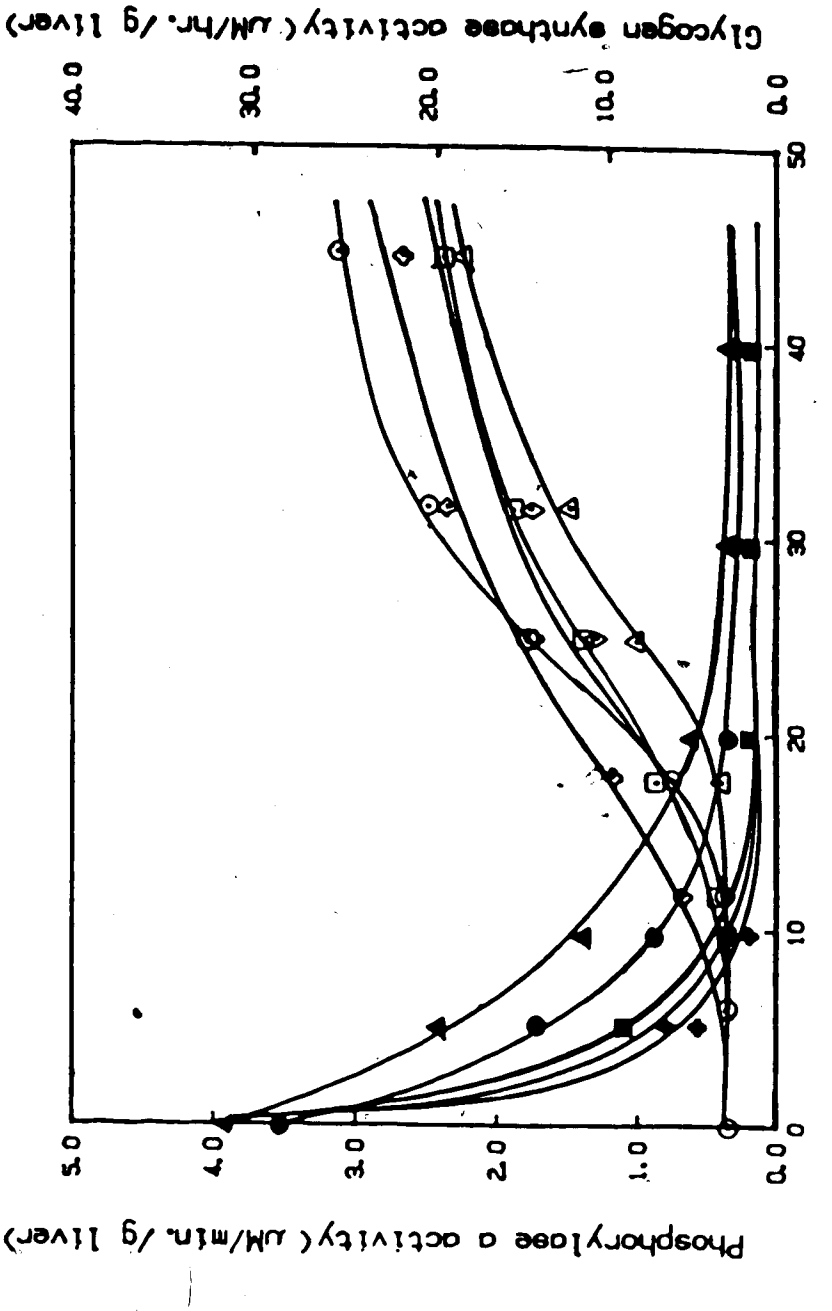

Glycogen synthase activity (μM/hr. /g liver)
40.0
30.0
20.0
10.0
0.0
Phosphorylase a activity (μM/min. /g liver)
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
Time (minutes)

FIGURE IV.5

Figure IV.6. Effect of added phosphorylase *a* on glycogen synthase activation in rabbit liver filtrates in the presence of glucose and caffeine.

Liver filtrates, obtained as described under Methods, were incubated in the presence of different combinations of ligands. About 15 min after initiating the activation (see arrow), 4 µl of buffer (to control) or stock muscle phosphorylase *a* solution (to others) was added. Aliquots of the activation mixture were withdrawn at indicated times and assayed for glycogen synthase activity as described in the text. Conditions of incubations were: Control-no additions (●–●), phosphorylase *a* (▲–▲), phosphorylase *a* + glucose (■–■), phosphorylase *a* + caffeine (◆–◆), phosphorylase *a* + glucose + caffeine (✖–✖). The amount of added phosphorylase *a* was 0.5 µg/ml incubation mixture and final concentrations of glucose and caffeine were 20 mM and 1 mM, respectively.

