

STIMULATION OF MAP KINASES AND S6 KINASES
BY SODIUM SELENATE AND VANADYL SULPHATE

by

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ABSTRACT

Insulin has a wide range of biological effects on mammalian cells including both metabolic and mitogenic actions. A phosphorylation cascade originating at the insulin-receptor and involving a number of different serine/threonine-protein kinases is believed to mediate, at least in part, some of these effects. The two best studied kinases within this phosphorylation cascade, MAP kinases and S6 kinases, are believed to play pivotal roles in insulin signal transduction. Both selenium and vanadium compounds have been shown to have insulin-mimetic effects on isolated rat adipocytes. In providing further evidence for their insulin-mimetic properties, their effects on the activity of MAP kinases and S6 kinases in isolated rat adipocytes were examined by measuring the phosphorylation of myelin basic protein (MBP) and Ribosomal S6 Protein, respectively.

Both MBP kinases and Ribosomal Protein S6 kinases were shown to be activated in response to insulin treatment of adipocytes thus confirming the suitability of this system for the investigation of insulin-mimetic agents. Sodium selenate and vanadyl sulphate treatment of cells led to dose and time-dependent stimulation of both MBP kinases and Ribosomal Protein S6 kinases. Maximal stimulation of MBP kinases by sodium selenate was ~2-fold control while Ribosomal Protein S6 kinases were stimulated to over 8-fold control. Vanadyl sulphate treatment led to higher levels of stimulation with MBP kinase activity being ~5-fold control and Ribosomal Protein S6 kinase activity reaching levels that were greater than 16-fold control. Anion-exchange chromatography of the crude cell extracts revealed several distinct peaks of MBP and Ribosomal Protein S6 kinase activity corresponding to previous reports in the literature, however no distinct kinase families were conclusively identified using immunological techniques.

Our results further confirm the insulin-mimetic properties of selenium and vanadium compounds. Both were shown to stimulate kinases within the signal transduction cascade of insulin to a greater degree than insulin itself. The distinct families of MAP- and S6 kinases

stimulated by these agents were not identified although the presence more than one family for each group of kinases was indicated.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
Arg	arginine
BCIP	5-bromo-4-chloro-indolyl phosphate
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CytPTK	cytosolic protein tyrosine kinase
DMF	N,N-dimethyl formamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol bis(b-aminoethyl ether-P-N, N, N',N'-tetraacetic acid)
ERK	extracellular signal-regulated kinase
GAP	GTPase activity protein
GDI	GDP dissociation inhibitor
GDP	guanine diphosphate
GDS	guanine nucleotide dissociation stimulator
Grb2	growth factor receptor-bound protein 2
GRF	guanine nucleotide releasing factor
GTP	guanine triphosphate
IR	insulin receptor
IRS-1	insulin receptor substrate-1
IRTK	insulin receptor tyrosine kinase
Lys	lysine
MAP	mitogen-activated protein

MAP-2	microtubule-associated protein-2
MBP	myelin basic protein
MEK	MAP kinase kinase or ERK kinase
MEKK	MEK kinase
min	minute
NBT	nitroblue tetrazolium
NGF	nerve growth factor
p42 ^{erk 2}	42-kDa MAP kinase encoded by ERK 2 gene
p44 ^{erk 1}	44-kDa MAP kinase encoded by ERK 1 gene
p44 ^{mpk}	44-kDa sea star MBP kinase
PAGE	polyacrylamide gel electrophoresis
PDGF	platelet-derived growth factor
PKA	cyclic AMP-dependent protein kinase
PKI	peptide inhibitor of PKA
PMSF	phenylmethylsulfonyl fluoride
PTPase	protein tyrosine phosphatase
RPM	rounds per minute
RSK	ribosomal S6 kinase
S6K	S6 kinase
SDS	sodium dodecyl sulphate
SOS	son of sevenless
STZ	streptozotocin
ser	serine
src	oncogene isolated from chicken <i>Rous Sarcoma</i> cells
thr	threonine
tyr	tyrosine

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DEDICATION

To my family.....

the *FARAHBAKHSIANS*

INTRODUCTION

I. VANADIUM

A. Background

Vanadium compounds were first recognized by Nils Gabriel Sefstrom in 1831. It is now known that vanadium is one of the most abundant minerals in the earth's crust with an average concentration of 100 ppm (Willsky, 1990).

Early on following its discovery, a significant amount of work was done on the physiological actions of vanadium. By the late 1800s vanadium was experimented with in the treatment of diseases ranging from tuberculosis to diabetes (Willsky, 1990). There was general lack of interest in the biological actions of vanadium for the greater part of the twentieth century. In 1977, Cantely *et al.* demonstrated that vanadate was a potent inhibitor of Na/K ATPase which led to a resurgence of interest in the biological effects of vanadium.

B. Forms

Vanadium is a group Vb element (Ramasarma and Crane, 1980). It may exist in oxidation states ranging from -3 to +5 (excluding -2), although only the +3 to +5 states are known to exist in biological systems (Butler, 1990). The toxicity of vanadium forms increases with increased valency (Willsky, 1990). The two forms with the most biological relevance are vanadate, +5 (VO_4^{-3}), and vanadyl (VO^{+2}). Both pH and concentration play a role in the determination of the forms present (Butler, 1990).

Vanadate is the form most likely to be present at physiological pH (pH 6-8) (Willsky, 1990). It exists as the monomeric H_2VO_4^- and HVO_4^{-2} ions in the μM concentration range, but can form the $\text{HV}_4\text{O}_{12}^{-3}$ at low mM concentrations (Willsky, 1990). Still higher

concentrations can lead to the formation of the $V_{10}O_{28}^{-6}$ decavanadate ion. Vanadyl is most stable in acidic pH (Ramasarma and Crane, 1980).

The V-O bond in the vanadates closely resembles the P-O bond which may account for some of the observed inhibitory effects of vanadium on ATPases (Ramasarma and Crane, 1980). The vanadyl ion, however, is not an effective inhibitor for ATPases. Following entry into the cell, vanadate is converted to vanadyl (Nechay *et al.*, 1986). This along with a series of studies showing insulin-mimetic properties of vanadium in adipocytes without inhibition of Na/K ATPase, suggests that this inhibition is not largely responsible for the insulin-mimetic effects of vanadium (Dubyak and Kleinzer, 1980).

C. Insulin-mimetic Effects

1. In Vitro

Tolman *et al.* (1979) were the first to show the insulin-mimetic properties of vanadium in isolated rat adipocytes. They reported the stimulation of glucose oxidation and transport into adipocytes following vanadium treatment. In addition, they showed the stimulation of glycogen synthase and inhibition of gluconeogenesis in the rat liver. Both the stimulation of glucose transport and glycogen synthase in adipocytes by vanadium have been determined to be time and dose dependent (Dubyak and Kleinzeller, 1980; Tamura *et al.*, 1983). Vanadium has also been shown to inhibit lipolysis (Degani *et al.*, 1981). In addition, vanadium compounds have been shown to stimulate amino acid transport (Munoz *et al.*, 1992), DNA synthesis (Hori and Oka, 1980; Canalis, 1985), cell proliferation (Lau *et al.*, 1988), and the cell cycle (Wice *et al.*, 1987). Vanadium compounds have also been shown to exert their insulin-mimetic effects on skeletal muscle preparations and isolated perfused livers (Clark *et al.*, 1985). However, of even greater interest are the insulin-mimetic effects of vanadium seen in intact animals.

2. *In Vivo*

Heyliger *et al.* (1985) were the first to show the insulin-mimetic properties of vanadium in the intact animal. Female wistar rats were made diabetic by a single I.V. injection of streptozotocin (STZ). STZ-diabetic rats were treated with vanadate over a four week period. Vanadium-treated STZ-diabetic rats had blood glucose levels similar to that of controls whereas those of the untreated diabetics remained more than two-fold higher than controls. The anti-diabetic effects of vanadium in STZ-diabetic rats following chronic treatment, including reductions in blood glucose, triglyceride, and cholesterol levels, have since been reported in numerous studies (Meyervitch *et al.*, 1987; Ramanadham *et al.*, 1989; Pederson *et al.*, 1989, Cam *et al.*, 1993). In addition, long-term vanadium treatment has been shown to prevent or improve the tissue alterations seen in the chronic diabetic state, such as decreased cardiac performance (Heyliger *et al.*, 1985; Ramanadham *et al.*, 1989), increased glycerol output from adipose tissue (Ramanadham *et al.*, 1989), and decreased glycogen synthase activity both in the muscle of partially pancreatectomized rat (Rossetti and Laughlin, 1989) and in the liver of STZ-diabetic rats (Bollen *et al.*, 1990).

Vanadium treatment of STZ-diabetic rats also doubles glucose uptake in muscle and liver tissue of these animals (Schechter, 1990). The improvements in the various blood parameters of the STZ-diabetic rats following vanadium therapy, correspond well to the *in vitro* stimulation of glucose uptake (Cam *et al.*, 1993; Dubyak and Kleinzeller, 1980), and inhibition of lipolysis (Degani *et al.*, 1981) seen in isolated rat adipocytes. This suggests that the *in vivo* actions of vanadium are brought about, at least in part, by vanadium itself and are not simply a result of the potentiation of insulin action. However, vanadium alone is not sufficient in keeping completely insulin-deficient rats alive (Battell *et al.*, 1992), indicating that not all of the effects of insulin are mimicked by vanadium or that not enough vanadium can be absorbed to fully replace insulin.

D. Mechanism of Action

It has been suggested that the insulin-mimetic actions of vanadium may be mediated via the insulin receptor. Tamura *et al.* (1984) were the first to show the phosphorylation of the 95-kDa subunit of the insulin receptor on its tyrosine residues following the treatment of intact cells with vanadium. In addition, they reported the stimulation of tyrosine kinase activity. Other groups have since reported dose-dependent activation of insulin receptor tyrosine kinase, IRTK, following treatment of intact adipocytes with vanadium compounds (Fantus *et al.*, 1990; Katoda *et al.*, 1987; Gherzi *et al.*, 1988). Vanadium was also shown to increase IR autophosphorylation and tyrosine kinase activity in purified IR preparations (Gherzi *et al.*, 1988). There is growing evidence, however, that the effects of vanadium observed in intact cells are not mediated through the insulin receptor. Adipocytes with very low numbers of insulin receptors are fully responsive to vanadium while being extremely insulin resistant (Green, 1986). A lack of correlation between vanadium's insulin-mimetic properties and IRTK activity in the rat adipocyte (Mooney *et al.*, 1989) and in the mouse diaphragm *in vivo* (Strout *et al.*, 1989) has also been shown. In addition, the presence of a cytosolic protein tyrosine kinase (CytPTK) in rat adipocytes (Shishiva and Schechter, 1992) which is specifically activated by vanadate but not insulin (Shisheva and Shechter, 1993) and whose activation correlates well with vanadium's stimulation of glucose oxidation and lipogenesis, suggests the presence of an IR-independent pathway for at least some of the effects of vanadium. Some of the other insulin mimetic effects of vanadium such as stimulation of glucose uptake and inhibition of lipolysis are not facilitated via this kinase (Shisheva and Shechter, 1993), indicating the presence of more than one pathway through which the insulin-mimetic actions of vanadium are brought about.

The activity of protein tyrosine phosphatases (PTPases) has been shown to be regulated by insulin as a possible means of modulating insulin-stimulated metabolic and growth-related effects (Meyerovitch *et al.*, 1992). Vanadium compounds are well known

PTPase inhibitors (Swarup *et al.*, 1982; Lau *et al.*, 1989). The dose and time-dependent inhibition of PTPases by vanadium compounds have been correlated with increased protein tyrosine phosphorylation and 3-O-methylglucose transport in isolated rat adipocytes (Shisheva and Shecter, 1993). It is therefore a possibility that the enhanced tyrosine phosphorylation of a series of proteins via the inhibitory effects of vanadium compounds on PTPases is involved in mediating the insulin-mimetic properties of vanadium compounds.

Protein tyrosine phosphorylations and tyrosine kinases are generally associated with the early steps in the signal transduction of insulin and other growth factors. Protein serine/threonine kinases, which account for a much greater percentage of the phosphorylation events that occur within the cell, are believed to be more directly involved in the mediation of the effects of insulin (Czech *et al.*, 1988). It is therefore possible that an insulin-mimetic compound such as vanadium may bring about some of its effects through the activation of serine/threonine protein kinases. The Mitogen Activated Protein kinases (MAP kinases) and the S6 protein kinases are two of the most widely investigated serine/threonine protein kinases (Pelech and Sangera, 1992). MAP kinases have been shown to activate at least one family of S6 kinases (Sturgill *et al.*, 1988). Vanadium compounds have been implicated in the *in vitro* activation of MAP and S6 kinases (Tobe *et al.*, 1992).

II. SELENIUM

Selenium is a group VI element which can exist in +6, +4, +2, and -2 oxidation states (Ursini and Bindoli, 1987). There are strong physical and chemical similarities between selenium and sulphur allowing the former to substitute for the latter in certain biological systems (Ursini and Bindoli, 1987). Selenium is considered to be an essential element with its deficiency resulting in a wide variety of effects ranging from compromised functioning of the immune system (Koller, 1986) to Keshan's disease, a cardiomyopathy of unknown etiology prevalent in some selenium-deficient areas of China (Chen *et al.*, 1980).

Selenium in association with glutathione peroxidase and vitamin E is believed to play an important anti-oxidant role (Ursini and Bindoli, 1987). A strong correlation between selenium levels and glutathione peroxidase activity and a negative correlation with lipid peroxide levels has been shown (Gromadzinka et al., 1988). Most studies on selenium have been centered around its anti-oxidant effects. Selenium, however, may also be necessary for the proper functioning of the immune system (Koller, 1986), mitogenic stimulation of lymphocytes (Parnham et al., 1983; Sheffy and Schultz, 1983), and prevention of pancreatic atrophy (Bunk and Combs, 1986). In addition, selenium is an essential nutrient for the growth of human fibroblasts in cell culture (McKeehan et al., 1976) and can support cell proliferation in cell free media.

Although the role of selenium in glutathione peroxidase activity is well established and a number of selenoproteins have been identified (Chen et al., 1980), the effects of selenium as an insulin-mimetic agent or as a growth factor are just beginning to be investigated. Ezaki (1990) was the first to report a number of insulin-mimetic effects of sodium selenate in isolated rat adipocytes, which included: the time and dose-dependent stimulation of glucose transport, translocation of glucose transporters to the plasma membrane, stimulation of cAMP phosphodiesterase activity, tyrosyl phosphorylation of a number of cellular proteins, and the phosphorylation of the ribosomal S6 protein. More recently, Pillay and Makgoba (1992) have shown the phosphorylation of the epidermal growth factor (EGF) receptor and the stimulation of its kinase activity in A431 cells by sodium selenate. In addition, they have shown that incubation of intact NIH-3T3-HIR3.5 cells with sodium selenate leads to enhanced phosphorylation of the insulin B-subunit and a 185 kDa, protein believed to be the insulin receptor substrate-1 (IRS-1), following insulin treatment. These *in vitro* insulin-mimetic effects are in part supported by work done on intact animals. McNeill *et al.* (1991) have reported the *in vivo* insulin-mimetic effects of sodium selenate in STZ-diabetic rats. They showed a decrease in plasma glucose levels, food intake, and fluid intake to near control levels in STZ-diabetic rats treated chronically with sodium

selenate. This appears to be the only report in the literature that is concerned primarily with the insulin-mimetic properties of selenium in the intact animal at this time.

III. MAP KINASES

Extracellular signal-regulated protein kinases (ERK) also known as mitogen activated protein kinases (MAP kinases) are a family of serine/threonine-protein kinases (ser/thr-protein kinases) believed to be essential in intracellular signaling processes. They are highly conserved and nearly ubiquitous in a diverse range of organisms from yeast to mammals (Nishida and Gotoh, 1993). Ray and Sturgill (1987) were the first to report the insulin stimulation of a soluble serine(ser)/threonine(thr)-protein kinase in serum-starved 3T3-L1 adipocytes that facilitated the phosphorylation of microtubule-associated protein 2 (MAP-2) *in vitro*. Further work resulted in the partial purification of a 40-42 kDa kinase requiring phosphorylation on both tyrosine and threonine residues for full activation (Ray and Sturgill, 1988a;b). In addition, MAP kinase was shown to phosphorylate and activate S6 kinase II, another insulin-stimulated kinase, obtained from *Xenopus laevis* (Sturgill *et al.*, 1988). The fact that MAP kinases were phosphotyrosine activated and were able to stimulate S6K II led some investigators to believe that they might serve as "switch kinases" transferring signals from tyrosine kinases associated with cell surface receptors to downstream ser/thr protein kinases thereby bringing about the metabolic and/or mitogenic effects (Sturgill *et al.*, 1988). However, other reports identifying the MAP kinase activator as a ser/thr kinase have refuted the "switch kinase" hypothesis (Kosaka *et al.*, 1992; Seger *et al.*, 1992).

At least six different MAP kinases ranging in molecular mass from 40 kDa to 64 kDa have been identified (for review see; Pelech and Sanghera, 1992). Extracellular signal-regulated kinase 1 (ERK 1) or p44^{erk1} was first purified as a 43 kDa protein from rat 1 HIRc B cells stimulated with insulin (Boulton *et al.*, 1991). ERK 2, with nearly 90% homology with ERK 1 was cloned from a rat brain cDNA library (Boulton *et al.*, 1990). A 54 kDa

MAP kinase was also obtained from the livers of rats treated with cycloheximide (Kryiakos and Avruch, 1990). It shares about 50% homology with ERK 2 in the catalytic domain (Pelech and Sanghera, 1992). Another MAP kinase, p44^{mpk} (maturation-activated myelin basic protein kinase), isolated from sea star oocytes (Sanghera *et al.*, 1990), shares nearly 77% homology with ERK 1 and ERK 2. In addition, there are several yeast protein kinases which show a relatively high degree of homology with ERK 1 and ERK 2 suggesting sequence conservation (Pelech and Sanghera, 1992).

As mentioned above, there are many members within the MAP kinase family present in a wide variety of species. All, however, are believed to share the common requirement of phosphorylation on both tyrosine and threonine residues for full activity, except for p44^{mpk} which is not threonine phosphorylated (Anderson *et al.*, 1990; Sanghera *et al.*, 1991; Scimeca *et al.*, 1991). The phosphorylation sites generally have the "TEY" (threonine-glutamic acid-tyrosine) amino acid sequence order with a threonine separated from tyrosine with a glutamic acid (Nishia and Gotoh, 1993). Thr-204 and Tyr-206 are phosphorylated in ERK 1 (Her *et al.*, 1991), whereas Thr-183 and Tyr-185 are phosphorylated for ERK 2 (Payne *et al.*, 1991). MAP kinases also share among themselves a similar substrate specificity and are regarded as proline-directed kinases (Hall and Vulliet, 1991). Myelin basic protein (MBP) and MAP-2 serve as excellent substrates for MAP kinases with the recognition determinant having the -Pro-X_{aa}-Ser/Thr-Pro (where X_{aa} represents one or two basic and/or neutral amino acids) motif (Gonzales *et al.*, 1991; Alvarez *et al.*, 1991). Both are often used as substrates for measuring MAP kinase activity. A number of substrates for MAP kinases have been identified in physiological systems, such as: p90^{rsk} from *Xenopus* oocytes (Ahn *et al.*, 1990), c-jun and c-Myc proteins (Alvarez *et al.*, 1991), Tau protein (Drewes *et al.*, 1992), EGF receptor (Alvarez *et al.*, 1991) cPLA₂ (Lin *et al.*, 1993), and many other proteins, most of which are involved in signal transduction. From the list of substrates above, it can be seen that MAP kinase might be involved in the regulation of protein kinases, proteins involved in transcription control, and membrane receptors. In addition, they are likely to play a role in

the induction of differentiation, expression of oncogenes, and G₀ to G₁ transition (Pelech and Sanghera, 1992). Due to the eclectic nature of the effects of MAP kinases and their targets, it is not surprising that a wide variety of substances stimulate MAP kinase activity. Included are most agonists whose receptors are coupled to tyrosine kinases such as growth factors and lymphokines, activators of protein kinase C, and inhibitors of protein-ser/thr and tyrosine phosphatases such as okadaic acid and vanadate (Pelech and Sanghera, 1992; Cobb *et al.*, 1991; Haystead *et al.*, 1990; Scimeca *et al.*, 1991).