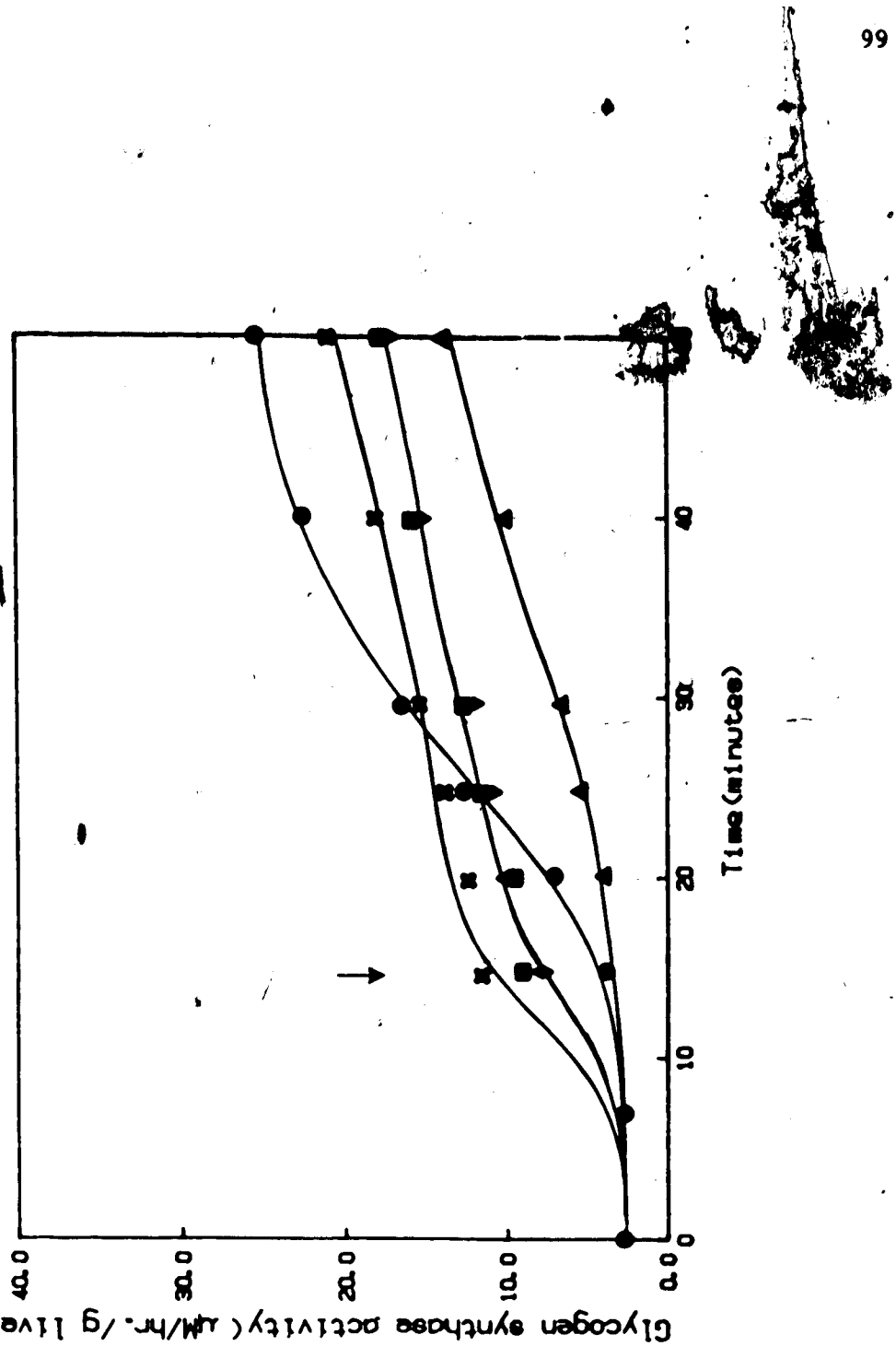

Glycogen synthase activity (μM/hr. /g liver)
0.0
10.0
20.0
30.0
40.0
0
10
20
30
40
Time (minutes)

FIGURE IV.6

activation over the control. The reason for the early plateau in glycogen synthase activity in the test incubations is undefined. Extension of the latency in glycogen synthase activation was also observed when exogenous phosphorylase *a* was added to the liver filtrates minutes after initiating the activation (Fig. IV.6), lending further support to the view that the lag could be attributed to the level of phosphorylase *a* activity.

D. Discussion

Despite numerous studies over the years, there is still no general agreement on the occurrence and characteristics of the latency in glycogen synthase activation reported by Hers, Stalmans, and their associates (43) to occur in rat liver filtrates. Some investigators claimed they could not detect the lag using protein-glycogen suspensions (7) although a lag is apparent in their figures. Demonstration of the latency required specific conditions such as the presence of anions such as sulfate, chloride and phosphate at non-physiological concentrations (44,45) and the lag period could not be detected with desalted preparations containing HEPES or imidazole only (44,45).

In this chapter, it is shown that the latency also occurs with rabbit liver filtrates under the conditions used. Glucose and caffeine, acting alone or together, shortened the lag period. Also, the response of the lag to the presence of phosphorylase *a* phosphatase inhibitors such

as AMP and ATP could be explained by the earlier reported effects of these ligands on purified rabbit liver protein phosphatases (33; see Chapter II). Thus, glucose and caffeine, acting independently and together, abolished the inhibition of phosphorylase *a* phosphatase activity in the liver filtrate and subsequently allowed for glycogen synthase activation. Taken with the effect of added phosphorylase, these results support the earlier observations on the importance of phosphorylase *a* in the modulation of glycogen synthase activation.

A combination of unphysiologically high concentrations of AMP (3 mM) and Mg^{2+} (5 mM) has been repeatedly shown to abolish the lag in glycogen synthase activation in rat liver filtrate (3). In a recent report, fructose 1-P was shown to have a similar effect (46). These observations were attributed to the ability of these ligands to render phosphorylase *a* 'non-inhibitory' to glycogen synthase phosphatase activity, implying that distinct phosphatases act on phosphorylase *a* and glycogen synthase *b*. A number of studies indicate that phosphorylase *a* inhibits glycogen synthase phosphatase activity *in vitro* (8,9,10). Thus, any accumulation of phosphorylase *a* would be expected to shut-down glycogen synthase activation irrespective of whether the same or different enzyme(s) is/are capable of acting on phosphorylase *a* and glycogen synthase *b*.

The results described in this chapter indicate that under conditions where phosphorylase *a* phosphatase activity

is inhibited (e.g. presence of AMP and ATP), the onset of glycogen synthase activation is suppressed. Therefore the explanation for the AMP (3 mM) and Mg^{++} (5 mM) or fructose 1-P effect on the latency seems improbable. The results presented here are consistent with the view that an indirect effect of glucose and caffeine, mediated through their binding to phosphorylase, could account for the effect of the ligands on glycogen synthase activation in the liver filtrate.

The liver filtrate is a complex mixture of proteins thus making it difficult to ascertain which of the proteins present are involved in the activation pattern. Thus, further understanding of the role of phosphorylase *a* in the regulation of hepatic glycogen metabolism by this mechanism requires that the interaction between phosphorylase *a*, glycogen synthase phosphatase(s), phosphorylase *a* phosphatase(s), and glycogen synthase *b* be demonstrated using purified proteins. Nonetheless, a combination of glucose and caffeine was quite effective in relieving the inhibition of glycogen synthase activation by phosphorylase *a* or inhibitors of phosphorylase *a* phosphatase activity in this study, strongly indicating that the inhibitory site on phosphorylase *a*, which was probed with caffeine, may be of physiological significance in hepatic glycogen metabolism.

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V. Phosphorylase a inhibition of glycogen synthase phosphatase activity of purified rabbit liver protein phosphatases

A. Introduction

Studies reported in Chapter IV of this thesis indicated the significance of phosphorylase *a* levels in the modulation of glycogen synthase activation by dephosphorylation in gel-filtered rabbit liver extract. The onset of glycogen synthase activation correlated with the level of phosphorylase *a* activity, occurring only after considerable depletion of this activity. The responses of the latency in glycogen synthase activation to the presence of glucose, caffeine and the nucleotides, ATP and AMP, could be explained by the observed effects of these ligands on the phosphorylase *a* phosphatase activity of purified protein-phosphatases-1 and 2A (Chapters II and III).

Dopere *et al*. (1) reported that the glycogen synthase phosphatase activity in rat liver results from co-operation between proteins termed the G- and S-components, which are preparable from the cytosolic fraction of glycogen-depleted liver. The G-component co-sediments with added particulate glycogen while the S-component does not, and the G-component by itself is unable to convert glycogen synthase *b* to the *a* form. By comparing their origins relative to the crude liver preparation, the G- and S-components are similar to protein phosphatases-1 and 2A, respectively (2).