IV. S6 KINASES

The role of the ribosomal S6 protein in cellular functioning is not yet clear, although it is believed to be involved in the initiation of protein synthesis. It is one of the many proteins that are activated through phosphorylation on serine residues following treatment of intact cells with various growth-factors such as epidermal growth factor, platelet-derived growth factor, and insulin (Smith *et al.*, 1980; Rosen *et al.*, 1981). The *in vivo* phosphorylation sites of the S6 protein have been shown to be Ser 235, Ser 236, Ser 240, Ser 244, and Ser 247 which reside in between Arg 231 and Lys 249 near the carboxyl terminus of the molecule (Bandi *et al.*, 1993). Two families of S6 kinases, the p90^{rsk} and the p70^{S6K} have been well characterized in a number of studies (for reviews see Maller, 1990; Erikson, 1991; Strurgill and Wu, 1991; Avruch *et al.*, 1991). Recently, a 31 kDa insulin-stimulated S6 kinase, distinct from p90^{rsk} and the p70^{S6K}, has been purified and characterized from rat skeletal muscle (Hei *et al.*, 1994). This kinase has been reported to be the main insulin-stimulated S6 kinase in rat skeletal muscle although it has not yet been cloned and sequenced (Hei *et al.*, 1993).

A. *rsk*

Erikson and Maller (1985) were the first to identify a protein kinase specific for ribosomal S6 protein with a molecular weight of approximately 92,000 from *Xenopus* eggs. This protein kinase was able to phosphorylate all of the sites in the S6 protein associated with growth-promoting stimuli in contrast to other protein kinases such as cAMP-dependent kinase which only phosphorylated some of the S6 protein sites (Erikson and Maller, 1985). This kinase was shown to be activated in *Xenopus* oocytes by progesterone and insulin, as well as by the microinjection of purified insulin-receptor tyrosine kinase (Cicirelli *et al.*, 1990; Stefanovic *et al.*, 1986). Subsequent purification of the kinase on a DEAE-Sephacel column yielded two peaks of activity which were termed S6 kinase I (eluted at 90 mM NaCl) and S6 kinase II (eluted at 160 mM NaCl) based on the order of elution (Erikson and Maller, 1986). Molecular cloning of S6 kinase II led to the discovery of two distinct cDNA fragments with 91 % homology, but more importantly, S6 kinase II was shown to have the unusual characteristic of having two apparent catalytic domains (Jones *et al.*, 1988). The amino terminal catalytic domain, believed to be the main site involved in phosphotransferase activity, showed similarity to catalytic sites of protein kinase C, cAMP-dependent protein kinase, and cGMP-dependent protein kinase, while the other catalytic domain resembled that of phosphorylase B (Jones *et al.*, 1988). S6 kinase II homologues were then found in avian and murine cDNA libraries (Alcorta *et al.*, 1989) and a 90 kDa protein that was functionally and immunologically related to S6 kinase II was found in chicken embryo fibroblasts (Sweet *et al.*, 1990). These homologous enzymes were placed in one family and referred to as ribosomal S6 protein kinases or *rsk*. Another member of this family isolated from insulin-stimulated rabbit skeletal muscle (Lavoinne *et al.*, 1990), has recently been shown to be 100% homologous to its mouse counterpart (Sutherland *et al.*, 1993). It is likely that more homologues are in the process of being identified at the present time.

B. p70^{S6K}

Although *Xenopus rsk* homologues are present in mammalian cells, another family of S6 kinases, referred to as p70^{S6K}, are believed to be the more dominant S6 kinase in this system. Early reports identified the presence of a 60-70 kDa insulin-stimulated kinase with a very high specificity towards ribosomal S6 protein in 3T3-L1 cells (Cobb, 1986). This kinase was determined to be distinct from several other protein kinases capable of *in vitro* phosphorylation of the S6 protein based on its substrate specificity and chromatic properties, in addition to its strong dependency on the presence of β -glycerophosphate in the homogenization buffer for the measurement of its activity (Cobb, 1986). An insulin-stimulated S6 kinase was also reported in rat hepatoma H4 cells (Nemenoff *et al.*, 1986). Blenis *et al.* (1987), reported the presence of a 65 kDa S6 kinase in developing chicken embryos and chicken embryo fibroblasts (CEF) which was activated by the tyrosine-specific protein kinase of Rous sarcoma virus (pp60^{src}), phorbol esters, and various growth factors. The above mentioned kinases are believed to be the same as the 70 kDa kinase identified and characterized in Swiss mouse 3T3 cells (Jeno *et al.*, 1988). This kinase was activated following treatment of the cells with epidermal growth factor (EGF), sodium orthovanadate, and serum. The tryptic phosphopeptide maps of the *in vitro* phosphorylation of ribosomal S6 protein by this kinase resembled those of the S6 protein phosphorylated *in vivo*, suggesting a physiological role for the kinase. Subsequent reports have identified an approximately 70 kDa kinase specific for ribosomal S6 protein in regenerating rat liver, cycloheximide-treated rat liver, and insulin-treated rabbit liver (Nemenoff *et al.*, 1988; Kozma *et al.*, 1989; Gregory *et al.*, 1989). In all cases, the enzymes had similar chromatographic properties, substrate specificity, and were extremely susceptible to inactivation by phosphatase 2A. In addition, activation of the enzyme was shown to be dependent on its specific phosphorylation (Price *et al.*, 1990). This family of approximately 70 kDa S6 kinases, p70^{S6K}, were shown to be distinct from the previously described p90^{rsk} S6 kinase family based on molecular size,

chromatographic properties, phosphopeptide maps, lack of immunological cross-reactivity, and molecular cloning (Chen and Blenis, 1990; Banerjee *et al.*, 1990). The molecular cloning of p70^{S6K} revealed the presence of one catalytic domain resembling that of protein kinase C which was 56% homologous to its *rsk* counterpart and showed that the two enzyme families are structurally quite distinct (Banerjee *et al.*, 1990).

As previously mentioned, MAP kinases have been shown to be the upstream activators of the *rsk* family (Sturgill *et al.*, 1988; Chung *et al.*, 1991). Gregory *et al.* (1989) reported the partial reactivation of p70^{S6K} inactivated with phosphatase 2A using active MAP kinase. This, however, is contrary to a number of different reports which suggest that MAP kinase is not the upstream activator of p70^{S6K} (Blenis, 1991; Ballou *et al.*, 1991; Chung *et al.*, 1992). Therefore the activator of the p70^{S6K} family remains elusive, although evidence suggests the involvement of a ser/thr kinase (Price *et al.*, 1990; Ferrari *et al.*, 1991).

V. UPSTREAM KINASES

Growth factor signal transduction is believed to involve a number of proteins such as membrane tyrosine kinases, Ras, Raf-1-kinase, MAP kinase kinase, MAP kinase, and *rsk*, interconnected in a complex and not necessarily linear network as shown in Figure I (Williams and Roberts, 1994). The activation of S6 kinases by MAP kinases (Sturgill *et al.*, 1988) was one of the first examples of a ser/thr kinase signal transduction cascade. Not surprisingly, these two groups of kinases have been used as a pivotal point from which other links in the signal transduction pathway of insulin and other growth-factors can be identified.

A. MAP Kinase Kinase

MAP kinase kinases or ERK kinases (MEKs) are believed to be the immediate upstream activators of MAP kinases and are activated through the phosphorylation of their tyrosine and threonine residues (Kosaka *et al.*, 1992; Seger *et al.*, 1992; Anderson *et al.*, 1990; Payne *et al.*, 1990). Previous experiments had already reported a factor which activated MAP kinase through tyrosine/threonine phosphorylation (Ahn *et al.*, 1991). Subsequent experiments demonstrated the sequential activation of MAP kinase activator, MAP kinase, and a S6 peptide kinase in rat liver following insulin injection (Tobe *et al.*, 1992). MEK was first purified from insulin-stimulated rabbit skeletal muscle as a ~ 45 kDa protein (Nakienly *et al.*, 1992). Other homologues including *Xenopus* oocyte and yeast homologues have also been identified suggesting that MEK is highly conserved (Matsuda *et al.*, 1992; Crews and Erikson, 1992). MEK is a novel enzyme in that it is the first dual specificity tyr/thr kinase identified (Nakienly *et al.*, 1992; Crews and Erikson, 1992). It is inactivated following treatment with phosphatase 2A, suggesting that it is activated through ser/thr phosphorylation (Matsuda *et al.*, 1992). MEK is the only known physiological substrate of Raf-1 kinase, a ser/thr kinase protooncogene (Kryiakos *et al.*, 1992). Raf-1 kinase is believed to be the main upstream activator of MEK (Kryiakos *et al.*, 1992; Dent *et al.*, 1992). Recently, a Ras(a GTP-activated protooncogene)-dependent MEK kinase (REK) which activates MEK in a Raf-1-independent manner has been identified (Itoh *et al.*, 1993). MEK can therefore be activated through at least two separate Ras-dependent pathways which may work in conjunction or separately. There are also Ras-independent pathways. For example, the proto-oncogene product Mos can also phosphorylate and activate MEK (Posada *et al.*, 1993).

B. raf Kinase Family

Raf-1 kinase, or c-raf-1, is the ubiquitously expressed ~74 kDa cellular homologue of the *v-raf* oncogene believed to be a mediator of growth factor and differentiation signal transduction (Bonner *et al.*, 1985; Morrison *et al.*, 1990). Two other members of the *raf* kinase family have been isolated, A-raf, a 68 kDa protein generally found in the epididymis and urogenital tissue, and 95 kDa B-raf, most abundant in brain and testicular tissue (Beck *et al.*, 1987; Ikawa *et al.*, 1988; Nishida *et al.*, 1988).

Raf-1 is the best studied member of the *raf* kinase family. Raf-1 kinase is a 648 amino acid protein divided into three conserved regions CR1, CR2, and CR3 (Heidecker *et al.*, 1990). CR1 is a cysteine-rich region homologous to the lipid-binding site of protein kinase C, while CR2 is a ser/thr-rich region possibly involved in raf-1 kinase activation (Ishikawa *et al.*, 1988). CR3 is the region responsible for the kinase activity (Hanks *et al.*, 1988). Truncation of both the CR1 and CR2 regions results in constitutively active kinase activity by CR3 (Stanton *et al.*, 1989; Heidecker *et al.*, 1990) suggesting some regulatory role for CR1 and CR2.

Morrison *et al.* (1988) were the first to show that Raf-1 kinase was an intermediate in the signal transduction cascade of growth factors. In their experiments they demonstrated that treatment of NIH-3T3-HIR cells with PDGF, FGF, and EGF led to hyperphosphorylation of Raf-1. In addition, they provided evidence that Raf-1 was downstream of PKC and the transforming oncogenes *v-src*, *v-sis*, and *v-ras* but upstream from nuclear oncogenes *v-fos* and *v-myc*. Insulin and other growth factors have also been shown to activate Raf-1 kinase by increasing its phosphoserine content in HeLa, NIH-3T3-HIR, and Chinese hamster cells overexpressing the human insulin receptor (Kovacina *et al.*, 1990). Further evidence for the importance of Raf-1 in signal transmission stemmed from experiments identifying Raf-1 as the upstream activator of MEK (Kryiakos *et al.*, 1992; Dent *et al.*, 1992). Cells transformed with the *v-Raf* oncogene displayed constitutively high levels of MEK activity while activated

Raf was able to phosphorylate and activate MEK over 30-fold *in vitro* (Kryiakos *et al.*, 1992). Other evidence suggests that the role of Raf-1 kinase in MEK activation and signal propagation may be tissue and cell-type specific. For example, rat PC12 cells, induced to express activated Raf, did not show significant activation of MAP kinases or *rsk* (Wood *et al.*, 1992). In addition *v-Raf* transformed rat fibroblasts also did not show constitutively high levels of MAP kinases while NIH-3T3-HIR mouse cells did (Gallepo *et al.*, 1992). In a more recent experiment with RCR cells mutated to block Raf-1 kinase expression, ERK1 and ERK2 were activated to the same extent as the NRK parent cell line discounting Raf-1 kinase as the only upstream activator of MAP kinases (Kizaka-Kondoh and Okayama, 1993). Another MEK kinase which activates MEK has also been identified in *Xenopus* oocytes suggesting the presence of more than one activator of MEK (Matsuda *et al.*, 1992). Interestingly, in the same study MAP kinase was also shown to phosphorylate MEK, although no activation occurred. MAP kinases have also been shown to phosphorylate Raf-1 kinase *in vitro* although no activation of Raf-1 was reported (Anderson *et al.*, 1991).

The cellular homologue of the oncogene *v-ras*, p21^{ras}, has been implicated in the activation of Raf-1 kinase (Blenis, 1993). Ras has been shown to lie upstream of Raf-1 with cells transformed with *v-ras* showing Raf-1 hyperphosphorylation (Morrison *et al.*, 1988). Other investigators have shown, however, that although ras increases the sensitivity of Raf-1 to growth factors, it alone is not sufficient to cause Raf-1 hyperphosphorylation (Reed *et al.*, 1991). Based on other experiments involving the activation of Raf-1 through *v-src* (a tyrosine kinase oncogene) in a ras-independent manner, it seems possible that activation of Raf-1 involves ras as well as a tyrosine kinase element (Williams and Roberts, 1994)

C. Ras

The Ras group of proteins have been implicated as key mediators of signals from receptor-associated tyrosine-kinases to downstream ser/thr-protein kinases and are therefore believed to play an important role in the regulation of cellular proliferation and differentiation (Sato *et al.*, 1992). At least four different highly homologous 21 kDa Ras proteins (ie. H-Ras, K4A-Ras, K4B-Ras, and N-Ras) have been found in humans (Sato *et al.*, 1992). Ras proteins show a high affinity for guanine nucleotides and are believed to be in an active state when bound to GTP (Ras-GTP), whereas the Ras-GDP complex is associated with the inactive state (Bourne *et al.*, 1990). Three classes of proteins are presently known to be involved in the control of Ras function via their role in the interconversion between Ras-GTP and Ras-GDP complexes. The first class, GTPase activating proteins (GAPs), negatively regulate Ras by stimulating the endogenous Ras GTPase activity thereby favouring the formation of the Ras-GDP complex (Bollag and McCormick, 1991). The second class of Ras regulators are known as guanine nucleotide dissociation stimulators (GDS) which allow the release of GDP from the inactive Ras-GDP complex and favour the formation of the active Ras-GTP state (Downward, 1992). The most recently identified group of Ras regulatory proteins are the Ras guanine nucleotide dissociation inhibitors (Ras GDI)(Bollag and McCormick, 1993).

Two Ras-specific GAPs that have been identified in mammalian tissue are p120 GAP and NF1 GAP (Trahey *et al.*, 1988; Xu *et al.*, 1990). Both GAPs have been shown to substantially increase Ras-GTPase activity but are believed to be differentially regulated (Bollaga and McCormick, 1991). Mutations in the GAP-binding region of Ras leading to lack of Ras binding to GAPs have led to the inhibition of Ras signal propagation suggesting that GAPs may also serve as downstream effectors of Ras (DeClue *et al.*, 1991). The binding of Ras to p120 is believed to cause conformational changes in the p120 molecule which are necessary for the transmission of signals to downstream targets of Ras (Martin *et al.*, 1992).

It therefore seems that GAPs serve the dual function of being both regulators of Ras function and mediators of its signal.

Another important class of Ras regulators are the GDS proteins which favour the release of GDP from the Ras-GDP complex and the formation of the active Ras-GTP form. A number of different GDSs have been identified in various organisms ranging from the yeast GDS, CDC25, and the *Drosophila* GDS, son-of-sevenless (SOS), to the human and mouse homologues of SOS, hSOS and mSOS1 and 2 (Jones *et al.*, 1991; Bowtell *et al.*, 1992). The translocation of Ras to the inner face of the plasma membrane, brought about through a series of posttranslational modifications including proteolysis, carboxylmethylation, palmitoylation, and farnesylation, is essential to Ras biological activity (Khosravi-Far *et al.*, 1992). It has therefore been suggested that the regulation of Ras via GDSs involves the translocation of the GDS proteins via adapter molecules such as growth-factor receptor-bound protein 2 (Grb-2) (Lowenstein *et al.*, 1992). Grb-2 is believed to be associated with cytoplasmic GDSs via the association of its two SH3 domains and the proline-rich present in GDSs (McCormick, 1993). Following the stimulation of receptor tyrosine kinases by their respective ligands and their subsequent autophosphorylation, the Grb-2-GDS complex associates with the autophosphorylated receptor or a membrane-bound substrate of the receptor, such as IRS-1 for the insulin receptor, thereby allowing the activation of membrane-associated Ras by GDS (McCormick, 1993; Tobe *et al.*, 1993). The third class of Ras regulators, GDI, are believed to be inhibitors of GDS proteins which do not significantly affecting GAP activity (Bollag and McCormick, 1993).

Ras-GTP formation has been shown to be brought about by the interaction of a number of different growth-factors with their respective receptors which can affect either/or GAP and GDS activity depending on the ligand and the cell system (for review; Khosravi-Far and Der, 1994). Following Ras activation, the signal from receptor tyrosine-kinases can propagate to the downstream ser/thr-protein kinases which have been discussed in the previous sections. Raf-1 kinase has already been mentioned as the initial ser/thr-protein

kinase in the cascade, and MAP kinase and *rsk* have been used as a pivotal point from which other kinases were investigated. There are, however, other Ras-independent means of activating MAP kinases and Ras undoubtedly has downstream targets other than ser/thr-protein kinases.

VI. RATIONALE

Insulin has a wide variety of biological functions ranging from its role in glucose homeostasis in the intact animal to its growth-factor and mitogenic effects in cell culture. Although no consensus has been reached on exactly how the actions of insulin are brought about, a ser/thr-protein kinase cascade, also present in the signal transduction pathway of other growth-factors, is believed to play a role in mediating at least some of the effects of insulin. MAP and S6 kinases are pivotal points within this pathway with their stimulation serving as a consistent step in growth-factor signal transmission. Both vanadium and selenium have insulin-mimetic properties in the intact animal as well as the isolated rat adipocyte. The mechanism of action of these two elements, however, has not yet been fully elucidated. Therefore, by investigating the effects of vanadyl sulphate and sodium selenate on the ser/thr-protein kinase phosphorylation cascade of isolated rat adipocytes through the measurement of MBP and Ribosomal Protein S6 kinase activity, we hope to gain some insight into the mechanism of action of vanadium and selenium as well as to provide further evidence for the insulin-mimetic properties of these two elements.

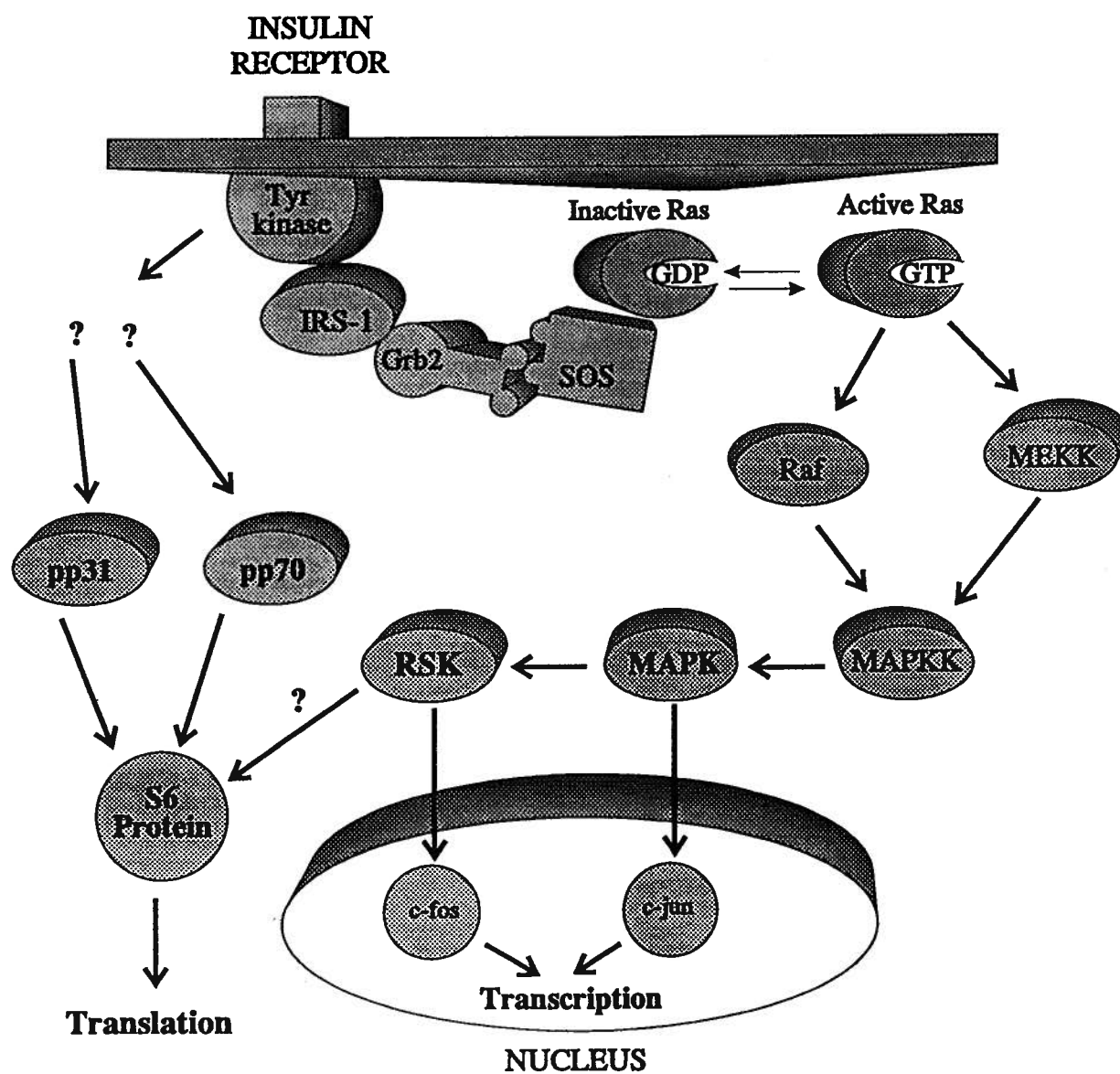
A. Hypothesis

The insulin-mimetic compounds vanadyl sulphate and sodium selenate will stimulate MAP and S6 kinases, key enzymes involved in the ser/thr-protein kinase signal transduction cascade of insulin, in the isolated rat adipocyte.

B. Objectives

- 1) To investigate the possible stimulation of MAP and S6 kinases in isolated rat adipocytes by sodium selenate and vanadyl sulphate in a time and dose-dependent manner.
- 2) To gain insight into the relative contribution of the different MAP kinase and S6 kinase families in the overall activity observed in the crude cell extracts by the use of Mono Q anion-exchange chromatography and immunoblotting.
- 3) To gain insight into the role of tyrosine kinases in the activation of MBP and Ribosomal Protein S6 kinases by vanadyl sulphate and sodium selenate using the tyrosine kinase inhibitor genistein.
- 4) To gain insight into the level of activity of the p70^{S6K} family of Ribosomal Protein S6 kinases by using the specific p70^{S6K} inhibitor rapamycin.

Insulin Signal-Transduction Cascade



MATERIALS AND METHODS

I. MATERIALS

Aprotinin, ATP, benzamidine, *bovine* insulin, *bovine* serum albumin (fraction V 98-99% albumin), collagenase type II, D-glucose, dithiothreitol (DTT), Dulbecco's Modified Eagle Media Base, EDTA, EGTA, leupeptin, MOPS, myelin basic protein (MBP), N,N-dimethylformamide (DMF), pepstatin A, peptide inhibitor of cAMP-dependent protein kinase (PKI), phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, soyabean trypsin inhibitor, β -glycerophosphate, and β -methyl-aspartic acid were purchased from SIGMA chemical company. Acrylamide, 5-bromo-4-choloro-indolyl phosphate (BCIP), ammonium persulfate, Bio-Rad protein reagent, Coomassie blue stain, L-glycine, N,N'-methylene-bis-acrylamide, nitroblue tetrazolium (NBT), nitrocellulose membrane (0.45 micron), sodium dodecyl sulphate, and β -mercaptoethanol were purchased from Bio-Rad Laboratories. Sodium selenate and vanadyl sulphate hydrate were purchased from Aldrich Chemicals. [$\gamma^{32}\text{P}$]ATP was purchased from ICN chemicals. S6-10 peptide (AKRRRLSSLRASTSKSESSQK) was generously provided by Dr. Ian Clark-Lewis (Biomedical Research Laboratories, Vancouver, Canada). Rapamycin was kindly provided by Dr. S. Sehgal of Wyeth-Ayersth Research.

The following anti-kinase rabbit antibodies were made in the laboratory of Dr. Steve Pelech using the synthetic peptides shown below:

GLAYIGEGAYGMVYKAC (GEGA);

EFQDFVNKCLVKNPAERADLKC (MAPKK-9);

PFEHQTYCQRTLREIQILLGFRHENVIGIRDILRAPGGC (R1);

CGGPFTFDMELDDLPERLKLKELIFQETARFQPGAPEAP (R2);

AMIVRNAKDTAHTKAERNILEEVKHPGGCC (S6K-III);

CLVKGAMAATYSALNSSKPTPQLKPIESSILAQRRVRKLPSTTL (rsk-CT).

A. Animals

Male Sprague-Dawley rats weighing 150-220g were used in all experiments. Animals were obtained either from Charles-River (Montreal, Canada) or U.B.C. animal breeding facilities. All animals were food-deprived overnight prior to the day of the experiment.

II. METHODS

A. Ribosomal Protein S6 kinase Substrate Preparation

The preparation procedure was based on that of Krieg *et al.* (1988) and is described below in detail.

1. Ribosomal 80S subunit preparation

One-hundred grams of frozen liver from food-deprived male Sprague Dawley rats were used in each preparation. The livers were ground to a powder using a mortar and pestle cooled with liquid nitrogen. The powder was then suspended in 200 ml of homogenization buffer (20 mM Tris-HCl (pH 7.4) 100 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1% Triton X-100, and 1% sodium deoxycholate), and disrupted with a polytron homogenizer (setting 5) for 20 second bursts until a homogeneous suspension was formed.

The homogenate was centrifuged at 9000xg for 20 minutes at 4°C. The supernatant was distributed in 29.5 ml aliquots into 38.5 Quick Seal Tubes (Beckman) containing 4 ml of buffer A (5 mM Tris-HCl (pH 7.4), 500 mM KCl, 2.5 mM MgCl₂, 0.5 M sucrose, 1 mM dithiothreitol, 1% Triton X-100, and 1% deoxycholate) and 5 ml of buffer B (same as buffer

A except for the sucrose concentration which was 1 M) was then carefully added to the top of the homogenate using a long glass pipet. The tubes were sealed and centrifuged for 16 hours at $\sim 200,000\times g$ and 4°C .

After the supernatant was removed and discarded, the tubes were inverted and centrifuged at 3000 RPM for 10 seconds in a table-top clinic centrifuge. This allowed for the separation of the ribosomal pellet, which remained firmly fixed to the bottom of the tube, from the extraneous jelly-like material that slid to the top of the tubes and was removed with Kimwipes. Following this removal, 1 ml of buffer C (20 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM MgCl_2 , and 1 mM dithiothreitol) and three glass beads were added to each tube. The tubes were placed on a table-top shaker and rotated at 100 RPM for 4 hours at 4°C in order to resuspend the pellets. The 80S suspensions were then pooled and stored at -70°C until they were used for 40S subunit preparation.

2. Ribosomal 40S subunit isolation

The isolation of the 40S ribosomal subunits involved the dissociation of the 80S ribosomal subunits followed by sucrose gradient centrifugation. The process is described below in detail. The dissociation cocktail was made by adding 3 ml of the 80S suspension to 20 ml of buffer C, 894 mg KCl, 30 mg puromycin and the volume made up to 30 ml with buffer C. The cocktail was then incubated at 37°C in a shaking water bath for 30 minutes. Stock sucrose solutions containing 7.5% and 37.5% sucrose dissolved in dissociation buffer (500 mM KCl (pH 7.4), 3 mM MgCl_2 , and 4 mM dithiothreitol) were used to make six 32.5 ml continuous sucrose gradients with the aid of a BRL gradient former. To each gradient tube, 4.8 ml of the dissociation cocktail was added and the tubes were centrifuged at $\sim 87,000\times g$ for 16 hours at 4°C .

Following the centrifugation, a hole was made into the bottom of each gradient tube and thirty 1 ml fractions were collected. From each fraction, 20 μl was added to 2 ml of

distilled water and absorbance measurements were made at 260 and 280 nm. When the A_{260}/A_{280} ratio was plotted versus the fraction number, two distinct peaks corresponding to 60S and 40S subunits were observed. The fractions containing the second and considerably smaller peak were pooled and centrifuged at 225,000xg for 4 hours at 4°C. The pellets were then resuspended in 1-3 ml of buffer C using a Teflon hand homogenizer. The protein content and the A_{260} of the suspension were measured in order to assess the purity of the preparation. The suspensions were stored at -70°C until used as substrate for Ribosomal Protein S6 kinase assays.

B. Adipocyte Isolation and Incubation

1. Adipocyte isolation

Rat adipocytes were isolated using a modified version of Rodbell's method (1964). Male Sprague Dawley rats (weighing 150-220g) were fasted overnight before being sacrificed either by a blow to the head followed by decapitation or with CO₂ gas. Their epididymal fat pads were quickly excised and placed in warm Dulbecco's Modified Eagle Media (DMEM) containing 2% BSA and 2 mM glucose (pH 7.4, 37°C).

The pads were then cut into pieces ≤ 5 mm in diameter and incubated in media containing 0.75 mg/ml crude collagenase (type II). The pieces were digested for 35-40 minutes while shaking at 80 cycles/minute in a 37°C water bath. The resultant slurry was then filtered through a 250 μ m nylon mesh in order to remove undigested fat and other debris. The filtrate was centrifuged at very low speed (~ 300 RPM) for 30 seconds forming a fat-cell layer above the aqueous infarnate. The infarnate was removed using a 19 gauge needle attached to a 20 ml syringe and warm media was added to the cells. The adipocytes were 'washed' twice more in this manner in order to remove any residual collagenase. The

cells were then equilibrated for 30 minutes in order to remove any effects that the digestion process may have had on them.

2. Time-course

All incubations were made in a 37°C shaking water bath using 290 µl of cell suspension and 10 µl of stock solution; 300 nM insulin, 30 mM sodium selenate, or 30 mM vanadyl sulphate. The final concentration of the agents and their respective time-points were as follows: 10 nM insulin; 0, 2.5, 5, 10, 15, 20, and 30 minutes; 1 mM sodium selenate; 0, 2.5, 5, 10, 13, 16, 19, and 27 minutes; and 1 mM vanadyl sulphate 0, 2.5, 5, 10, 20, and 30 minutes. All time-points contained untreated controls. The incubations were stopped with the addition of 1.25 ml of ice-cold homogenization buffer (25 mM MOPS (pH 7.2), 10 mM EGTA, 2 mM EDTA, 75 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and the following protease inhibitors; 1 mM PMSF, 3 mM benzamidine, 10 µM leupeptin, 0.75 µM aprotinin, and 100 µg/ml soybean trypsin inhibitor) followed by the disruption of the cells using a polytron (setting 5, 3 seconds). The cellular extracts were centrifuged for 60 minutes at 19,000xg and the supernatant was stored at -70°C to be assayed later for kinase activity and protein content.

3. Dose-response curve

Isolated rat adipocytes were incubated with different concentrations of insulin, sodium selenate, and vanadyl sulphate for 5, 10, and 20 minutes, respectively. These optimal time-points for each agent were based on the results obtained from the time-course experiments. As before, all incubations were done in a 37°C shaking water bath using 290 µl cell suspension and 10 µl stock solutions and were stopped with the addition of 1.25 ml of ice-cold homogenization buffer followed by cell disruption. The final concentrations of

insulin used were 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, and 100 nM. The sodium selenate concentrations used were 100 nM, 1 μ M, 10 μ M, 100 μ M, 1 mM, and 10 mM. The vanadyl sulphate concentrations were 31.6 μ M, 100 μ M, 316 μ M, 1 mM, 3.16 mM, and 10 mM.

4. Genistein and Rapamycin experiments

During these experiments, adipocytes were incubated for 30 minutes with 200 nM genistein or 50 ng/ml rapamycin prior to a 20 minute incubation with either 1 mM sodium selenate or 1 mM vanadyl sulphate. All other aspects of these experiments were the same as those described above.

C. Phosphocellulose Paper Assays

Paper assays were used for measuring MBP kinase and S6-peptide kinase activity. The assay cocktail contained 20 mM MOPS (pH 7.2), 25 mM β -glycerophosphate, 5 mM EGTA, 2 mM EDTA, 20 mM MgCl_2 , 2 mM sodium orthovanadate, 1 mM dithiothreitol, 500 nM protein kinase inhibitor, and one of the following substrates; myelin basic protein (1 mg/ml) or S6-10 peptide (0.25 mg/ml). The following procedure was used in all phosphocellulose paper assays. Five μ l of cellular extract (5-10 μ g) was added to 15 μ l of the cocktail. The reactions were initiated with the addition of 5 μ l of $[\gamma^{32}\text{P}]\text{ATP}$ (250 μ M, specific activity 2000 cpm/pmol) and allowed to proceed for 10 minutes at 30°C. The reactions were stopped by spotting 20 μ l of the reaction mixture onto a P-81 phosphocellulose paper square (2X2 cm). The paper squares were then repeatedly washed with 1 % phosphoric acid in order to remove non-specific ATP binding and counted using liquid scintillation counting.

D. Ribosomal Protein S6 Kinase Assays

The assay cocktail used was identical to that described above except for the use of 0.35 μM purified rat liver 40S ribosomal subunit as substrate. The reactions were started with the addition of 5 μl of [$\gamma^{32}\text{P}$]ATP (15 μM , specific activity 10,000 cpm/pmol). After being allowed to proceed for 30 minutes at 30°C, the reactions were stopped with the addition of 20 μl of SDS sample dilution buffer (10% SDS, 20% glycerol, and 5 % β -mercaptoethanol dissolved in 0.125 mM Tris-HCl, pH 6.8). The samples were then boiled for 4 minutes and loaded onto 12.5% polyacrylamide mini-gels. Gel electrophoresis was conducted using a Bio-Rad Protein II cell. The bands were visualized using Coomassie brilliant blue stain and the 32-kDa S6 protein band was excised and counted using liquid scintillation counting.

E. Protein Assays

All protein assays were performed using either the Bio-Rad protein reagent or the Sigma Lowry assay kit. In either case, a standard curve range between 13.9 μg -141 μg was used and 20 μl of cellular extract were measured in duplicate for each sample.

1. Lowry protein assays

All tubes containing either protein standard or sample were diluted to 1 ml with distilled water. One milliliter of Lowry reagent was then added and the tubes were allowed to stand at room temperature for 20 minutes. With rapid and immediate mixing, 0.5 ml of Folin & Ciocalteu's Phenol Reagent Working Solution were added to each tube and colour was allowed to develop for 30 minutes. Absorbance values were measured at 750 nm and protein concentrations were determined using a standard curve.

2. Bio-Rad reagent

All tubes were made into a final volume of 0.1 ml using distilled water. The reaction was started with the addition of 5 ml of diluted Bio-rad dye reagent to each test tube. The tubes were vortexed and allowed to stand for at least 10 minutes at room temperature before absorbance measurements at 595 nm were made. Protein concentrations were again measured using a standard curve.

F. Chromatography

A Pharmacia LKB Biotechnology Inc. FPLC (fast protein liquid chromatography) and a Pharmacia MonoQ column (HR5/5) were used at 4°C for all adipocyte cell extract chromatographic fractionations. Two to three mg of protein were applied at a rate of 1 ml/min to the column which was equilibrated with buffer A (10 mM MOPS, 25 mM β -glycerophosphate, 5 mM EGTA, 2 mM EDTA, 2 mM sodium orthovanadate, and 2 mM dithiothreitol). The column was developed at the same flow rate with a 15 ml linear NaCl gradient (0-800 mM) in buffer A. Sixty 250 μ l fractions were collected and used for kinase activity measurements and for immunoblotting.

G. Western Blotting

1. BCIP/NBT coloring reagent method

Selected fractions from MonoQ runs of 1 mM vanadyl sulphate, 1 mM sodium selenate, or control crude extracts were digested with an equal volume of SDS sample buffer for 4 minutes at 100°C and applied to 11% polyacrylamide gels. Gel electrophoresis was

conducted overnight using a Bio-Rad Protein II cell at 15 mA per gel. The proteins in the gels were then transferred onto nitrocellulose membranes in a Hoefer transfer cell at 300 mA for 3 hours. The membranes were briefly stained with Ponceau stain in order to visualize molecular weight standard proteins before being incubated for 3 hours at room temperature with blocking solution (20 mM Tris-base (pH 7.5), NaCl 500 mM, 5% Skim milk, and 0.1% NaN₃) using a Bellco orbital shaker. They were then washed 2 times with TTBS (20 mM Tris-base (pH 7.5), 500 mM NaCl, and 0.05% Tween-20) before being incubated overnight with one of the following primary antibodies; S6K70III, S6K-rsk-CT, R1, R2, or GEGA. The membranes were again washed 2 times with TTBS and incubated with the secondary antibody (Bio-Rad goat anti-rabbit IgG alkaline phosphatase diluted 1:3000) for 2 hours. Following this incubation, the membranes were washed 2 more times with TTBS and rinsed with TBS (20 mM Tris-HCl (pH 7.5) and 500 mM NaCl). A BCIP (5-bromo-4-chloro-indoyl phosphate dissolved first in N,N-dimethylformamide) and NBT (nitroblue tetrazolium dissolved first in 70% N,N-dimethylformamide) containing color development solution (100 mM NaHCO₃ (pH 9.8) and 100 μ M MgCl₂.6H₂O) was used to incubate the membranes for 2-3 hours. Following the colour development, the membranes were washed with distilled water and dried in between two pieces of blotting paper.

2. ECL

Immunoblots were developed using reagents and protocol provided in the Amersham Life Sciences ECL starter kit.

H. Statistical Analysis

Some data are expressed as mean $\pm$ standard error of the mean (S.E.M.). Statistical significance was determined by one way analysis of variance (ANOVA) followed by Fischers LSD test used for comparing results for a given set of groups or using the Student

"t" test. A probability of less than 0.05 ($p < 0.05$) was used as the level of statistical significance.

RESULTS

I. MAP KINASES

A. Time-Course

1. Insulin

A 40% suspension of isolated rat adipocytes was incubated with 10 nM insulin for 0-30 minutes in order to establish a time-course for the stimulation of MBP kinase activity (Fig. 1). Initial activation of MBP kinase activity appeared to occur at 2.5 minutes with a ~1.3-fold stimulation over control although this was not statistically significant. At 5 minutes, maximal stimulation at ~1.7-fold over control was observed. MBP kinase activity was not observed at any of the later time-points, thus confirming the transient nature of insulin action in this system as previously reported in the literature (Haystead *et al.*, 1990).

2. Sodium selenate

A time-course of the stimulation of MBP kinase activity by selenium was carried out by incubating adipocytes with 1 mM sodium selenate for 0-30 minutes (Fig. 2). Initial activation occurred at 2.5 minutes with a ~1.3-fold increase over control. Maximal stimulation was observed after 10 minutes of incubation with nearly a two-fold increase over control. In contrast to insulin, the MBP kinase stimulation by sodium selenate was relatively long-lasting with ~1.5-fold increase over control remaining even after 27 minutes of incubation.

3. Vanadyl sulphate

Rat adipocytes were treated with 1mM vanadyl sulphate for 0-30 minutes. After 10 minutes of incubation activity was ~4.5-fold control and at 20 minutes maximal stimulation occurred with a ~5.5-fold increase over control (Fig. 3). Activity decreased after 30 minutes of incubation, however it remained over 2.5-fold that of control, and was higher than that of either insulin or sodium selenate at any time-point (Fig. 4).

B. Dose-Response

1. Insulin

Based on the time-course, 5 minutes was chosen as the optimal time-point for observing MBP kinase activation in isolated rat adipocytes by insulin. To obtain a dose-response curve, adipocytes were incubated for 5 minutes with the following concentrations of insulin; 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, and 100 nM (Fig. 5). Significant stimulation was only observed at 10 and 100 nM with a ~1.5-fold increase over control.