However, in further studies reported recently by the same group of investigators [Mvumbi *et al.* (ref. 3)], the G-component was able to convert glycogen synthase *b* to the *a* form, a finding that disagrees with the earlier report. In the recent report, phosphorylase *a* inhibited the activity of the G-component (as present in protein-glycogen complex) acting on glycogen synthase *b* whereas the S-component was not affected. Further, the dephosphorylation (activation) of rabbit skeletal muscle glycogen synthase *b* (phosphorylated by glycogen synthase kinase-3) by partially purified G-component in the presence of rabbit skeletal muscle phosphorylase *a* was preceded by a lag period corresponding to the time required to reduce the phosphorylase *a* activity.

Ingebritsen *et al.* (4), in assessing the physiological roles of the different protein phosphatases involved in glycogen metabolism, concluded that protein phosphatases-1 and/or 2A may be the enzyme(s) responsible for the activation of glycogen synthase in the early studies of Hers and co-workers. Other studies indicated that protein phosphatase-1 and 2A account for virtually all the phosphorylase *a* and glycogen synthase *b* (sites-2 and 3 phosphorylated) phosphatase activities in dilute extracts from different mammalian tissues including rabbit skeletal muscle and liver (2,5).

This section of the thesis reports on some of the numerous studies done to demonstrate the inhibition by phosphorylase *a* of the glycogen synthase phosphatase

activities of the different rabbit liver protein phosphatases, particularly with a view to identifying which protein phosphatase(s) is/are involved. Glucose and caffeine showed additive activation of the phosphorylase *a* phosphatase activity of the different phosphatase forms (Chapter II), and significantly affected the glycogen synthase activation patterns seen with gel-filtered rabbit liver extract (Chapter IV), hence the effects of these ligands were investigated with purified proteins to further clarify the concept of phosphorylase *a* inhibition of glycogen synthase activation.

B. Materials and Methods

Materials

Phosphocellulose (P11) was obtained from Whatman and Sepharose 4B was from Pharmacia. Sources of other materials used have been reported in the preceding chapters.

Protein preparation

Procedures for the purification and preparation of the M_r 35 000 phosphatase, phosphatases-1, $2A_1$ and $2A_2$, and phosphorylase *a* were as reported in Chapter II.

Glycogen synthase *a* was purified from fresh rabbit skeletal muscle by an unpublished procedure (Dr. B. Osterlund, in our laboratory), which was adapted from the work of Shaltiel and Er-El (6) and Nimmo *et al.* (7), and

employed $(NH_4)_2SO_4$ fractionation and sequential chromatography using aminobutyl agarose and Sepharose 4B. The synthase preparation was further purified on phosphocellulose (P11) as described by Soderling *et al.* (8) to remove contaminating kinase and phosphatase activities. At this stage, the protein preparation had a specific activity of 3-5 U/mg, using the assay method of Thomas *et al.* (9) as described by Nimmo *et al*, (7). It had an activity ratio [low G-6-P/high G-6-P assay of Guinovart *et al.* (10)] of about 0.8-0.9.

Phosphorylation of the glycogen synthase *a* was by the general procedure reported by Embi *et al.* (11) using the catalytic subunit of cAMP-dependent protein kinase. The phosphorylation mixture contained 2 mg/ml glycogen synthase *a*, 10 mM sodium glycerol 1-P (pH 6.9), 0.4 mM EDTA, 0.1 mM EGTA, 0.9 mM $Mg(OAc)_2$, 0.1 mM ATP or [γ-^{32}P]-ATP and the kinase (200 U/ml ; see page 57 for definition of U). After incubation at 30°C for up to 2.5 hours, the reaction was terminated by the addition of 0.1 volumes of 250 mM EDTA and 500 mM NaF. An equal volume of ice-cold saturated $(NH_4)_2SO_4$ was then added and, after storing at 4°C for 15 min, the protein pellet was collected by centrifugation at 8 000 g for 15 min. The pellet was dissolved in buffer containing 50% (v/v) glycerol and passed through a Sephadex G-25 (fine) column to remove excess nucleotide and NaF. Fractions with $A_{260/280}$ ratios of 0.58-0.59 (12) were pooled and concentrated using CX-30 ultrafiltration units. The

activity ratio (low/high G-6-P assay; see above) of the glycogen synthase *b* varied from 0.15 to 0.30 and incorporation of phosphate was from 0.8 to 2 mole/mole protein (M_r 88 000). The protein was stored at -70°C as it lost activity rapidly at 4°C.

Rabbit liver glycogen synthase *b* was prepared by a modification of the method described by Killilea and Whelan (13). The homogenizing buffer and glycogen pellet resuspension buffer contained 1 mM benzamidine, 1 mM PMSF, and 1 μg/ml of pepstatin and leupeptin. The first glycogen pellet was used and glycogen present was partially digested by incubation of the suspension at 30°C with human saliva α-amylase. The activity ratio (activity without divided by that with 10 mM G-6-P) for two different preparations up to the DE 52 step was 0.01-0.03.

Glycogen synthase phosphatase assay

Glycogen synthase phosphatase activity was measured under various conditions using either of two general methods. Specific conditions of the incubation mixture are stated with the figure legends but those reported in this section represent the sets of condition most frequently used.

Method 1: Phosphatase activity was determined by release of ^{32}P from labelled glycogen synthase *b* at 30°C. The incubation mixture contained 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.5 mM EDTA, 10% (v/v) glycerol, 0.2 mg/ml ^{32}P-labelled

glycogen synthase *b* , and the phosphatase in a total of 500 µl. The substrate and effectors, in 250 µl, were preincubated for 5 min before initiating the reaction with an equal volume of suitably diluted, preincubated phosphatase preparation. At the indicated times, 50 µl aliquots of the mixture were transfered into 175 µl of ice-cold 10% TCA and 25 µl of ice-cold 25 mg/ml BSA was added. Further processing of the mixture was as described for the phosphorylase *a* phosphatase assay (see Chapter II).

Method 2: Phosphatase activity was determined by the activation of glycogen synthase (glycogen synthase *b* to *a* conversion) at 30°C. A standard incubation mixture contained 50 mM HEPES (pH 7.1), 0.1 M NaCl, 10% (v/v) glycerol, glycogen synthase *b* and suitably diluted phosphatase preparation. The reaction was initiated as described for Method 1 above, and at indicated times, aliquots were withdrawn into a substrate mixture and assayed as described in Chapter IV.

Phosphorylase a assay

Phosphorylase *a* activity was determined using the inorganic phosphate release assay described in Chapter IV.