2. Sodium selenate

Adipocytes were incubated for 10 minutes with the following concentrations of sodium selenate; 100 nM, 1 μ M, 10 μ M, 100 μ M, 1 mM, and 10 mM (Fig. 6). The 10 minute time-point was chosen because significant stimulation of both MBP kinases and Ribosomal Protein S6 kinases was present. MBP kinase activation by sodium selenate was only observed at 1 and 10 mM. At both concentrations the stimulation was approximately 2-fold higher than control.

3. Vanadyl sulphate

The following concentrations of vanadyl sulphate were used in obtaining a dose-response curve for the effects of vanadyl sulphate on MBP kinase activity; 10 μ M, 36 μ M, 100 μ M, 316 μ M, 1 mM, 3.16 mM, and 10 mM. Maximal stimulation of MBP kinases by vanadyl sulphate occurred at 1 mM with a ~5-fold increase over control (Fig. 7). No effects were observed at lower concentrations. At 3.16 mM, MBP kinase activity dropped to ~4-fold control and at 10 mM there was a further drop of activity to slightly more than 2-fold control. Therefore, there appears to be a very narrow range of concentrations at which the effects of vanadyl sulphate on adipocyte MBP kinases can be observed.

C. Mono Q Anion-Exchange Chromatography and Immunoblotting

Cellular extracts from adipocytes stimulated with 1 mM sodium selenate, 1 mM vanadyl sulphate, or untreated controls were applied to a Mono Q anion-exchange column and the collected fractions were assayed for MBP kinase activity using myelin basic protein as the substrate. In addition, the fractions were probed with MAP kinase-specific antibodies.

1. Sodium selenate

Five peaks of MBP kinase activity were present in the Mono Q elution profile of sodium selenate-stimulated samples (Fig. 8). The first peak was centered around fraction 17, 226 mM NaCl, with 9 pmol/min/ml of activity. Peak two was centered around fraction 26 (346 mM NaCl) with 45 pmol/min/ml of activity. Immediately following it was peak three, which was centered around fraction 29 (386 mM NaCl) with 27 pmol/min/ml of activity. Peaks four and five were 9 pmol/min/ml each and were centered around fractions 38 (505 mM NaCl) and 40 (532 mM NaCl), respectively.

Peak fractions with MBP kinase activity from the sodium selenate-stimulated samples were probed with the R1 antibody against MAP kinases (Fig 9). A 52 kDa band was present in fractions 26, 27, 28, and 29 corresponding to the two major peaks of MBP kinase activity. A 50 kDa band was also present in fractions 26 and 27. No other bands were present in any other fractions. A separate set of immunoblots were subjected to the much more sensitive ECL detection system, although no bands were visualized (data not shown).

2. Untreated control

The Mono Q profile of extracts from untreated adipocytes had four peaks of interest (Fig. 8). Peak one corresponded with the second peak of sodium selenate-treated samples with 10 pmol/min/ml of activity centered around fraction 27 (359 mM NaCl). Peak two with 9 pmol/min/ml of activity was centered around fraction 32 (426 mM NaCl). Peak three, centered around fraction 48 (638 mM NaCl) was the most prominent peak in the control Mono Q profile with 31 pmol/min/ml of MBP kinase activity. This peak was not present in the sodium selenate-treated samples. Peak four, centered around fraction 58 (771 mM NaCl) was the second largest control peak with 16 pmol/min/ml of activity.

3. Vanadyl sulphate

Four major peaks of MBP kinase activity were present in the Mono Q profile of the vanadyl sulphate-stimulated samples (Fig. 10). Peak one was centered around fraction 18 (239 mM NaCl) with 10 pmol/min/ml of activity. Peaks two and three both had 56 pmol/min/ml of activity and were centered around fractions 26 (346 mM NaCl) and 29 (386 mM NaCl), respectively. Peak four was centered around fraction 41 (545 mM NaCl) with 9 pmol/min/ml of activity. All four peaks corresponded to the major peaks observed in the sodium selenate-stimulated samples.

4. Vanadyl sulphate time-course

Cellular extracts from adipocytes incubated for 10, 20, or 30 minutes and untreated control cells were applied to a Mono Q anion-exchange column (Fig. 11). A single major peak of activity was present for all time-points which were centered around fraction 26 (345 mM NaCl). The 10 minute samples had 125 pmol/min/ml of activity while at 20 minutes maximal MBP kinase stimulation was present with 174 pmol/min/ml. MBP kinase activity was 118 pmol/min/ml for the 30 minute samples.

In the control samples, a broad peak ranging from fraction 6 (69 mM NaCl) to fraction 24 (166 mM NaCl) was observed with fraction 23 (152 mM NaCl) having the maximal MBP kinase activation, 79 pmol/min/ml, was present. This peak was not reproduced in other experiments suggesting that it represented an experimental artifact.

Mono Q fractions of vanadyl sulphate-stimulated samples from the time-course were probed with R1 and R2 antibodies against MAP kinase (Fig. 12). With the R1 antibody, a dark 44 kDa band and a light 42 kDa band were visible in the pooled fractions 17-23. In addition, a 42 kDa band was present in the pooled fractions 24-29. Two bands were visible for the pooled fractions 17-23 with the R2 antibody, a very dark relatively broad 42 kDa band and a lighter 38 kDa band. No bands were visible for the pooled fractions 24-29. With the use of the ECL detection system, no relevant bands were visualized (data not shown).

II. RIBOSOMAL PROTEIN S6 KINASES

A. Time-Course

1. Insulin

Isolated rat adipocytes were incubated with 10 nM insulin for 0-30 minutes. A slight stimulation of Ribosomal Protein S6 kinase activity was observed after 2.5 minutes of incubation (Fig. 13) and increased activity continued with longer incubation times until a maximal was reached at 10 minutes, with a greater than 2-fold increase over control. At 15 and 20 minutes of incubation, a 1.5-fold control level of activity remained, but after 30 minutes of incubation no difference from control samples was apparent.

2. Sodium selenate

Adipocytes were incubated with 1 mM sodium selenate for 0-27 minutes. Initial activation of Ribosomal Protein S6 kinases by sodium selenate occurred after 10 minutes of incubation with a 3-fold increase over controls (Fig. 14). By 13 minutes, activity had risen slightly and it continued to increase at 16 minutes with nearly a 5-fold increase over control, but it was not until 19 minutes that the maximal stimulation of ~8.5-fold control was observed. The activity decreased after 27 minutes but remained over 4-fold that of control.

3. Vanadyl sulphate

The time points chosen for adipocytes treated with 1 mM vanadyl sulphate were 0-30 minutes. Significant activation of Ribosomal Protein S6 kinases by vanadyl sulphate was not observed until 10 minutes of incubation with an ~8-fold increase over control (Fig. 15).

Maximal stimulation occurred after 20 minutes with a 17-fold increase over control. Activity decreased after 30 minutes of incubation but remained 11-fold higher than control. As with MBP kinases, the Ribosomal Protein S6 kinase stimulation of vanadyl sulphate was higher than insulin and sodium selenate (Fig. 16).

B. Dose-Response

1. Insulin

Isolated rat adipocytes were incubated with the following concentrations of insulin; 1 pM, 10 pM, 100 pM, 1 nM, 10 nM, and 100 nM (Fig. 17). Ribosomal Protein S6 kinase activity was present at insulin concentrations of 1, 10 , and 100 nM with 1.5, 1.6, and 1.9-fold increase over control, respectively. The lowest concentration at which Ribosomal Protein S6 kinase activity could be detected was 10 times lower than that for which MBP kinase activity could be observed. This seems an unlikely possibility if the MBP kinases were directly upstream of the activated Ribosomal Protein S6 kinases activated and may perhaps point to the p70^{S6k} family as being responsible for the activity observed.

2. Sodium selenate

Adipocytes were incubated with 100 nM, 1 μ M, 10 μ M, 100 μ M, 1 mM, and 10 mM sodium selenate. The lowest concentration of sodium selenate causing Ribosomal Protein S6 kinase activation in adipocytes was 100 μ M with a stimulation of over 2-fold control (Fig. 18). At the two higher concentrations used, 1 and 10 mM, a nearly 3-fold control level of activity was present. As was the case with insulin, Ribosomal Protein S6 kinase stimulation occurred at a 10 times lower concentration for MBP kinase.

3. Vanadyl sulphate

The following concentrations of vanadyl sulphate; 10 μ M, 36 μ M, 100 μ M, 316 μ M, 1 mM, 3.16 mM, and 10 mM, were used in obtaining a dose-response curve of Ribosomal Protein S6 kinase activity (Fig. 19). Maximal stimulation occurred at a concentration of 1 mM with a 14-fold increase over control and decreased to 9-fold control at 3.16 mM. Unlike the MBP kinase dose response curve, which retained a 2-fold control activity when the concentration was increased to 10 mM, no activity was detected at this high concentration. In addition, as was the case with insulin and sodium selenate, Ribosomal Protein S6 kinase activation occurred at a 10 times lower concentration, 316 μ M, than that required to stimulate MBP kinase activity with a 2-fold increase over control.

C. Mono Q Anion-Exchange Chromatography

Cellular extracts from adipocytes incubated with 1 mM sodium selenate, 1 mM vanadyl sulphate, and untreated controls were applied to a Mono Q anion-exchange column. Collected fractions were assayed for S6-peptide kinase and Ribosomal Protein S6 kinase activity using a synthetic S6 peptide and 40S ribosomal subunits as the respective substrates.

1. Sodium selenate

a. S6-peptide kinase

Four peaks of S6-peptide-kinase activity in the Mono Q profile of the Selenium-stimulated samples were of interest (Fig. 20). Peak one, centered around fraction 17 (226 mM NaCl) was the largest with 22 pmol/min/ml of activity. Peaks two and three were centered around fraction 28 (372 mM NaCl) and 31 (412 mM NaCl) with 10 pmol/min/ml of

activity each. Peak four was centered around fraction 45 (599 mM NaCl) with 9 pmol/min/ml of S6P-kinase activity.

b. Ribosomal Protein S6 kinase

Four relevant peaks of Ribosomal Protein S6 kinase activity were present in the Mono Q profile of selenium-treated samples (Fig. 21). Peak one, corresponding to peak one of the S6P-kinase profile, had the highest activity with 357 fmol/min/ml centered around fraction 17 (226 mM NaCl). Peaks two and three had 107 fmol/min/ml of activity each and were centered around fractions 24 (319 mM NaCl) and 27 (359 mM NaCl), respectively. Peak four was centered around fraction 36 (479 mM NaCl) with 142 fmol/min/ml of activity.

2. Untreated control

a. S6 peptide kinase

The predominant peaks of S6P-kinase activity for the control samples were centered around fractions 44 (585 mM NaCl) and 46 (612 mM NaCl) with 16 pmol/min/ml of activity each (Fig. 20). These peaks correspond to peak four in the Mono Q profile of the selenium-stimulated samples although their level of activity was nearly 2-fold higher.

b. Ribosomal Protein S6 kinase

Similar to the sodium selenate-treated samples, untreated control samples also had a peak centering around fraction 17 with 71 fmol/min/ml of activity and at fraction 24 with 143 fmol/min/ml (Fig. 21). A 71 fmol/min/ml peak corresponding to peak three of selenium-stimulated samples was centered around fraction 28, 372 mM NaCl. The largest control peak

was centered around fraction 44 (585 mM NaCl) with 286 fmol/min/ml of activity and was not present in selenium-treated samples.

3. Vanadyl sulphate time-course

a. S6-peptide kinase

At least six major peaks were present in the S6P-kinase Mono Q profiles of cellular extracts from adipocytes incubated with 1 mM vanadyl sulphate for 10, 20, or 30 minutes (Fig. 22). Peak one was centered around fraction 5 (55 mM NaCl) with the 20 minute samples having the highest activity (48 pmol/min/ml) followed by the 10 minute samples, 24 pmol/min/ml, and the 30 minute and control samples with 12 pmol/min/ml of activity each. Peak two was centered around fraction 8 (97 mM NaCl) for the 20 minute samples with 42 pmol/min/ml of activity. It centered around fraction 9 (110 mM NaCl) with 28 pmol/min/ml of activity for the 30 minute samples and was not present for the 10 minute and control samples. The 20 minute samples had the highest activity for peak three with 48 pmol/min/ml, centered around fraction 19 (249 mM NaCl). The 30 minute samples had the next highest activity with 42 pmol/min/ml centered around fraction 29 (235 mM NaCl). Peak three was centered around fraction 22 (290 mM NaCl) for the 10 minute samples with 27 pmol/min/ml of activity. Peak four, was centered around fraction 26 (345 mM NaCl) for the vanadyl sulphate-stimulated samples with 31 pmol/min/ml for the 10 minute samples, 49 pmol/min/ml for the 20 minute samples, and 37 pmol/min/ml for the 30 minute samples. Peak four was not present in control samples. Peak five, centered around fraction 28 (373 mM NaCl) for all groups, was the largest S6P-kinase peak with 15 pmol/min/ml for controls, 27 pmol/min/ml for the 10 minute samples, 53 pmol/min/ml for the 20 minute samples, and 22 pmol/min/ml for the 30 minute samples. Peak six was only present in the 20 and 30

minute samples, centering around fraction 32 (428 mM NaCl) with 33 pmol/min/ml of activity.

b. Ribosomal Protein S6 kinase

The Mono Q profile of the vanadyl sulphate-treated adipocyte extracts had a single major peak of activity encompassing fractions 17-24 (Fig. 23). The 10 minute samples had the largest peak with 892 fmol/min/ml of activity centered around fraction 21 (276 mM NaCl). The 20 minute samples had 654 fmol/min/ml also centered around fraction 21. The 30 minute samples had 536 fmol/min/ml of activity centering around fraction 20 (262 mM NaCl).

D. Immunoblotting

Selected Mono Q fractions from sodium selenate-stimulated and vanadyl sulphate-stimulated samples were probed with S6K-III and S6K-CT antibodies against S6 kinases. Bands were visualized using the BCIP/NBT coloring reagents or the enhanced chemiluminiscence (ECL) detection system.

No bands were present in the blots of Mono Q fractions from sodium selenate or vanadyl sulphate-stimulated samples probed with S6K-III antibody and developed using BCIP/NBT coloring reagents (data not shown). A series of bands were observed on the selenium blot probed with the S6K-CT antibody (Fig. 24A). Fraction 18, associated with peak one of S6 kinase activity in the Mono Q profile, had a band at 52 kDa. Fractions 19 and 20, also associated with peak one, had a band at 55 kDa. A 64 kDa band was present in all other fractions.

With the vanadyl sulphate-stimulated samples probed with S6K-CT, a 55 kDa band was visible for fractions 17-23 using the BCIP/NBT coloring reagents (Fig. 24B). Due to the

detection limits of this system, large amounts of protein were loaded onto gels which led to overloading and poor resolution.

Much lower concentrations of proteins were used for the series of immunoblots visualized using the ECL detection system. Selenium-stimulated samples probed with the S6K-III antibody had a 68 kDa band present for fractions 17,18,19, and 20 with fraction 19 having the heaviest band (Fig. 25A). Using the S6K-CT antibody, a 62 kDa band which was heavy for fractions 17, 18, and 19 and light for fractions 20 and 21 was present (Fig. 25B). In addition, a light 54 kDa band was present for fractions 17-20. Vanadyl sulphate stimulated samples yielded a 70 kDa band for fractions 17-21 when probed with the S6K-III antibody (Fig. 26A). Fraction 18 had the heaviest band while fraction 21 had the lightest. Using the S6K-CT antibody, a 69 kDa band was present in fractions 17, 18, 19, and 20 with fraction 18 having the darkest band (Fig. 26B). . In addition, a faint 73 kDa band was present in fraction 16 and a 59 kDa band in fractions 18 and 39. The ~70 kDa bands present in figure 28A-D are similar enough in molecular mass and their pattern of appearance as to suggest that they represent one band. These bands were visualized with the Ponceau stain used to mark the molecular weight standards suggesting that they may represent a contaminating protein such as BSA (molecular mass 66.2 kDa) present in high concentrations in all the samples as opposed to trace amounts of S6 kinases.

III. GENISTEIN EXPERIMENT

Genistein, a tyrosine-kinase inhibitor, was used to gain further insight into the activation of MAP (Fig. 27) and Ribosomal Protein S6 kinases (Fig. 28) by insulin, sodium selenate, and vanadyl sulphate.

A. MBP kinases

The minor activation of MBP kinases by insulin, ~1.5-fold control, was not affected by the presence of 200 nM genistein (Fig. 27). Sodium selenate alone had modest effects, 1.5-fold control, which were abolished with genistein treatment. There was also a ~36% reduction in the stimulation of MBP kinases by vanadyl sulphate from 2.5-fold control to 1.6-fold control in the presence of genistein.

B. Ribosomal Protein S6 kinases

Insulin stimulation of Ribosomal Protein S6 kinase activity was reduced from 2-fold control to control levels in the presence of genistein (Fig. 28). There was only a small reduction in the effects of sodium selenate. The 6-fold control Ribosomal Protein S6 kinase activity following vanadyl sulphate treatment was reduced to only 2-fold control in the presence of genistein.

IV. RAPAMYCIN EXPERIMENTS

The macrolide rapamycin selectively blocks the activation of the pp70^{S6K} kinase family without affecting pp90^{rsk} kinases or the MBP kinases (Fingar *et al.*, 1993). In order to help elucidate the contribution of the two above mentioned Ribosomal Protein S6 kinase families in extracts from adipocytes stimulated with sodium selenate and vanadyl sulphate, rapamycin was used in the next series of experiments.

A. MBP kinases

Rapamycin did not block the activation of MBP kinases incubated with either 1 mM sodium selenate or 1 mM vanadyl sulphate (Fig. 29). Sodium selenate alone caused a 1.8-fold increase over control and in the presence of 50 ng/ml rapamycin a 1.7-fold increase over control was observed. Likewise, vanadyl sulphate caused a 1.5-fold increase in the absence and a 1.4-fold increase in the presence of rapamycin.

B. Ribosomal Protein S6 kinases

Ribosomal Protein S6 kinase activity was affected by the presence of rapamycin. Sodium selenate caused a 1.9-fold increase in Ribosomal Protein S6 kinase activity which was reduced to 1.4-fold in the presence of rapamycin (Fig. 30). Vanadyl sulphate produced a 2.2-fold increase over control which was not significantly reduced with rapamycin.

Figure 1

Time-Course for the Activation of MBP kinases by Insulin.