C. Results

Based on the finding that protein phosphatase-2A represents the major phosphorylase *a* and glycogen synthase *b* phosphatase activities in tissue extracts including rabbit liver (2), initial efforts were directed at the M_r 35 000

phosphatase and phosphatases-2A$_1$ and 2A$_2$. An experiment representative of those employing rabbit skeletal muscle glycogen synthase *b*, ^{32}P-labelled with the catalytic subunit of cAMP-dependent protein kinase, is shown in Fig. V.1. There was no appreciable effect of added rabbit skeletal muscle phosphorylase *a* on the dephosphorylation of the glycogen synthase *b* by protein phosphatase-2A$_1$. Similar patterns were observed for the other type-2 phosphatase forms (not shown). The additional presence of a combination of glucose (20 mM) and caffeine (1 mM) did not affect the dephosphorylation patterns.

These findings were surprising especially since in these experiments phosphorylase *a* was used at concentrations up to 24-fold molar excess to the synthase. The K_m obtained for the rabbit liver M_r 35 000 phosphatase acting on glycogen synthase *b* from the same tissue was about 0.12 μM (14). When compared with the K_m's reported in Chapter II of this thesis for the different type-2 protein phosphatases, this indicates a higher affinity for the synthase for this phosphatase form.

However, K_i's of about 1 μM and 7 μM, respectively, were obtained for thiophosphorylase *a* inhibition of the phosphorylase *a* phosphatase activity of the M_r 35 000 phosphatase and phosphatase-2A$_1$ from rabbit liver using rabbit skeletal muscle proteins (unpublished experiments), indicating that the K_m's for the protein phosphatases reflect more than their affinity for the substrate. Also, the

Figure V.1. Effects of phosphorylase *a*, glucose and caffeine, on the dephosphorylation of ^{32}P-labelled glycogen synthase *b* by protein phosphatase-2A$_1$.

Phosphatase-2A$_1$ activity on ^{32}P-labelled rabbit skeletal muscle glycogen synthase *b* was determined by Method 1 as described in the text. Conditions were: Control (no additions) (●–●); phosphorylase *a* (0.1 mg/ml) (■–■); phosphorylase *a* (0.1 mg/ml) + glucose (20 mM) + caffeine (1 mM) (▲–▲); phosphorylase *a* (5.4 mg/ml) (◆–◆); phosphorylase *a* (5.4 mg/ml) + glucose (20 mM) + caffeine (1 mM) (▼–▼). Rabbit skeletal muscle phosphorylase *a* was used in this and subsequent figures, and the buffer was 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.5 mM EDTA, and 10% (v/v) glycerol. Data in this and subsequent figures in this chapter are from duplicate incubation mixtures for single experiments representative of several repeated trials.

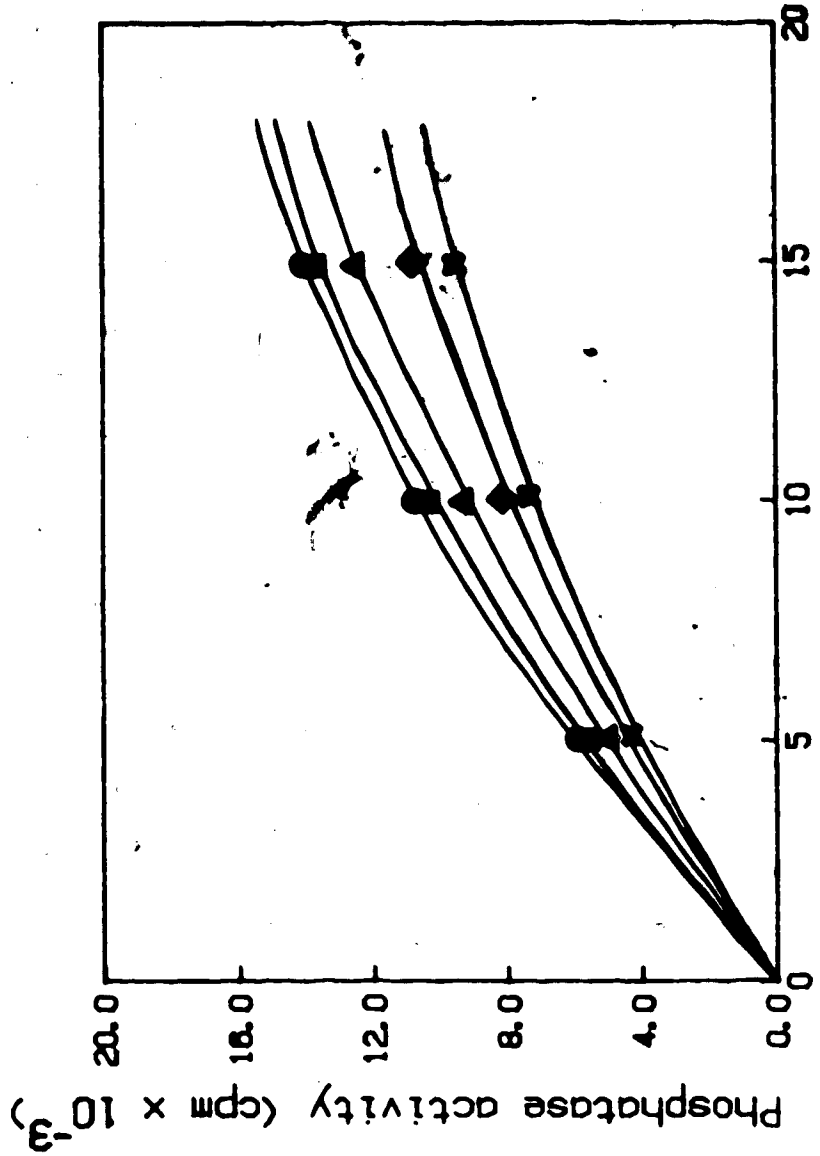
Phosphatase activity (cpm x 10^{-3})
20.0
16.0
12.0
8.0
4.0
0.0
0
5
10
15
20
Time (minutes)

FIGURE V.1

concentration dependency of the phosphorylase *a* inhibition of the synthase phosphatase activity has led numerous investigators to suggest that the inhibition results from direct competition (15,16). The results obtained in the present study with the type-2 protein phosphatases disagrees with this notion and suggests that some poorly understood intrinsic feature of these phosphatases could result in the desensitization of their synthase phosphatase activity to inhibition by phosphorylase *a*.

The report by Mvumbi *et al*. (3) directed the project towards investigating the involvement of protein phosphatase-1. Representative data from one of the numerous experiments done with this phosphatase form is shown in Fig. V.2. The dephosphorylation of glycogen synthase *b* was significantly inhibited by added rabbit skeletal muscle phosphorylase *a*. Of significance is the fact that the inhibition was seen at a phosphorylase *a* concentration much lower than that which did not affect the type-2 phosphatases (see above). However, no lag in dephosphorylation was detected. Several other experiments (not reported), in which the conditions of incubation were varied to represent more closely the conditions used by Mvumbi and co-workers, were carried out with no success.

A different approach in assaying the glycogen synthase phosphatase activity was employed (Method 2 above).

Figure V.2. Effects of phosphorylase *a*, glucose and caffeine, on the dephosphorylation of glycogen synthase *b* by protein phosphatase-1.

Phosphatase-1 activity on ^{32}P-labelled rabbit skeletal muscle glycogen synthase *b* was determined by Method 1 as described in the text, [% dephosphorylation (open symbols) and phosphorylase *a* activity (closed symbols)]. Conditions of incubations were: Control (no additions) (⊙–⊙); phosphorylase *a* (25 μg/ml) (□–□); phosphorylase *a* (25 μg/ml) + glucose (20 mM) + caffeine (1 mM) (△–△). Buffer: 50 mM imidazole-chloride (pH 7.4), 0.5 mM DTT, 0.5 mM EDTA, and 0.25 mM sucrose. All incubations contained 1 mM free Mg^{2+} and 5 mM $(NH_4)_2SO_4$.

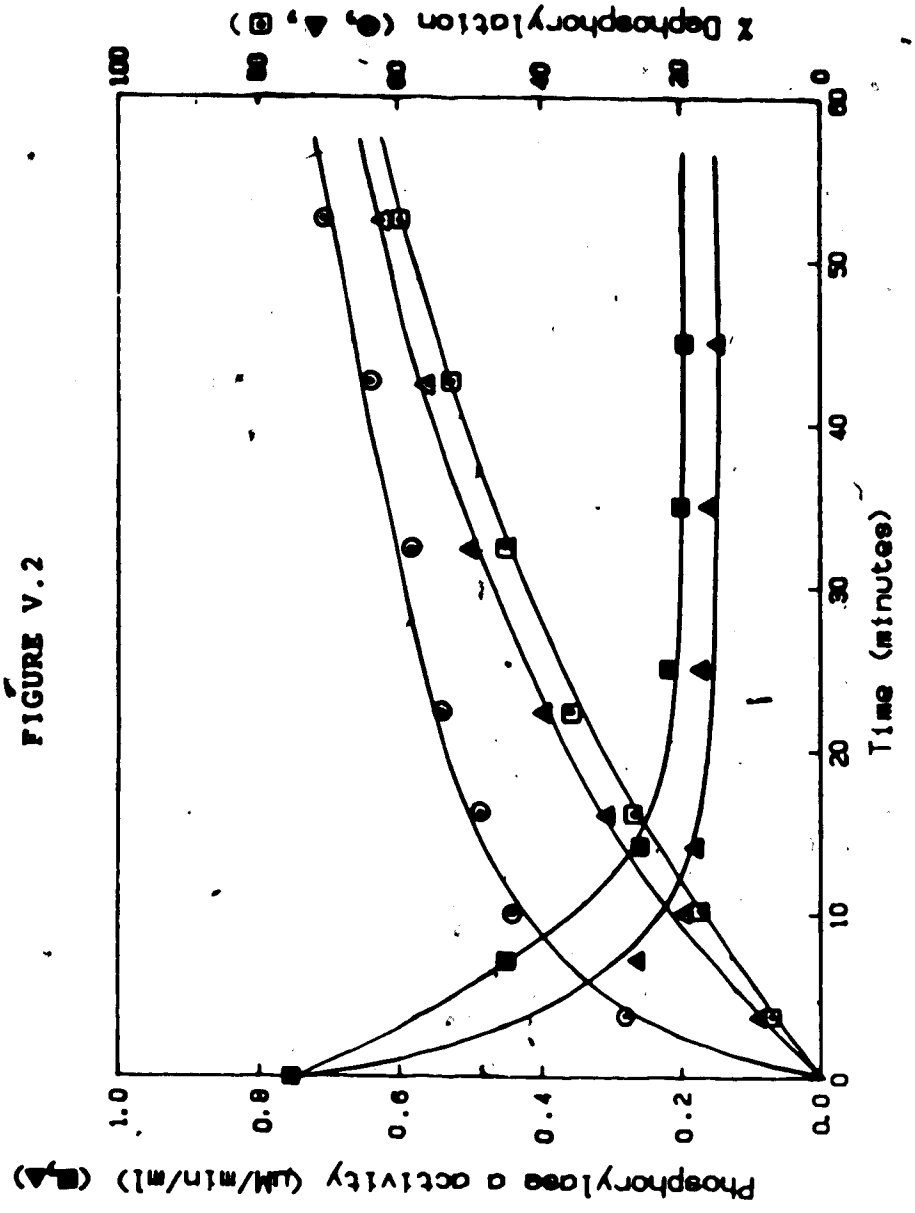
% Dephosphorylation (●, ▲, ■)
Phosphorylase a activity (μM/min/ml) (■, ▲)
Time (minutes)

FIGURE V.2

Fig. V.3 shows data in which a mixture of purified rabbit liver phosphatases-1 and $2A_1$ was used as phosphatase source. The added phosphorylase *a* (10 μg/ml) significantly inhibited the activation of the rabbit skeletal muscle glycogen synthase *b* by phosphatase action, but no latency was observed.

Rabbit liver extract obtained as described in Chapter IV (up to glasswool filtration) was incubated at 30°C in the presence of 20 mM glucose and 1 mM caffeine, to ensure depletion of phosphorylase *a* and complete conversion of glycogen synthase *b* to *a*. The treated extract was then put through Sephadex G-25 (fine) and the filtrate used as phosphatase source in some experiments. Fig. V.4 shows data representative of such experiments. As with previous results, phosphorylase *a* inhibited glycogen synthase activation. However, the latency in the activation could not be demonstrated. Further, the presence of a combination of glucose and caffeine did not alter the patterns observed.

It was envisaged that the inability to demonstrate the latency in glycogen synthase activation may be due to absence of particulate glycogen which may serve as an anchor for the interacting proteins. Experiments were then performed in the presence of 1% (wt/v) rabbit liver glycogen. As shown in Fig. V.5, the pattern of glycogen synthase activation did not differ from previous results. Phosphorylase *a* was inhibitory, latency in activation was not observed, and there was no difference seen whether

Figure V.3. Effects of phosphorylase *a*, glucose and caffeine, on the activation of glycogen synthase *b* by a mixture of protein phosphatases-1 and $2A_1$.

Activation of rabbit skeletal muscle glycogen synthase *b* by a mixture of rabbit liver protein phosphatases-1 and $2A_1$ was determined by Method 2 as described in the text. Conditions were: Control (no additions) (●—●); phosphorylase *a* (10 μg/ml) (■—■); phosphorylase *a* (10 μg/ml) + glucose (20 mM) + caffeine (1 mM) (▲—▲). Buffer: 50 mM HEPES (pH 7.1), 0.1 M NaCl, and 5% (v/v) glycerol.