Isolated rat adipocytes were prepared by the method of Rodbell (1964) using collagenase digestion of epididymal fat pads. A 40% suspension of the cells was incubated with 10 nM insulin for 0-30 minutes. The incubations were terminated with the addition of ice-cold homogenization buffer and cellular extracts prepared and assayed for MAP kinase activity, as described in Methods, using MBP as substrate. Each time-point was expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 24 pmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of MBP Kinases by 10 nM Insulin

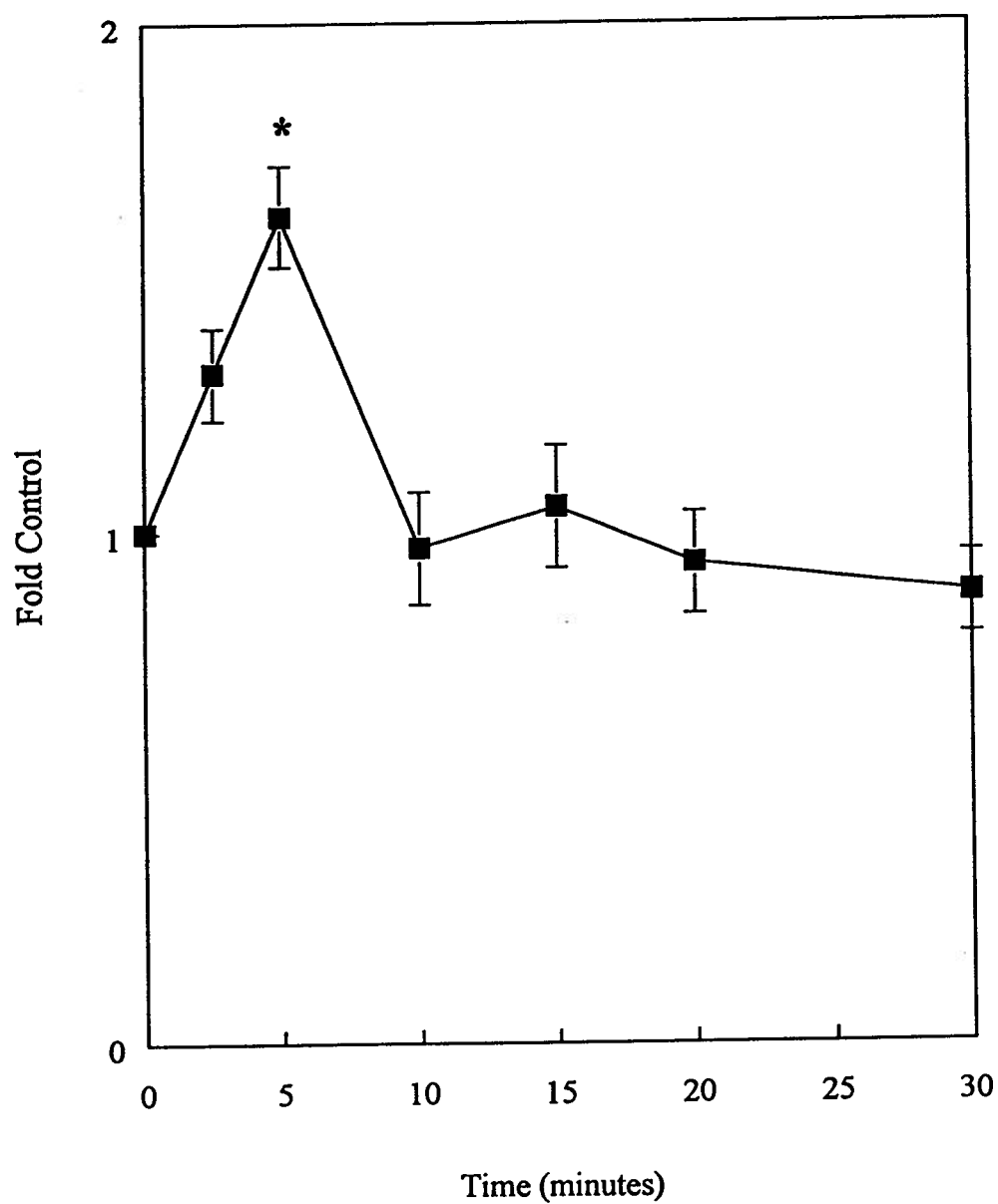


Figure 2**Time-Course for the Activation of MBP kinases by Sodium Selenate.**

Adipocytes were prepared and assayed for MBP kinase activity as described in Fig. 1 following their incubation with 1 mM sodium selenate for 0-30 minutes. Each time-point was expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 27 pmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of MBP Kinases by 1 mM Sodium Selenate

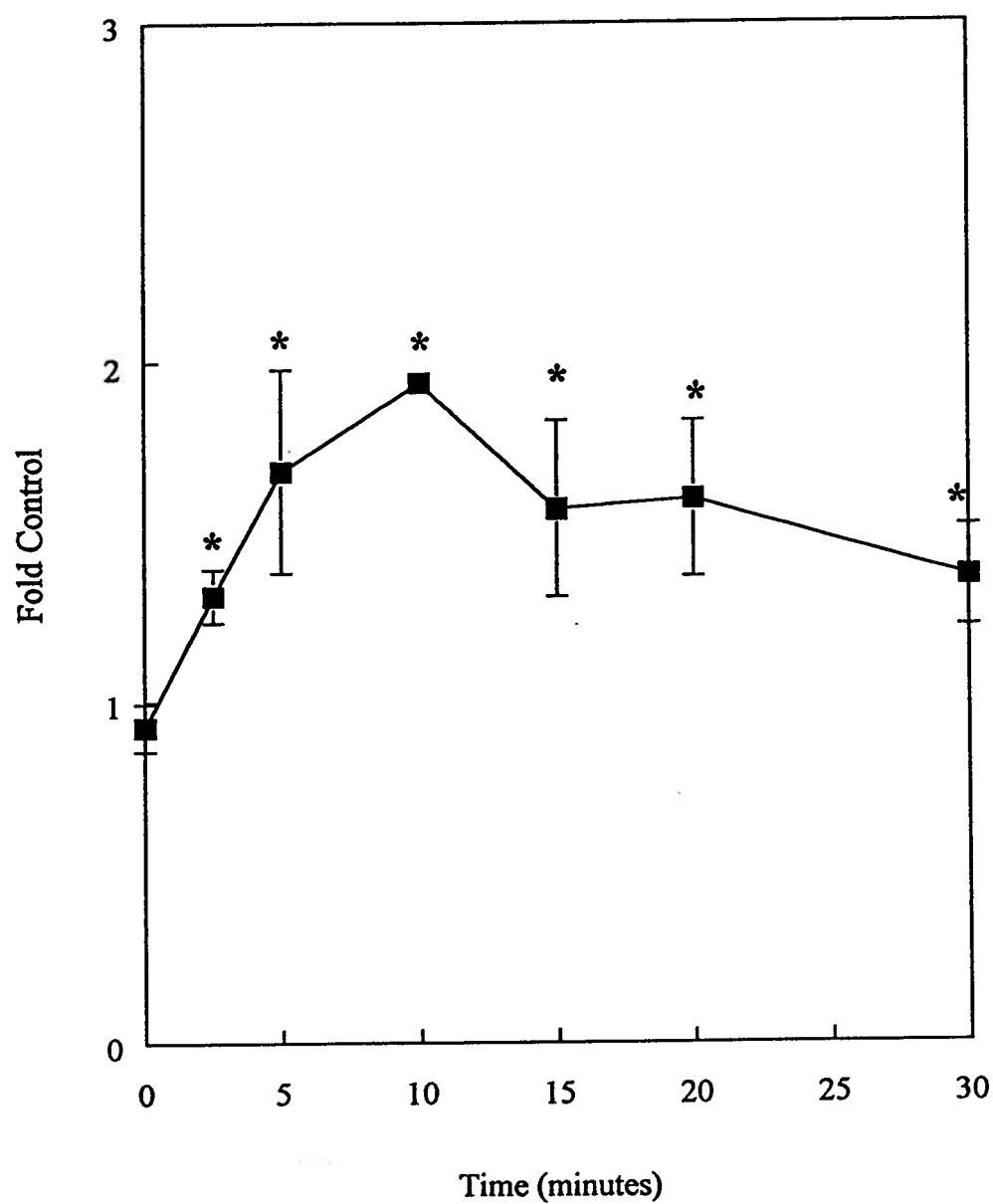


Figure 3**Time-Course for the Activation of MBP kinases by Vanadyl Sulphate.**

Adipocytes were prepared and assayed for MBP kinase activity as described in Fig. 1 following their incubation with 1 mM vanadyl sulphate for 0-30 minutes. Each time-point was expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 16 pmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of MBP Kinases by 1 mM Vanadyl Sulphate

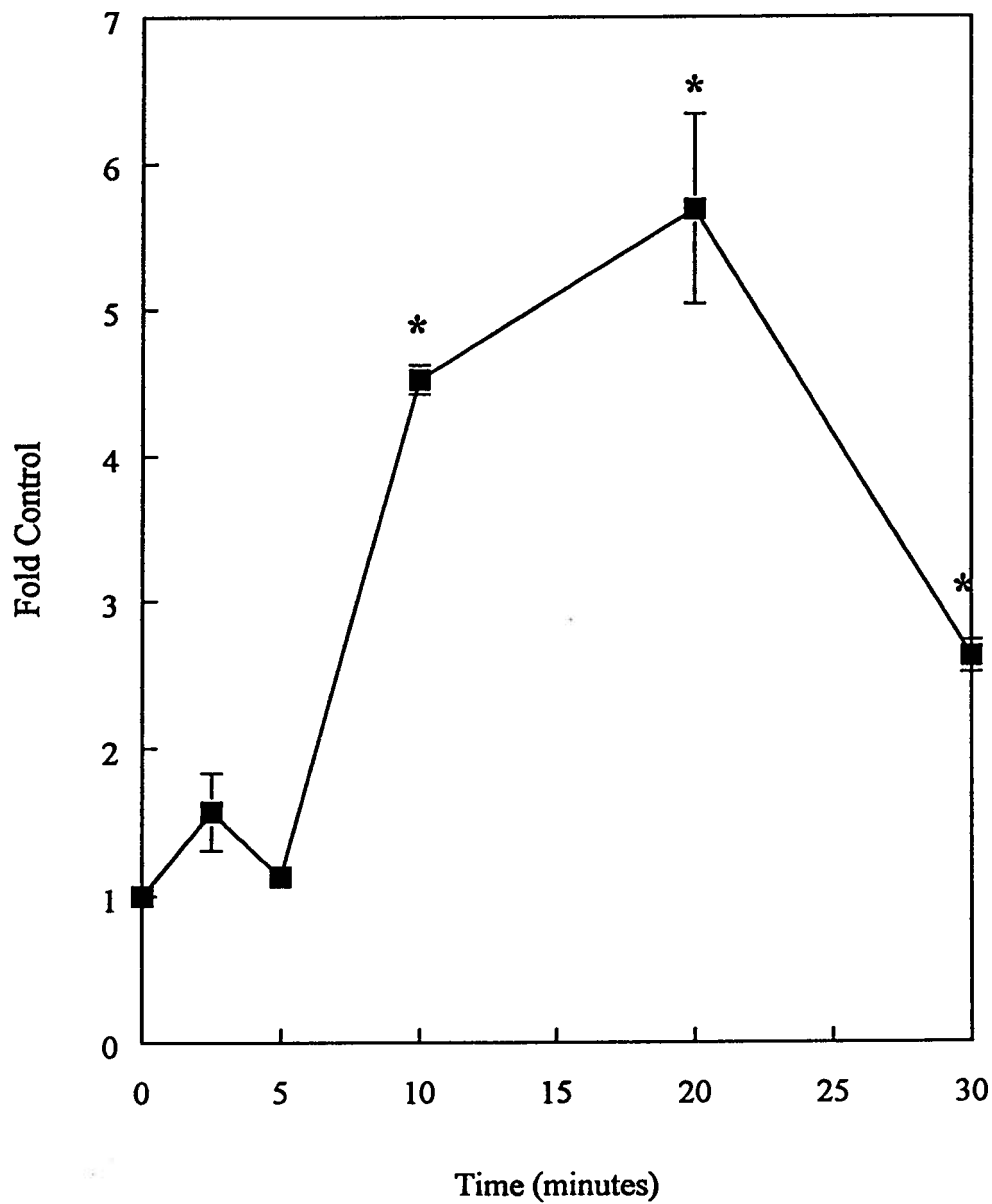


Figure 4

**Comparison of the Time-Course of Activation of MBP kinase
by 10 nM insulin, 1 mM Vanadyl Sulphate, and 1 mM Sodium Selenate.**

Compilation of figures 1-3. * denotes significant difference from control ($p < 0.05$)

MBP Kinase Time-Course Comparisons

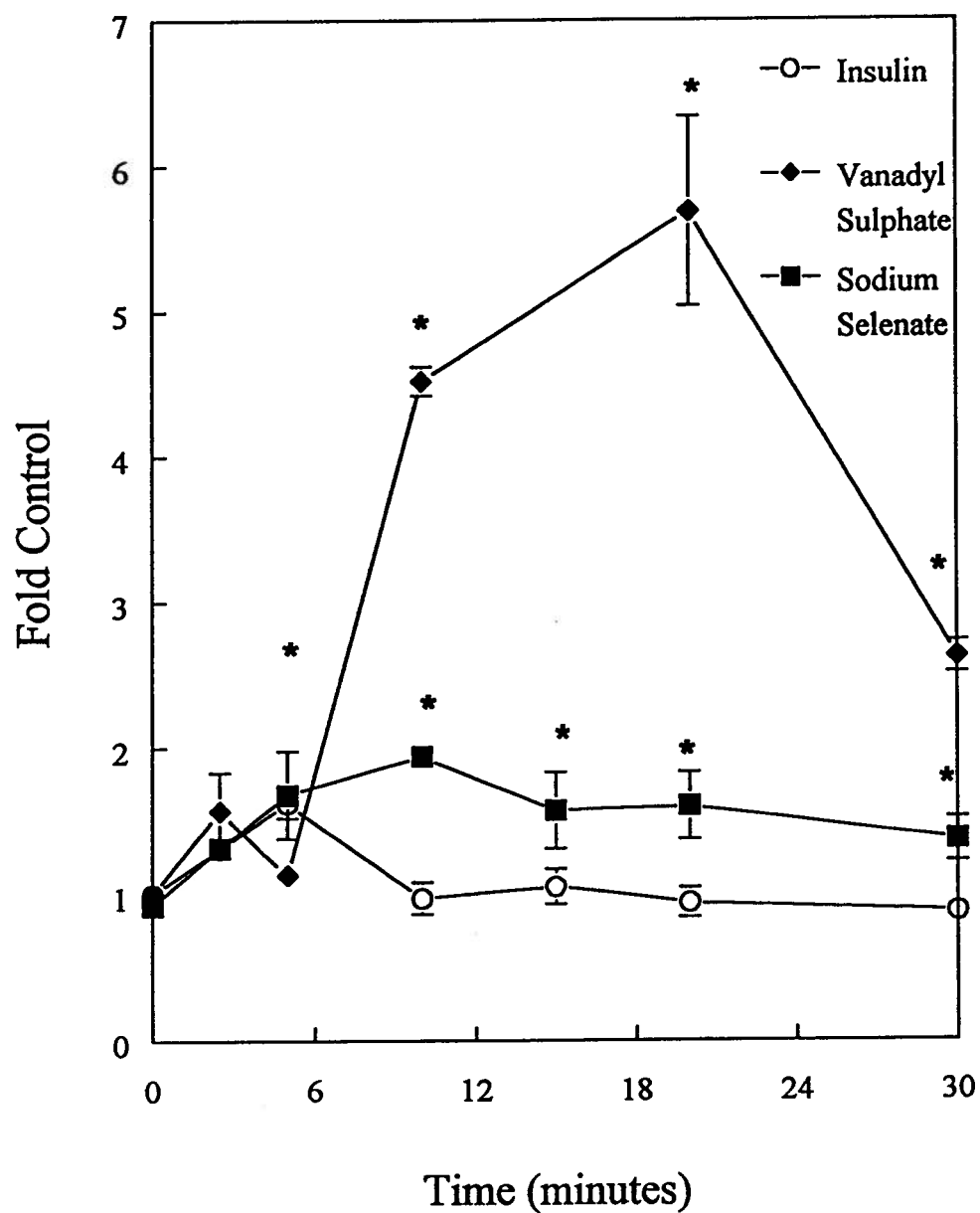


Figure 5**Dose-response for the Activation of MBP kinases by Insulin.**

Isolated rat adipocytes were incubated for 10 minutes with an insulin concentration range of 1 pM to 100 nM. Cellular extracts were prepared and assayed for MBP kinase activity as previously described. Each time-point was expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 24 pmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of MBP kinases by Insulin

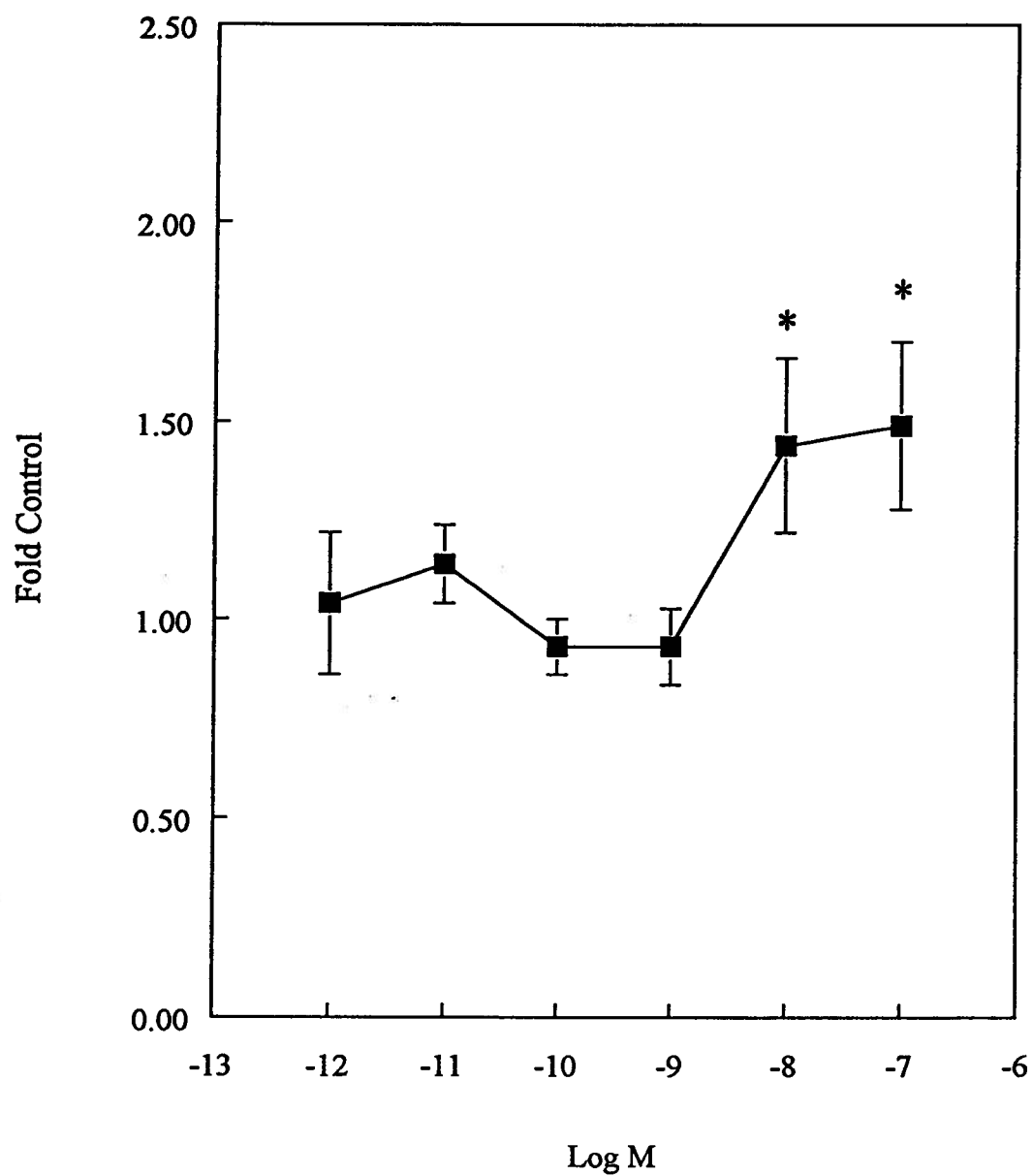


Figure 6**Dose-response for the Activation of MBP kinases by Sodium Selenate.**

Isolated rat adipocytes were incubated for 10 minutes with sodium selenate concentrations ranging from 100 nM to 10 mM. Cellular extracts were prepared and assayed for MBP kinase activity as previously described. Each time-point was expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 27pmol/min/mg. * denotes significant difference from control ($p < 0.05$)

Activation of MBP Kinases by Sodium Selenate

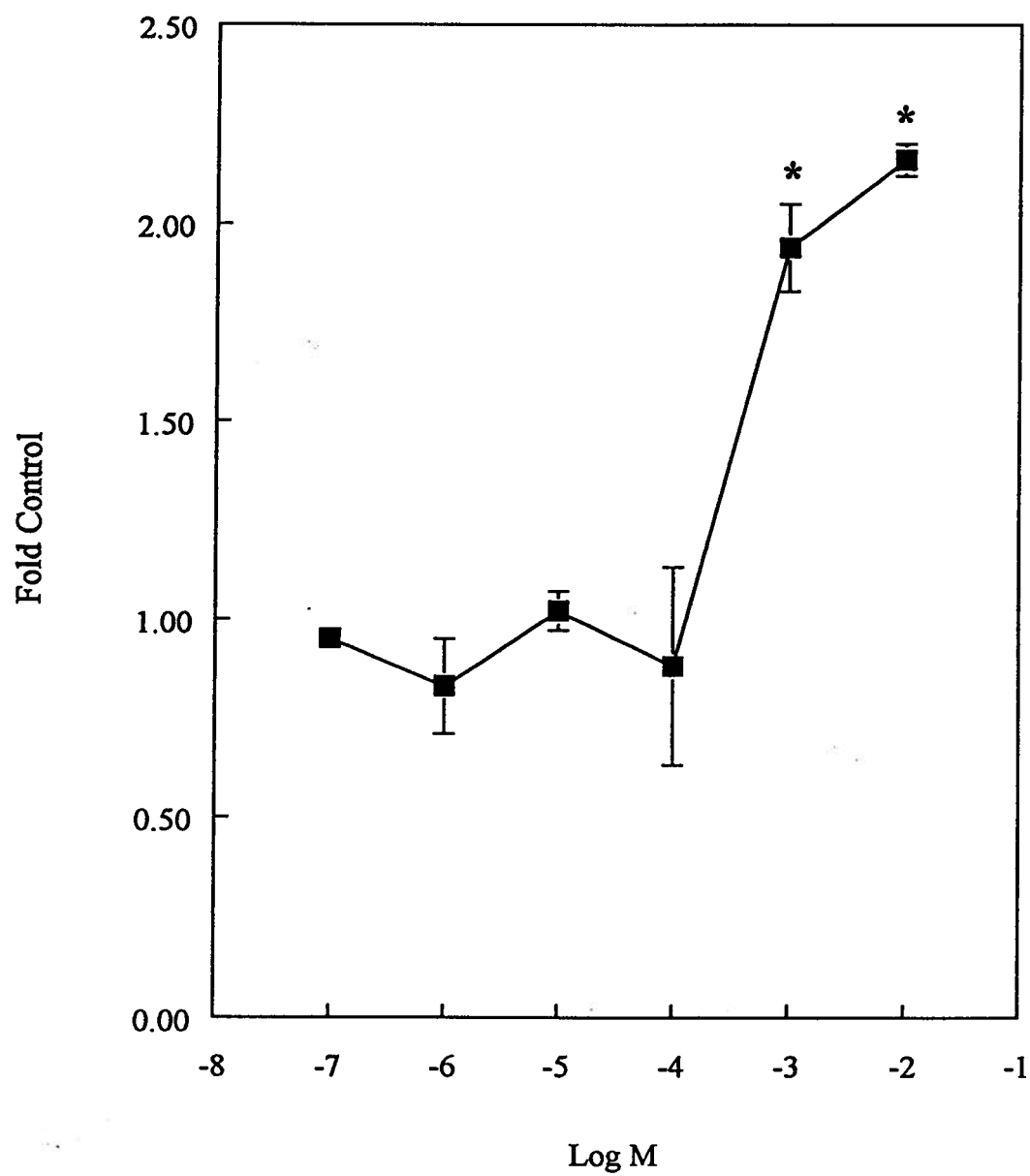


Figure 7**Dose-response for the Activation of MBP kinases by Vanadyl Sulphate.**

Isolated rat adipocytes were incubated for 20 minutes with vanadyl sulphate concentrations ranging from of 10 μ M to 10 mM. Cellular extracts were prepared and assayed for MBP kinase activity as previously described. Each time-point was expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 13 pmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of MBP Kinases by Vanadyl Sulphate

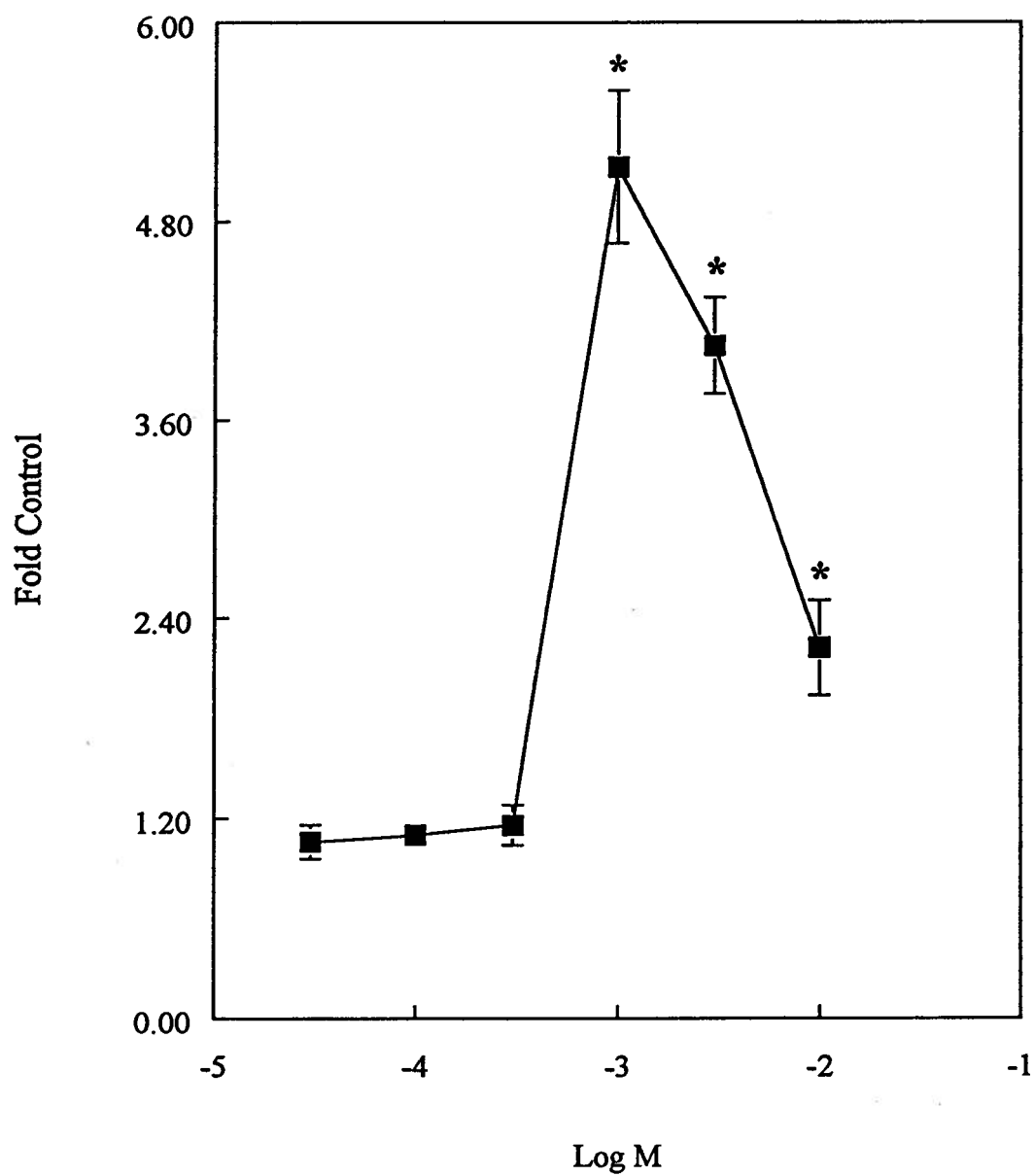


Figure 8**Elution Profile of Sodium Selenate-stimulated MBP kinase Activity**
Resolved on a Mono Q Anion-Exchange Column.

Extracts made from isolated rat adipocytes incubated for 20 minutes with 1 mM sodium selenate and untreated controls were applied to a Mono Q anion exchange column and developed using a linear NaCl gradient from 0 to 800 mM in 60 fractions with a volume of 0.25 ml each. Fractions were assayed for MBP kinase activity. The experiment was repeated two times although the data points represent a single experiment.

MBP Kinase Mono Q Profile Sodium Selenate

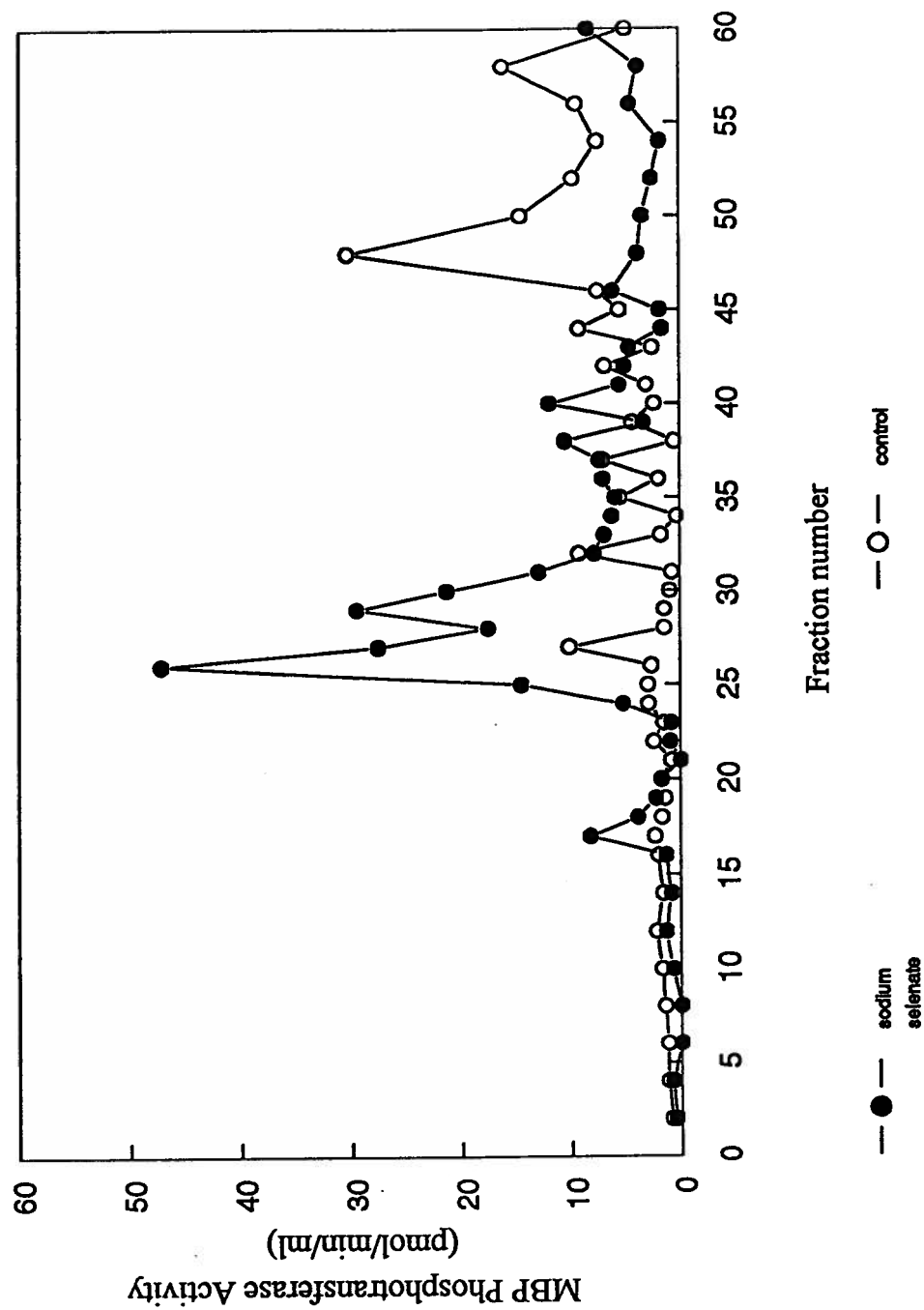


Figure 9

Immunoblotting of Mono Q fractions from Sodium Selenate-stimulated samples with MAP kinase-specific Antibodies.

Selected fractions from the elution profile of sodium selenate-stimulated samples were resolved on 11% SDS-polyacrylamide gels, transferred to nitrocellulose paper, and probed with the R1 antibody against MAP kinases. The blots were developed using alkaline phosphatase as the secondary antibody and BCIP/NBT colouring reagents. A 52 kDa band was present in fractions 26-29 corresponding to peaks one and two of MBP-kinase activity from Fig. 8. Lane assignments are shown below.

<u>Lane</u>	<u>Fraction Number</u>
1	molecular weight standards
2	16
3	17
4	18
5	25
6	26
7	27
8	28
9	29
10	30
11	31
12	32
13	40
14	41
15	42

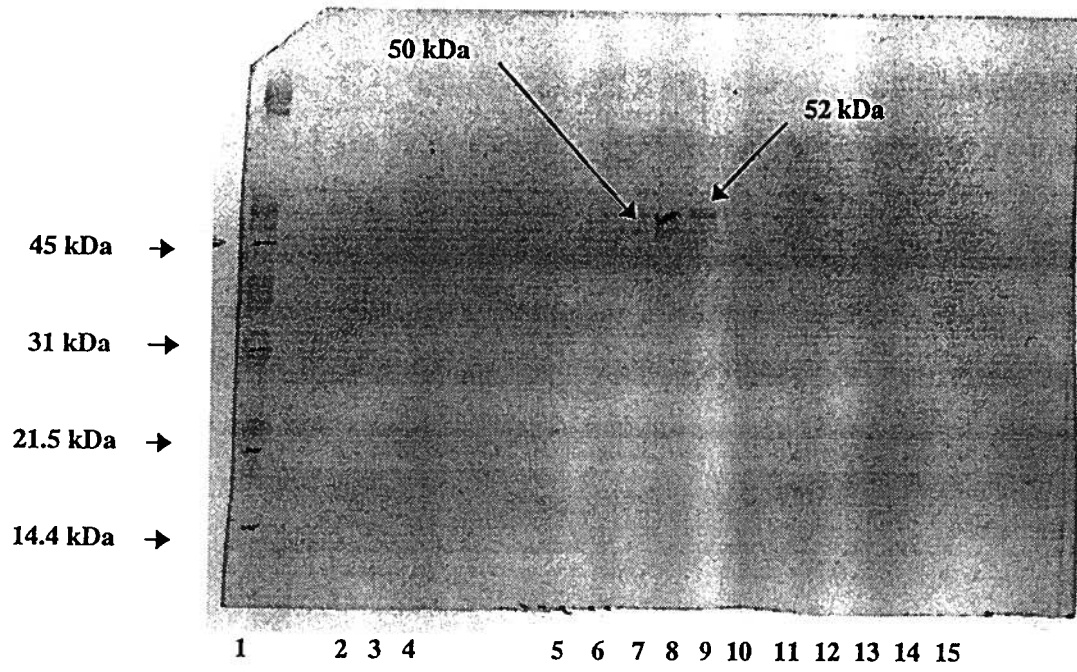


Figure 10

**Elution Profile of Vanadyl Sulphate-stimulated MBP kinase Activity
on Mono Q anion exchange Column.**

Extracts of isolated rat adipocytes incubated for 20 minutes with 1 mM vanadyl sulphate or untreated controls were resolved and assayed as described in Fig. 8. Two major peaks at 346 and 386 mM NaCl, corresponding to the peaks seen for the sodium selenate-stimulated samples, were present with 56 pmol/min/ml of activity each. The experiment was repeated two times although the data points represent a single experiment.

MBP Kinase Mono Q Profile

Vanadyl Sulphate

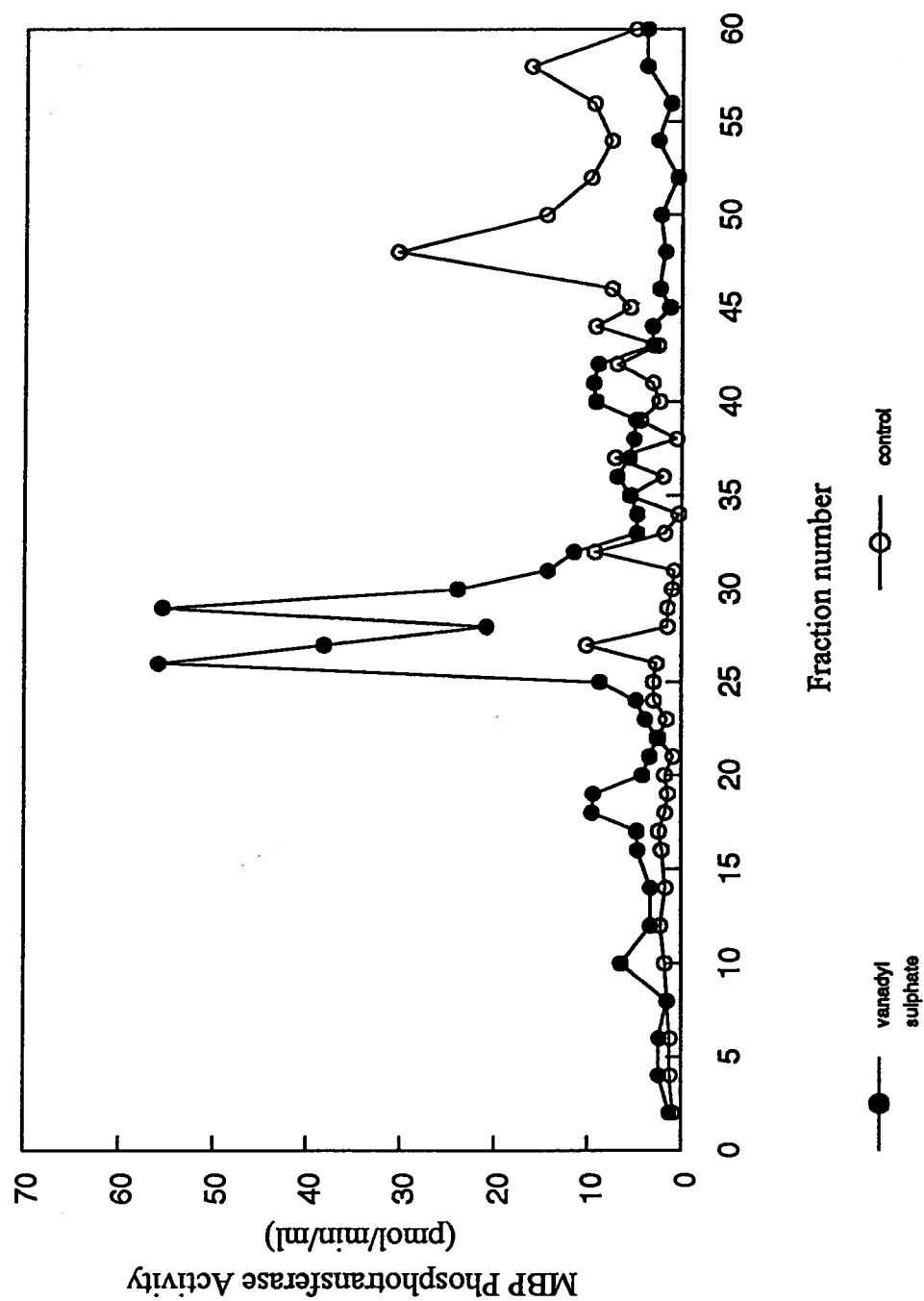


Figure 11**Mono Q Elution Profile of Vanadyl Sulphate-stimulated
MBP kinase Activity Time-Course.**

Extracts from isolated rat adipocytes incubated with 1 mM vanadyl sulphate for 10, 20, 30, or untreated controls were applied to a Mono Q anion-exchange column and developed using a linear NaCl gradient of 0-800 mM (60 fractions with 0.2 ml volume each). Fractions were assayed as previously described. Data points represent a single experiment.