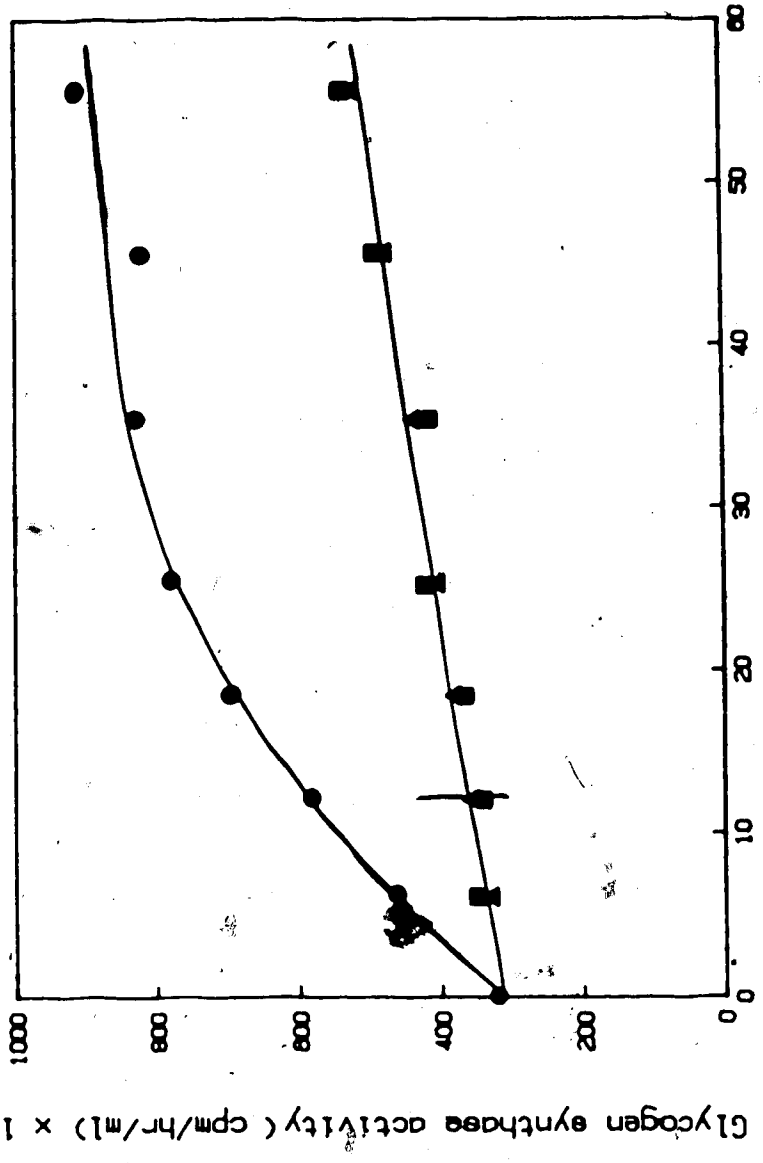
Glycogen synthase activity (cpm/hr/ml) x 10^{-2}
1000
800
600
400
200
0
0
10
20
30
40
50
60
Time (minutes)

FIGURE V.3

Figure V.4. Effects of phosphorylase *a*, glucose and caffeine, on the activation of glycogen synthase *b* using glucose/caffeine-treated extract as phosphatase source.

Activation of rabbit skeletal muscle glycogen synthase *b* was determined by Method 2 using glucose/caffeine-treated extracts as described in the text. Conditions were: Control (no additions) (●–●); phosphorylase *a* (50 μg/ml) (■–■); phosphorylase *a* (50 μg/ml) + glucose (20 mM) + caffeine (1 mM) (▲–▲). Buffer condition was same as in Figure V.3.

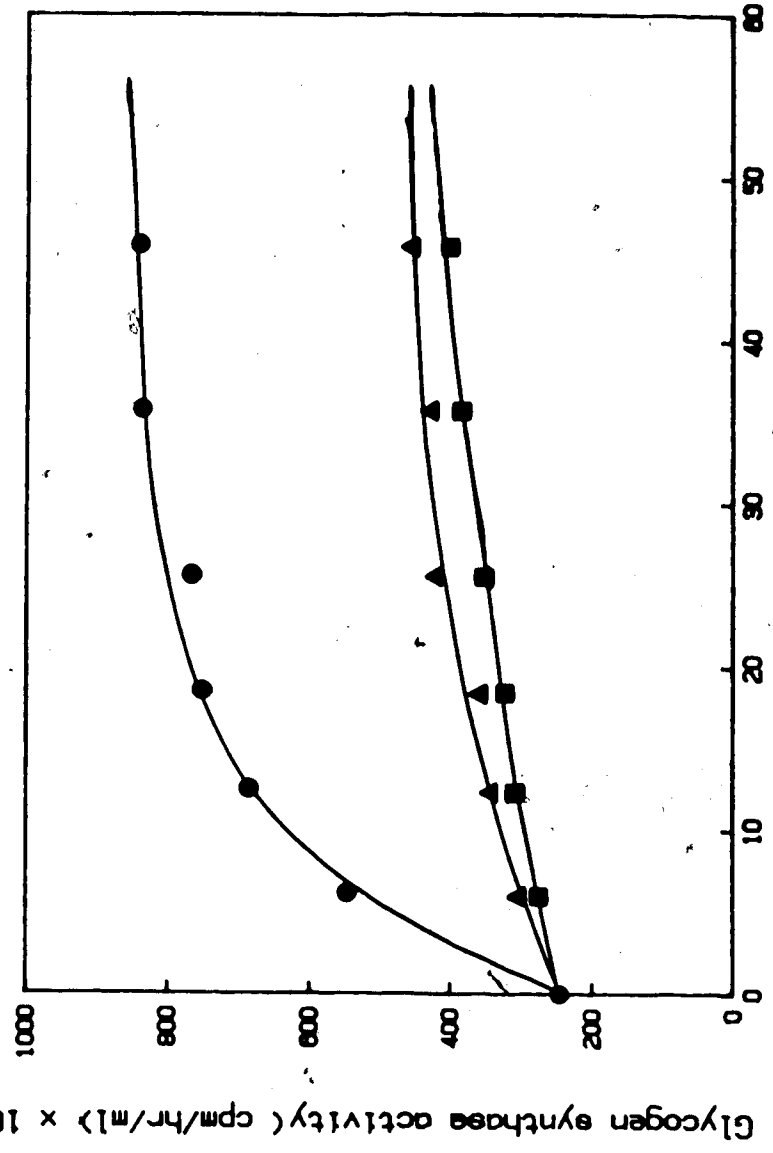
Glycogen synthase activity (cpm/hr/ml) x 10 -2
1000
800
600
400
200
0
0
10
20
30
40
50
60
Time(minutes)

FIGURE V.4

Figure V.5. Effects of phosphorylase *a*, glucose and caffeine, in the presence of added glycogen, on the activation of glycogen synthase *b* by protein phosphatase-1.