Activation of MBP Kinases by Vanadyl Sulphate

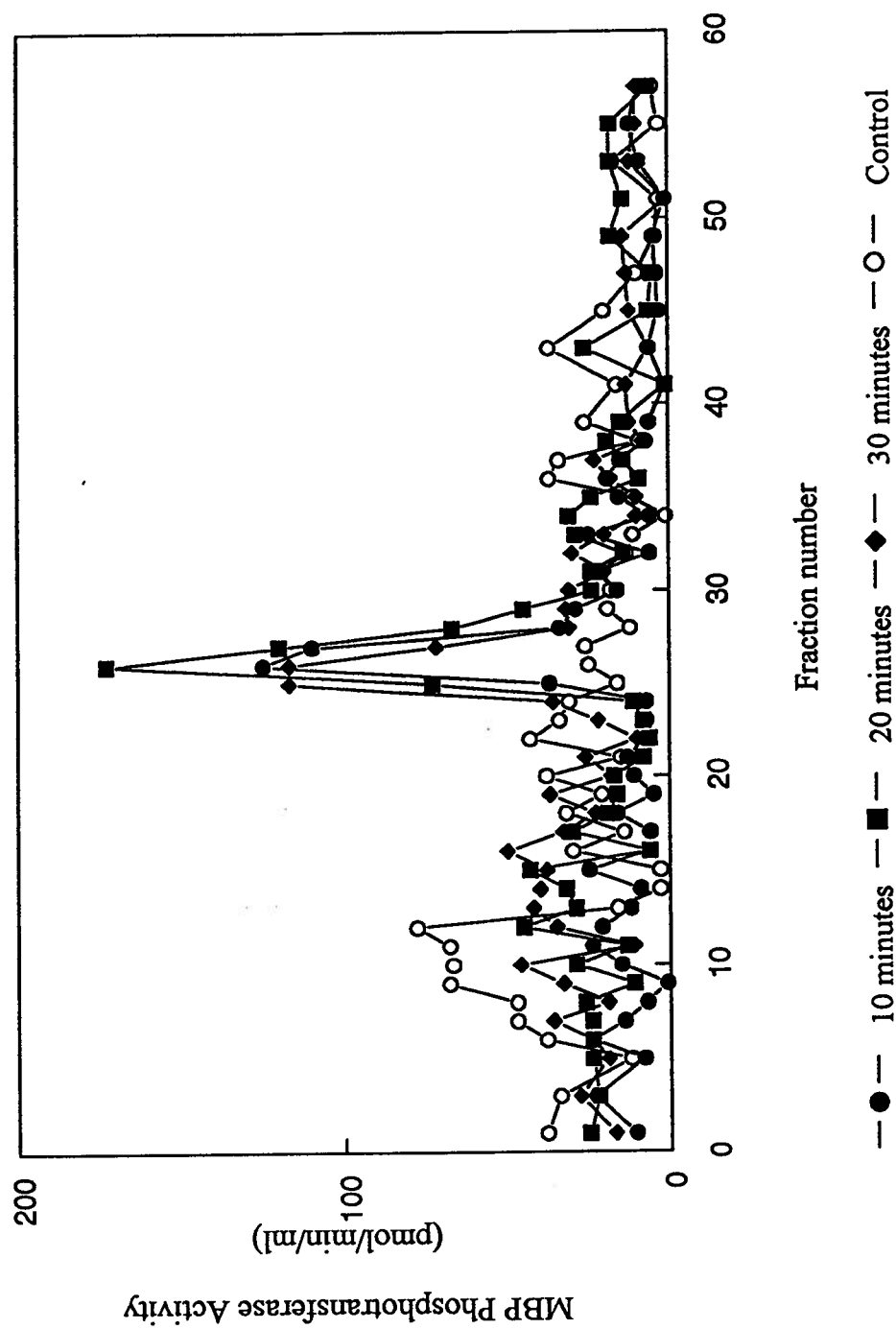


Figure 12

Immunoblotting of Mono Q Fractions **from Vanadyl Sulphate-stimulated Samples.**

Selected Mono Q fractions from the 20 minute sample of the time-course experiment were resolved as described in Fig 9. Both R1 and R2 primary antibodies were used to probe the fractions. With the R1 antibody, a light 42 kDa band was present in pooled fractions 17-23 and 24-29. In addition, a heavy band was present at 44 kDa for pooled fractions 17-23. With the R2 antibody, a heavy 42 kDa band and a lighter 38 kDa band were present for fractions 17-23. Both sets of pooled fraction corresponded to the peak of MBP-kinase activity seen in Fig. 11.

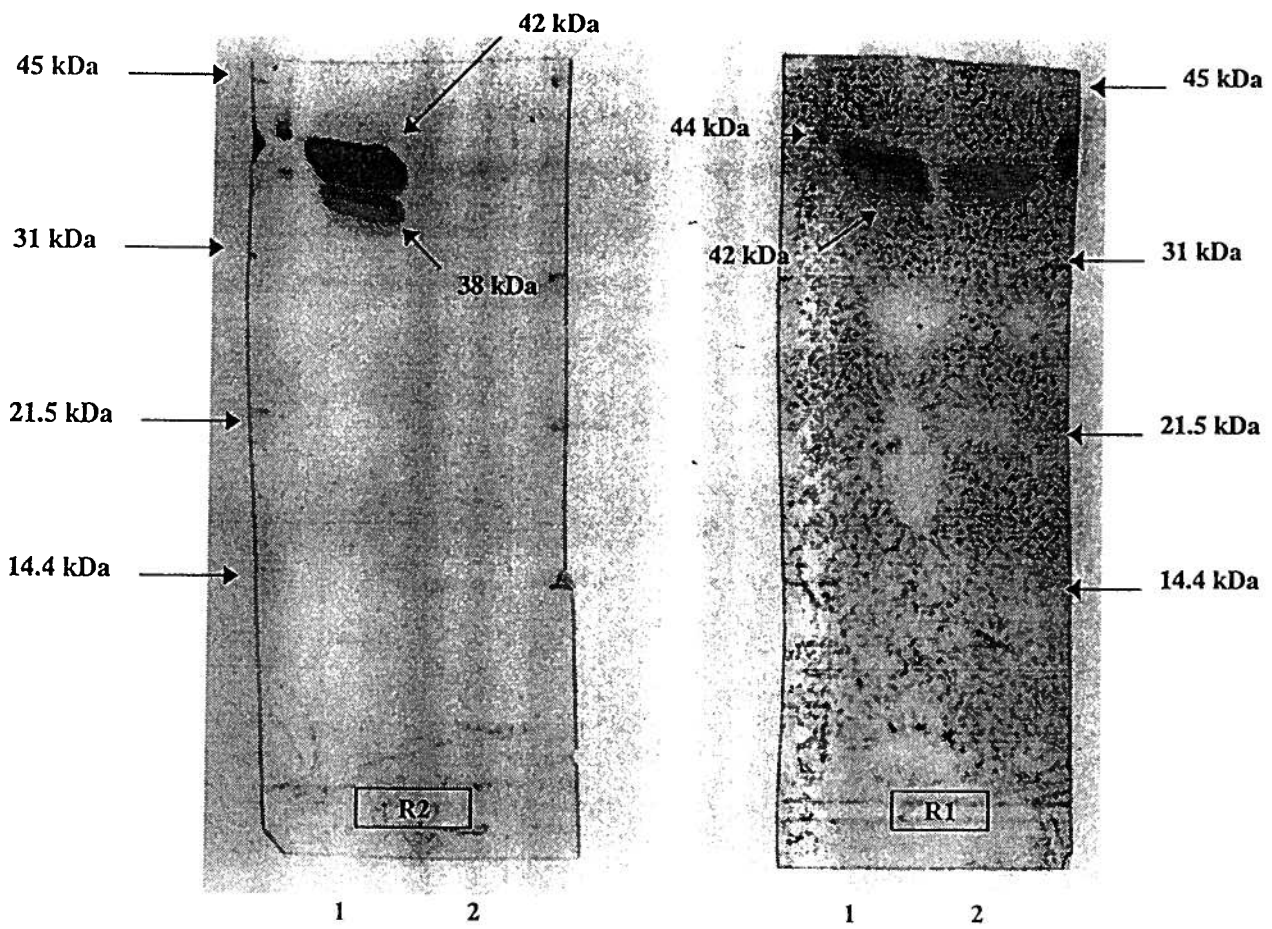


Figure 13**Time-Course for the Activation of Ribosomal Protein S6 kinases by Insulin.**

Isolated rat adipocytes were incubated with 10 nM insulin for 0-30 minutes. The incubations were stopped with the addition of ice-cold homogenization buffer and cellular extracts prepared and assayed for Ribosomal Protein S6 kinase activity, as described in Methods, using 40S ribosomal subunits as substrate. Time-points represent the average of two measurements.

Activation of Ribosomal Protein S6 kinases by 10 nM Insulin

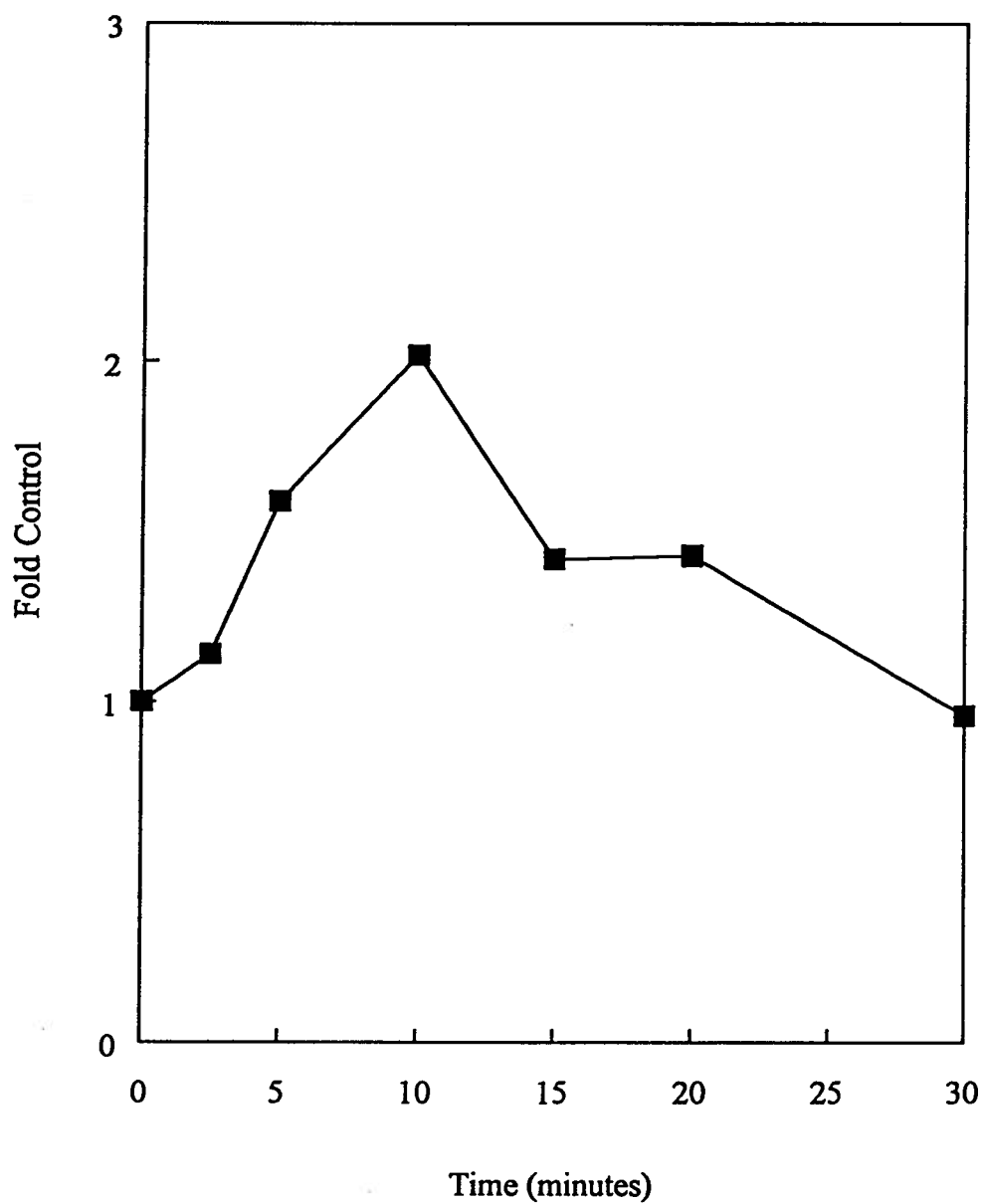


Figure 14**Time-Course for the Activation of Ribosomal Protein S6 kinases by Sodium Selenate.**

Isolated rat adipocytes were incubated with 1 mM sodium selenate for 0-27 minutes. Cellular extracts were prepared and assayed for Ribosomal Protein S6 kinase activity as previously described.. Each time-point is expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 51 fmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of Ribosomal Protein S6 kinases by 1 mM Sodium Selenate

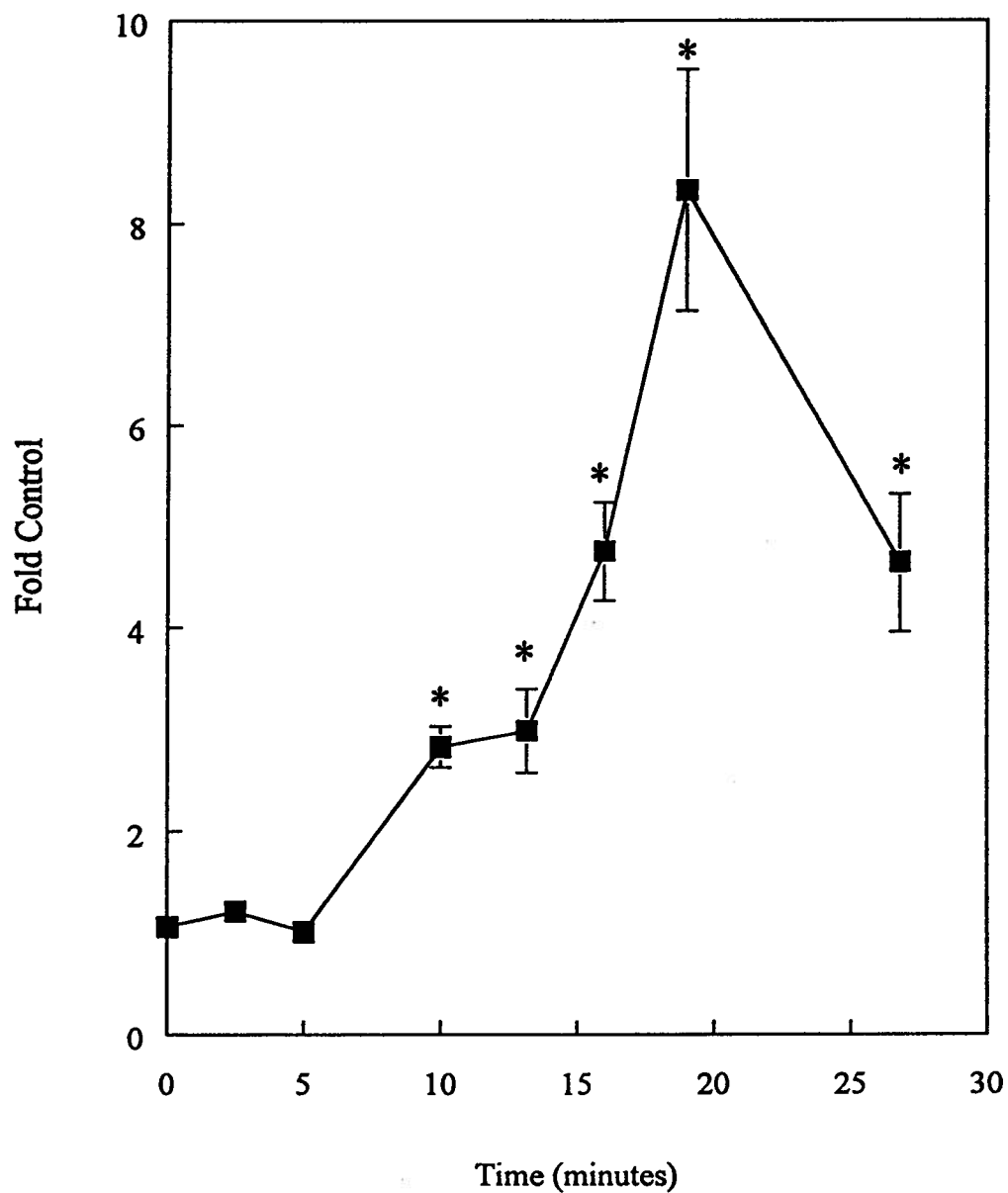


Figure 15**Time-Course for the Activation of
Ribosomal Protein S6 kinases by Vanadyl Sulphate.**

Isolated rat adipocytes were incubated with 1 mM vanadyl sulphate for 0-30 minutes. Cellular extracts were prepared and assayed for Ribosomal Protein S6 kinase activity as previously described. Each time-point represents the measurement of three separate samples. Each time-point is expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 87 fmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of Ribosomal Protein S6 kinases by 1 mM Vanadyl Sulphate

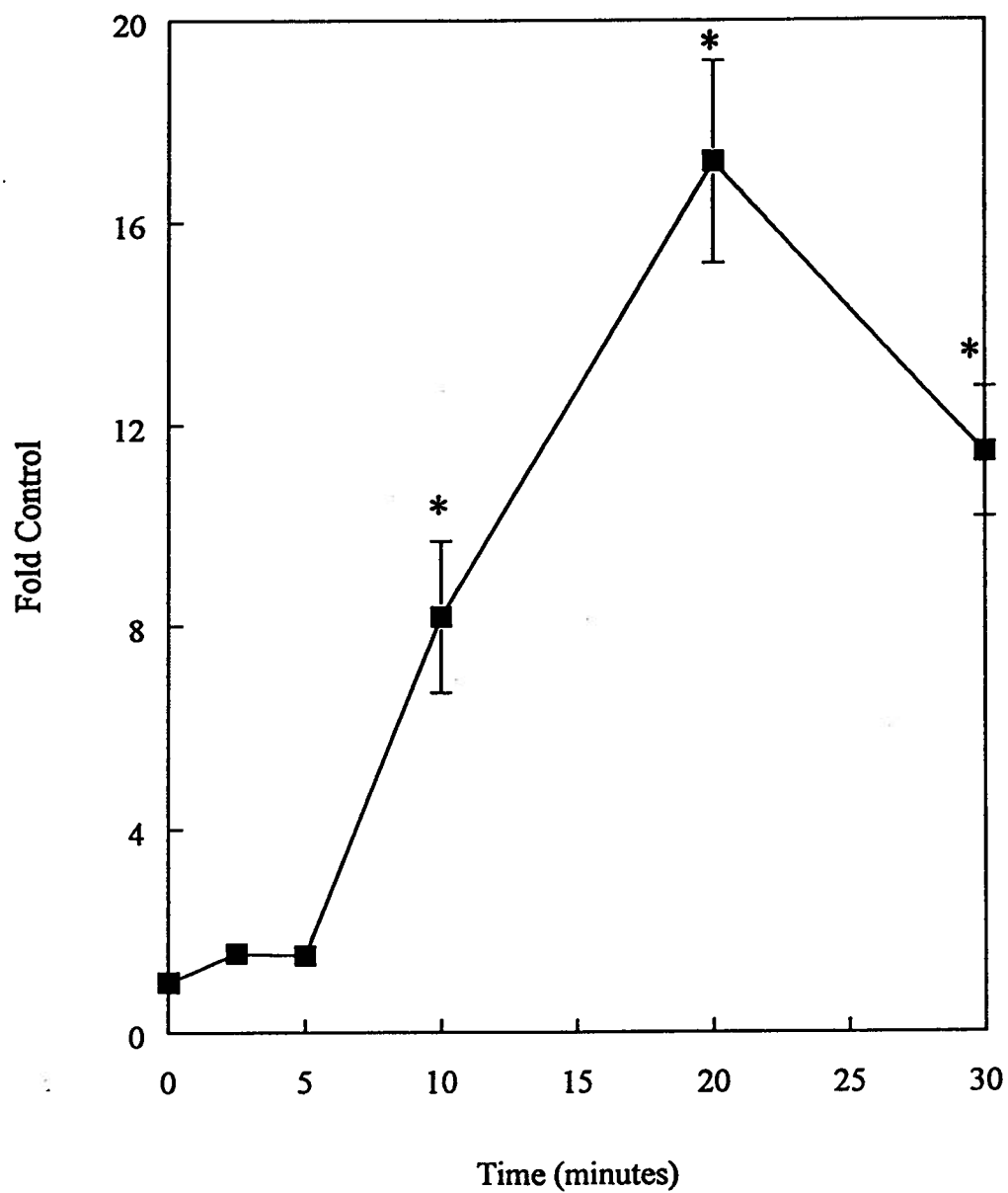


Figure 16

**Comparison of the Time-Course of Activation of Ribosomal Protein S6 kinase
by 10 nM insulin, 1 mM Vanadyl Sulphate, and 1 mM Sodium Selenate.**

Compilation of figures 13-15. * denotes significant difference from control ($p < 0.05$)