Activation of rabbit skeletal muscle glycogen synthase *b* by rabbit liver protein phosphatase-1 was determined by Method 2, described in the text, in the presence of 2.2 mg/ml rabbit liver glycogen. Conditions were: Control (no additions) (●—●); phosphorylase *a* (10 μg/ml) (■—■); phosphorylase *a* (10 μg/ml) + glucose (20 mM) + caffeine (1 mM) (▲—▲). Buffer used was same as in Figure V.3.

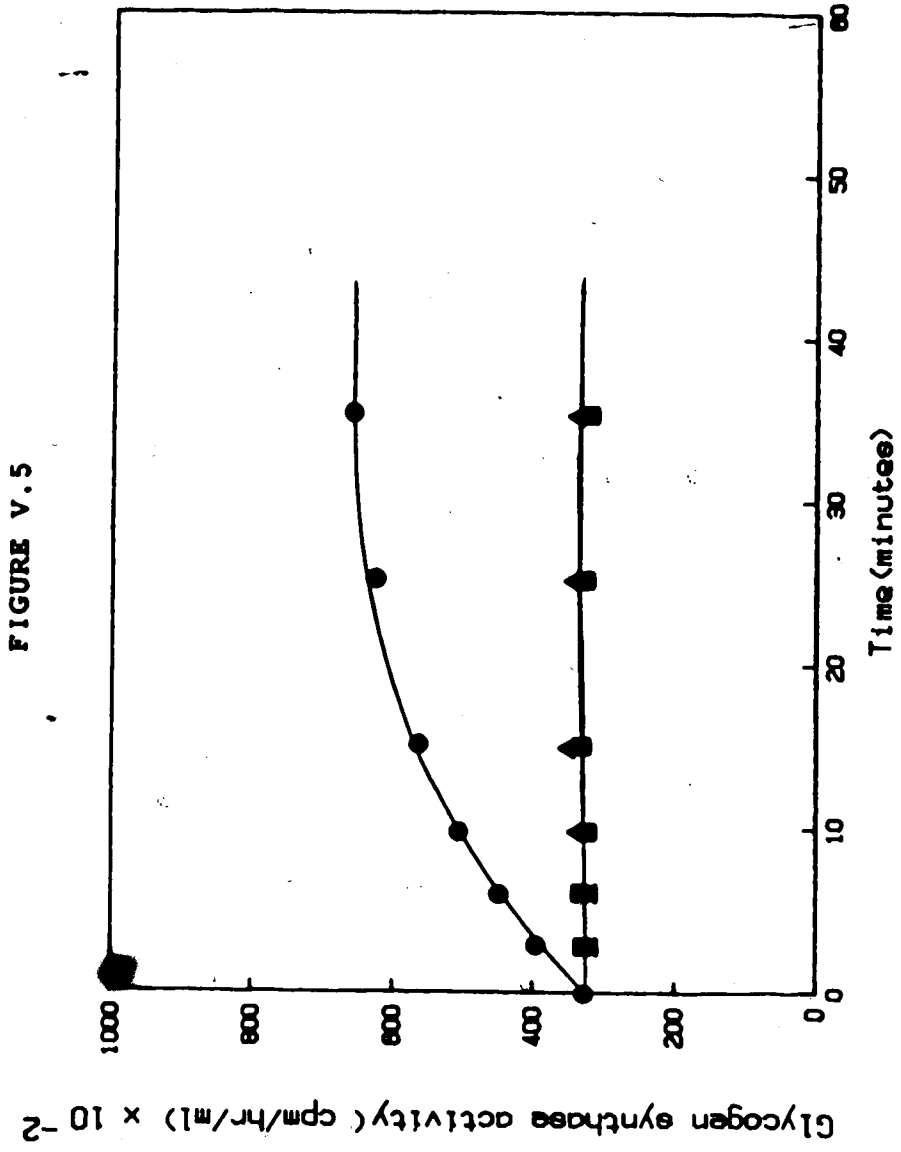
Glycogen synthase activity (cpm/hr/ml) x 10 -2
1000
800
600
400
200
0
0
10
20
30
40
50
60
Time(minutes)

FIGURE V.5

a combination of glucose and caffeine was present or not.

In other experiments (not shown), protein phosphatase-1 did not convert any rabbit liver glycogen synthase *b* to *a*. The G-component reported by Dopere *et al.* (1) behaved similarly.

Phosphorylase *a* activity levels were not monitored during the experiments shown in Figs. V.3-5 due to practical limitations. However, control experiments (not shown) with rabbit skeletal muscle phosphorylase *a* and the phosphatase preparations under similar conditions showed that the phosphatase activity used in each case was sufficient to lead to about 80% decrease in phosphorylase *a* activity within 10 min incubation time.

D. Discussion

Although the results of the studies reported in this chapter are inconclusive, in that the demonstration of the latency in glycogen synthase activation observed in previous studies using gel-filtered liver extract (see **Chapter IV**) could not be achieved using purified proteins, some interesting observations were obtained.

The inhibition of glycogen synthase phosphatase activity by phosphorylase *a* was evident for protein phosphatase-1 and not with phosphatases-$2A_1$ and $2A_2$, and the 35 000 M_r phosphatase under the condition used. These findings agree with those reported by Mvumbi *et al.* (3), given the similarities between their G- and S-components and

protein phosphatases-1 and 2A, respectively.

The inability to demonstrate the latency in this report could stem from a number of factors. In a recent report (17), Ca^{2+} and glycogen were shown to act synergistically as inhibitors of rat liver glycogen synthase phosphatase using crude extract. Similar results were also obtained with the G-component, as present in the protein-glycogen complex or in partially purified form, acting on purified liver glycogen synthase *b*. The major phosphatase source used by Mvumbi *et al*. in their studies was from the protein-glycogen complex, suggesting that perhaps the latency observed was due to inhibition of the phosphatase activity by glycogen. High concentrations of glycogen are known to inhibit the protein phosphatase activity against glycogen synthase *b* (18). The situation is further complicated by the fact that the protein-glycogen suspensions used was not gel-filtered, hence the possibility of 'interference' by small molecules in the activation experiment. Furthermore, the possibility that the G-component is not equivalent to protein phosphatase-1 exists, as the former consists of phosphatase activities co-sedimenting with particulate glycogen added to glycogen-depleted liver cytosol preparations. However, Mvumbi and co-workers were able to show the latency when partially purified G-component was acting on rabbit skeletal muscle glycogen synthase *b* phosphorylated by kinase F_A [similar to glycogen synthase kinase-3 (19)], suggesting that perhaps the difference in phosphorylation sites may be a

factor in the differences in this report.

A major set-back for the phosphorylase *a* threshold in the activation of glycogen synthase is the observation that the latency could not be demonstrated with liver preparations from starved animals (20). It has been shown that the livers of starved rats contain larger amounts of glycogen synthase *a* than livers from fed animals despite the presence of high levels of phosphorylase *a* in livers from fed animals (21). It is well known that most of the enzymes of glycogen metabolism are closely associated with particulate glycogen, thus depletion of glycogen by fasting would result in dissociation of these enzymes leading to probable 'loss' of the control features inherent in the protein-glycogen complex.

As is presently known, protein phosphatase-1 is the only phosphatase activity in protein-glycogen complex from livers of well-fed rabbits while the type-2 phosphatases involved in glycogen metabolism, phosphatase-2A (comprising phosphatase-$2A_0$, $2A_1$ and $2A_2$) are located exclusively in the cytosol (2). Also phosphatase-2A accounts for over 60% of the phosphorylase *a* and glycogen synthase *b* phosphatase activities in rabbit liver (4). Thus, with negligible glycogen concentrations in liver preparations due to fasting, glycogen synthase *b* would become susceptible to high phosphatase action from both phosphatases-1 and 2A, a consequence of the apparent 'solubilization' of these proteins. This situation could lead to the uncoupling of

phosphorylase *a* inhibition of glycogen synthase phosphatase activity, and may be one of the factors responsible for the absence of latency in glycogen synthase activation in liver preparations from starved animals. In light of the above discussion, the significance of the findings in studies in this chapter in which only protein phosphatase-1 action on glycogen synthase *b* was appreciably inhibited by phosphorylase *a* becomes apparent.

The observation that the combined presence of glucose and caffeine at concentrations that would increase the rate of dephosphorylation of added phosphorylase *a* by phosphatase action did not affect the results of the experiments reported here seems surprising. A plausible explanation for this could be that the phosphorylase *a* level is too low to allow detectable differences between the incubations with and without the activators.

The results indicate that protein phosphatase-1 activity on glycogen synthase *b* is strongly inhibited by phosphorylase *a*; thus further exploration of this finding in relation to the latency of glycogen synthase activation should be directed towards this phosphatase form. There is no doubt that a reconstituted system in which the latency in this activation is demonstrable would shed more light on our understanding of the role of phosphorylase *a* and the functional site on phosphorylase *a* that binds caffeine in the regulation of hepatic glycogen metabolism.

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VI. General Discussion and Conclusions

Following the initial description of the inhibitor site on rabbit muscle phosphorylase *a* monomer specific for nucleosides and purine derivatives such as caffeine (1,2), a number of studies have been done to ascertain the functional significance of this site in the regulation of glycogen metabolism (reviewed in ref 3). Caffeine preferentially binds to this inhibitor site with apparent K_i's of 0.1 mM and 0.4 mM for the rabbit skeletal muscle and liver phosphorylase *a*, respectively, and has served as a functional probe for the site. Caffeine was shown to act synergistically with glucose in inhibiting phosphorylase *a* from both rabbit skeletal muscle and liver (2). Also, in combination with glucose, caffeine further decreased the latency in glycogen synthase activation in rat hepatocytes (4).

Studies reported in Chapter II of this thesis (see also ref. 5) indicate that glucose and caffeine had independent and additive activating effects on four molecular forms of rabbit liver phosphorylase *a* phosphatase activity: the 'catalytic' subunit (M_r 35 000) phosphatase and phosphatases-1, $2A_1$ and $2A_2$. The caffeine effect was seen at μM concentrations suggesting a significant role for the action of ligands that bind to the nucleoside site on the phosphorylase *a* monomer. In addition, both glucose and caffeine counteracted the inhibition of phosphorylase *a* phosphatase activity by nucleotide inhibitors (ATP and AMP)

and other inhibitors tested.

In Chapter III, using the natural substrate (phosphorylase *a*) and two alternate substrates (histone and a tetradecapeptide encompassing residues 5 to 18 in phosphorylase *a*), distinction was made between substrate and/or enzyme-directed effects of some physiologically important ligands. Glucose, caffeine, AMP, ADP, P_i and glucose 1-phosphate showed substrate-directed inhibition for the four phosphatase forms. ATP exerted both substrate and enzyme-directed effects for the 35 000 M_r phosphatase and phosphatases-1 and $2A_1$ but only a substrate-directed effect for phosphatase-$2A_2$ suggesting that the γ-subunit of the type-2 phosphatases may be involved in ATP binding. The results in this chapter suggest an important role for substrate-directed control by ligands that affect phosphorylase *a* phosphatase activity.

The molecular basis for the Hers' hypothesis, in which the activation of glycogen synthase by phosphatase action is modulated by the level of phosphorylase *a* activity, was further investigated using rabbit liver filtrates. Chapter IV shows that glucose and caffeine, acting alone or together, relieved phosphorylase *a* inhibiton of glycogen synthase activation in the filtrates. These ligands opposed the effects of phosphorylase *a* phosphatase inhibitors such as ATP and AMP. Also, exogenous phosphorylase *a* extended the latency in glycogen synthase activation. Studies with purified proteins (Chapter V) showed that the dephosphorylation

or activation of rabbit skeletal muscle glycogen synthase, phosphorylated by cAMP-dependent protein kinase, by protein phosphatase-1 was severely inhibited by rabbit skeletal muscle phosphorylase *a*. However, the activities of the M_r 35,000 phosphatase and phosphatases-$2A_1$ and $2A_2$, on the same substrate, were not appreciably affected by phosphorylase *a* concentrations several fold higher than that which inhibited the phosphatase-1 activity. The results agree with reports of other workers (6, see Chapter IV).

Although the latency in glycogen synthase activation was not demonstrated using purified proteins under the reported conditions, the results indicate that phosphorylase *a* inhibits phosphatase action on glycogen synthase *b* and further studies with purified proteins would aid our understanding of the role of phosphorylase *a* in the regulation of the activation process.

The results from the independent but related studies in this thesis are consistent with the view that phosphorylase *a* activity level modulates the activation of glycogen synthase although the involvement of additional factor(s) could not be ruled out. Furthermore, the additive effects of glucose and caffeine with respect to the inhibitory conditions observed in these studies, support the contention that glucose alone may not be the sole physiological regulator of glucose homeostasis through its action on hepatic phosphorylase *a*. Thus, the nucleoside site on phosphorylase *a*, which binds caffeine and other purine

derivatives, may be of physiological significance. There in no doubt that the isolation and characterization of the natural ligand(s) for this site would be valuable in clarifying the functional role of this site in the regulation of glycogen metabolism.

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