Ribosomal Protein S6 kinase Comparisons

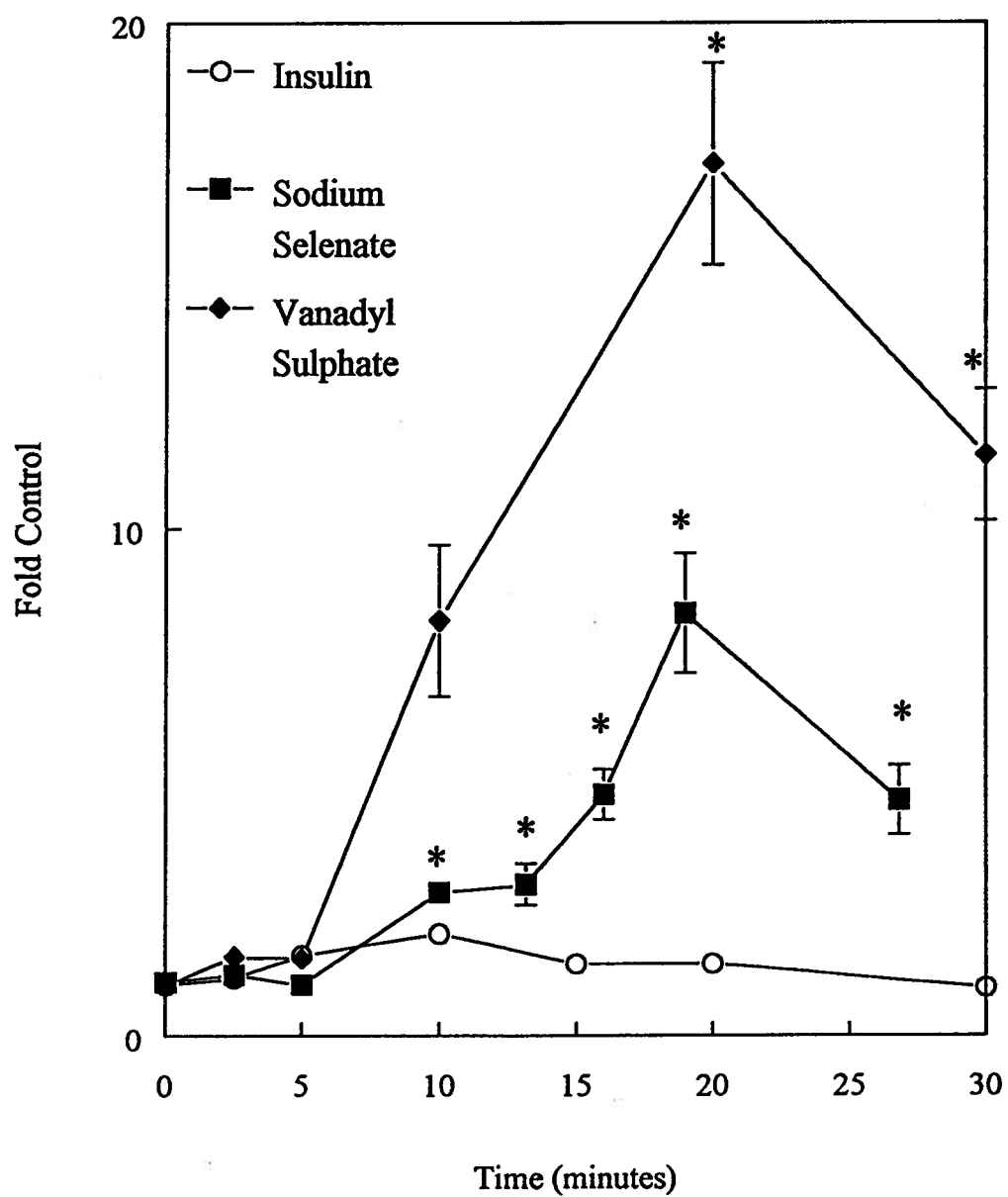


Figure 17**Dose-Response for the Activation of Ribosomal Protein S6 kinases by Insulin.**

Isolated rat adipocytes were incubated for 10 minutes with the insulin concentrations 1 pM-100 nM. Extracts were prepared and assayed as previously described in Fig. 13. Significant stimulation of Ribosomal Protein S6 kinase activity was present at 1, 10 and 100 nM concentrations. Each time-point was expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 4 fmol/min/mg. * denotes significant difference from control ($p < 0.05$)

Activation of Ribosomal Protein S6 kinases by Insulin

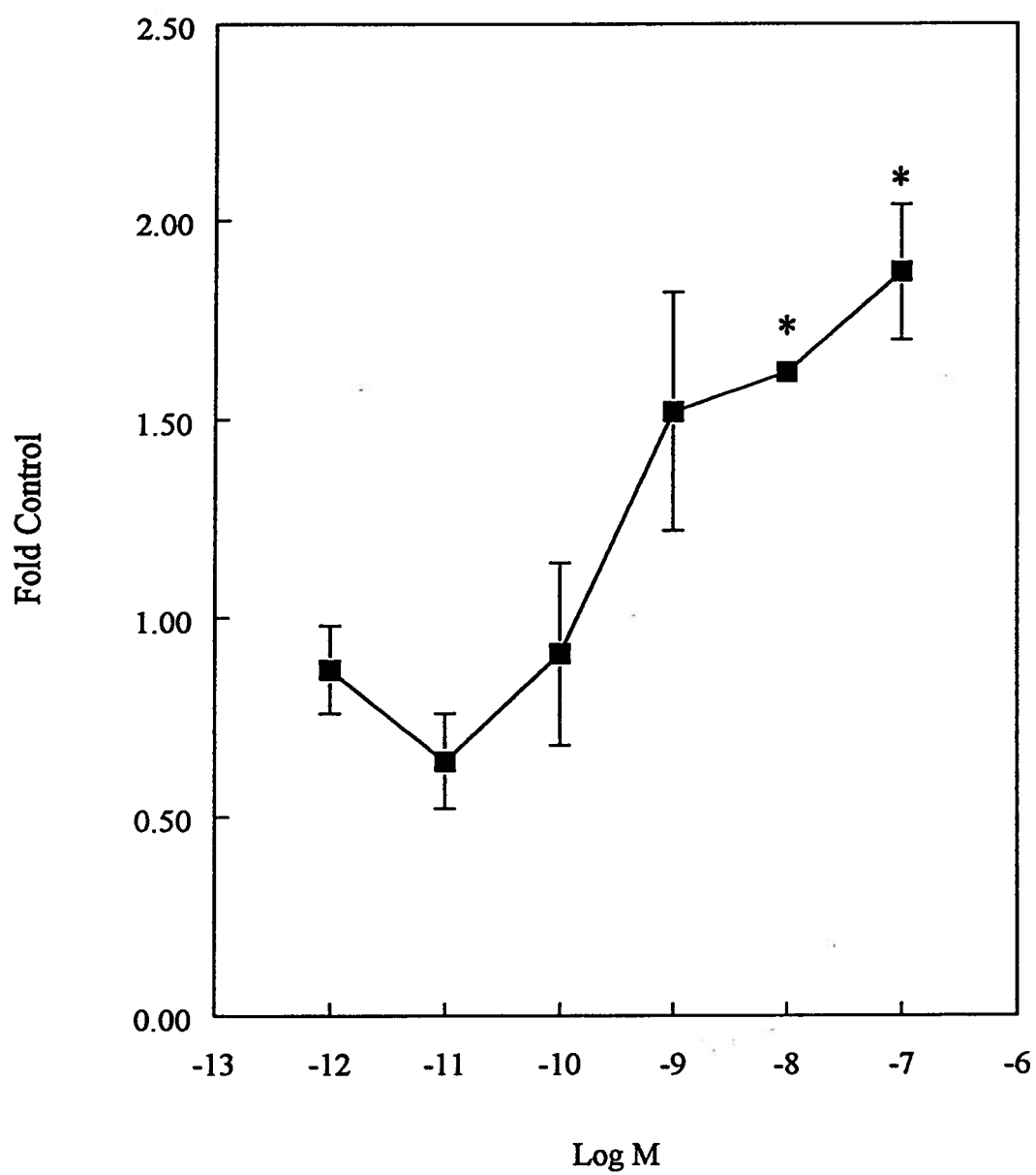


Figure 18**Dose-Response for the Activation of
Ribosomal Protein S6 kinases by Sodium Selenate.**

Isolated rat adipocytes were incubated for 10 minutes with 100 nM-10 mM sodium selenate. Extracts were prepared and assayed as previously described. Ribosomal Protein S6 kinase activity was present at 100 μ M, 1 mM, and 10 mM sodium selenate concentrations. Each time-point was expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 157 fmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of Ribosomal Protein S6 kinases by Sodium Selenate

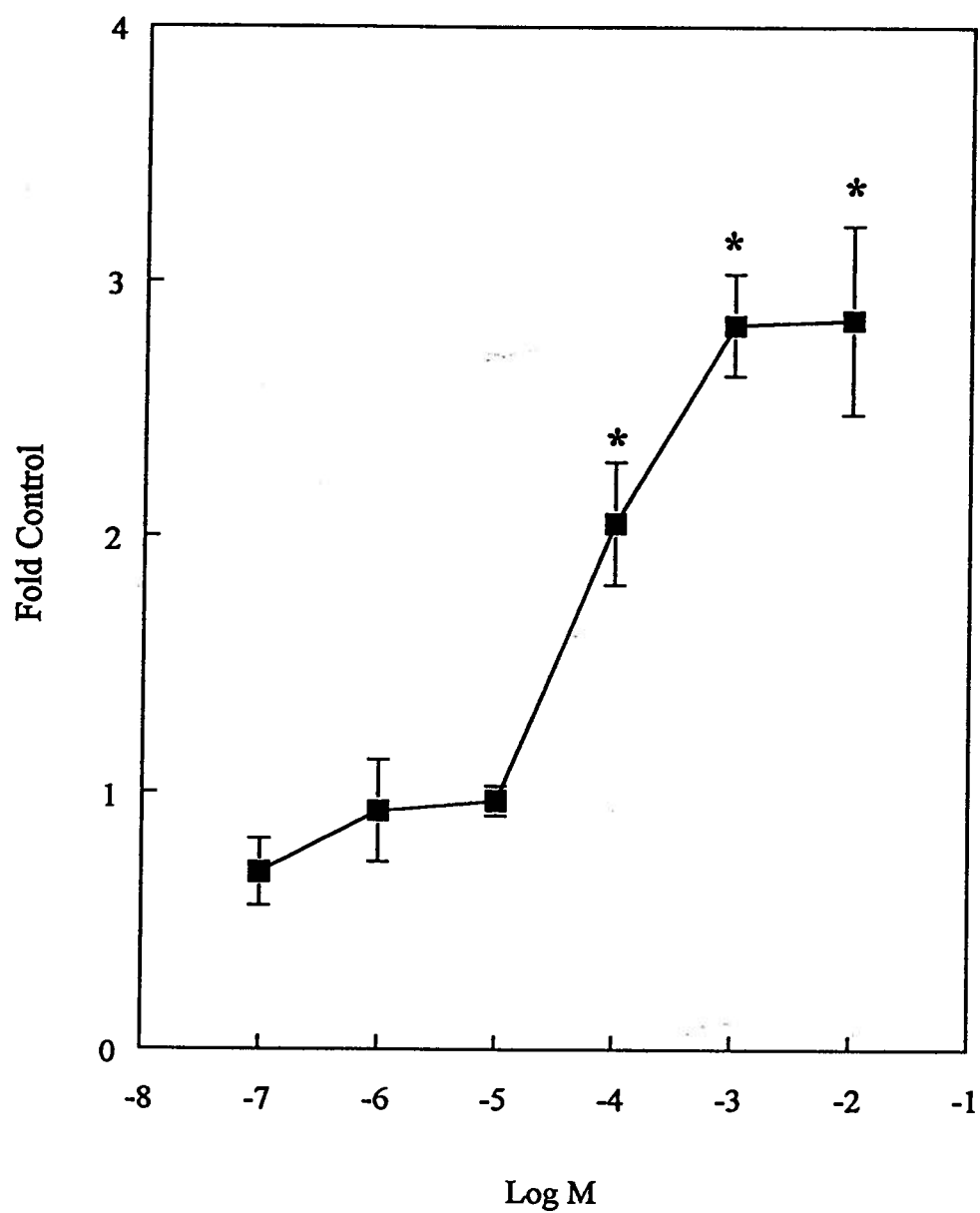


Figure 19**Dose-Response for the Activation of
Ribosomal Protein S6 kinases by Vanadyl Sulphate.**

Isolated rat adipocytes were incubated for 20 minutes with vanadyl sulphate concentrations ranging from 10 μ M-10 mM. Ribosomal Protein S6 kinase activity followed a similar pattern to MBP kinase dose-response curve in that it was not present at less than 1 mM vanadyl sulphate. Higher concentrations again resulted in lower activity. Each time-point is expressed as mean $\pm$ S.E.M. of fold-control activity (n=3). The actual control value was 87 pmol/min/mg. * denotes significant difference from control (p<0.05)

Activation of Ribosomal Protein S6 kinases by Vanadyl Sulphate

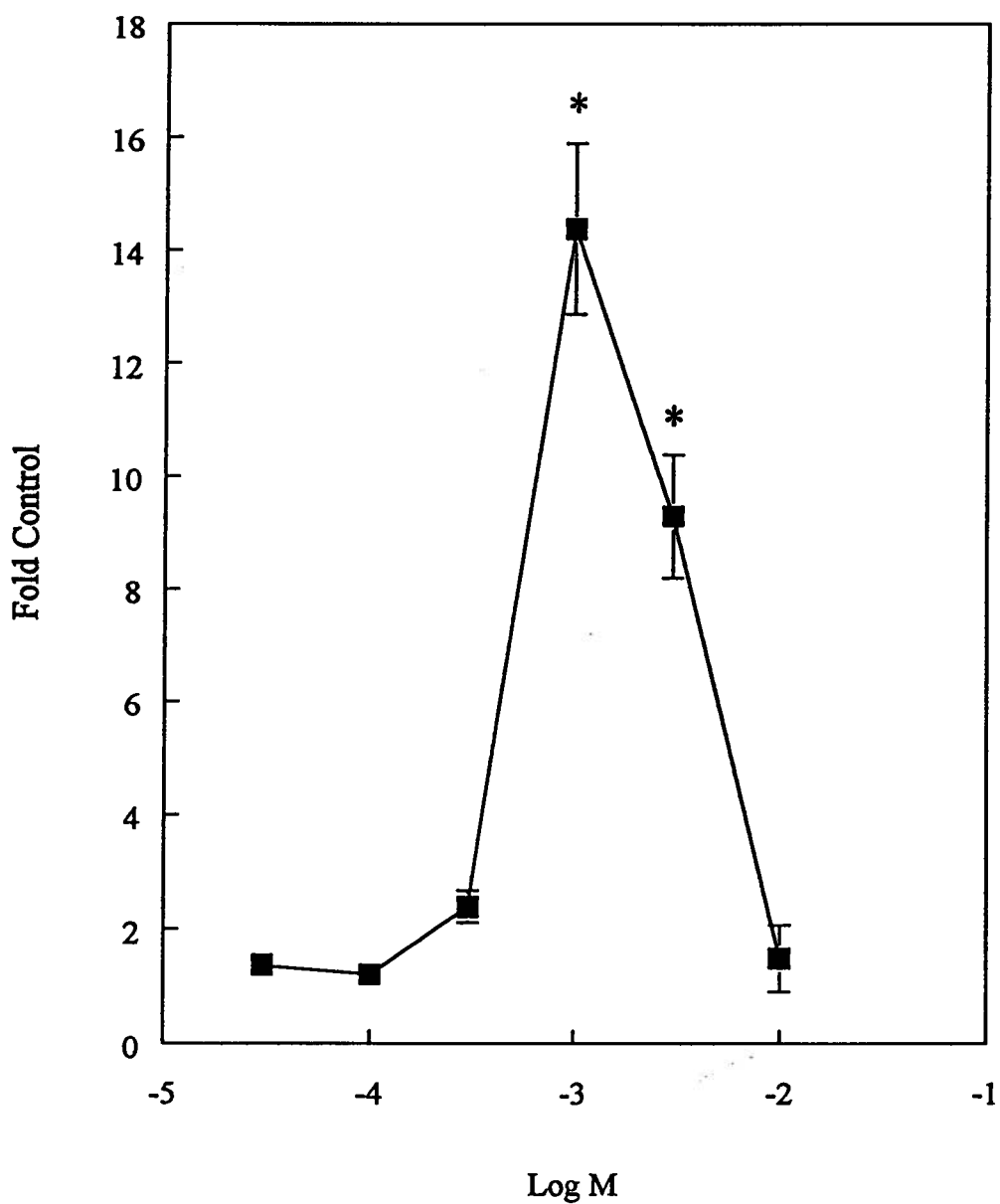


Figure 20

Mono Q Elution Profile of Sodium Selenate-stimulated S6-peptide kinase Activity.

Mono Q fractions from both sodium selenate-treated and control samples obtained in Fig. 8 were assayed for S6-peptide kinase activity using a phosphocellulose paper assay with a synthetic S6-peptide as substrate. Peak one was the largest peak observed with 22 pmol/min/ml of activity. Data points represent a single experiment.

S6-peptide Kinase Mono Q Profile

Sodium Selenate

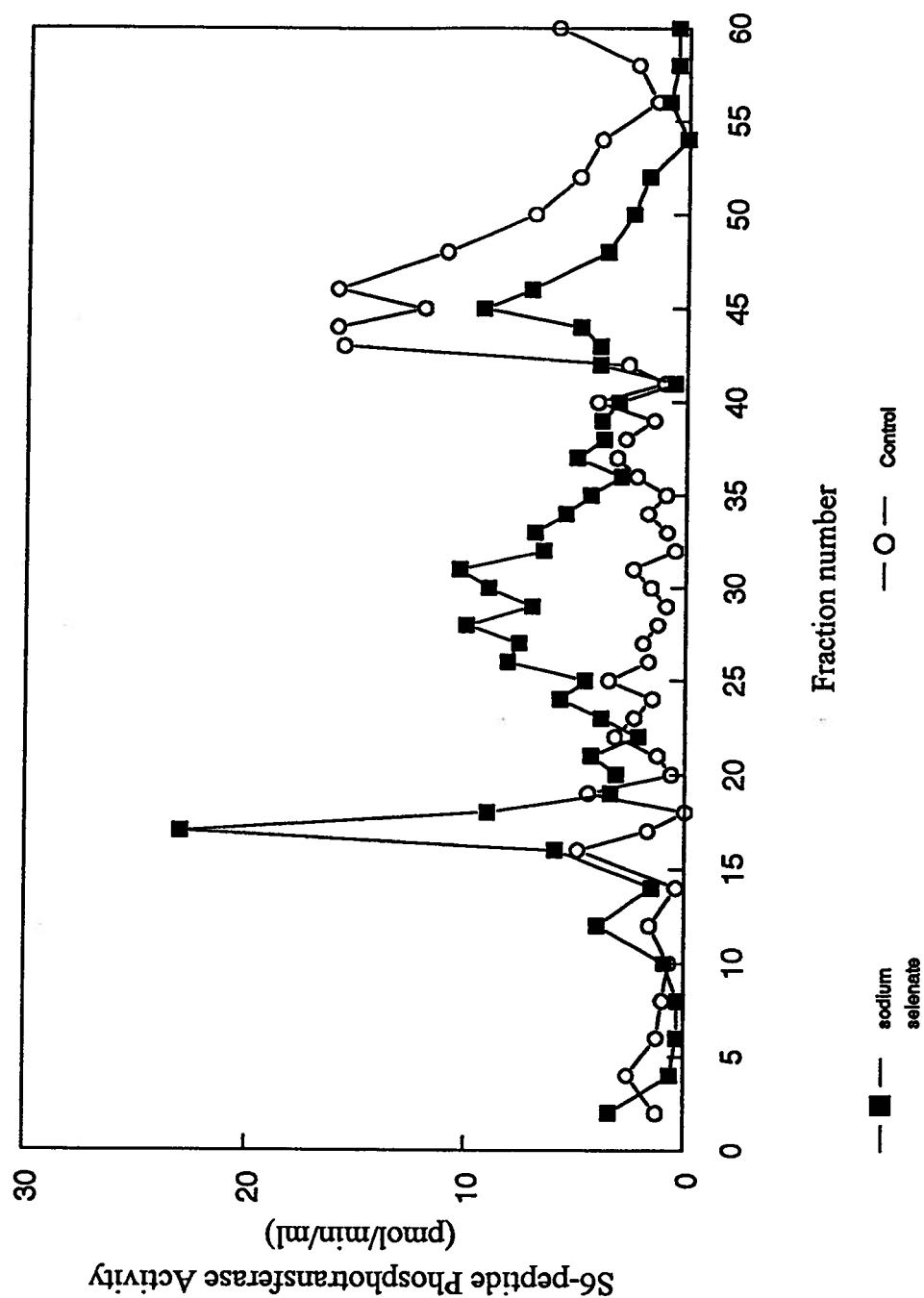


Figure 21**Mono Q Elution Profile of Sodium Selenate-stimulated
Ribosomal Protein S6 kinase Activity.**

The fractions from figure 20 were assayed for Ribosomal Protein S6 kinase activity using ribosomal 40S subunits as substrate. Data points represent a single experiment.

Activation of Ribosomal Protein S6 kinase by Sodium Selenate

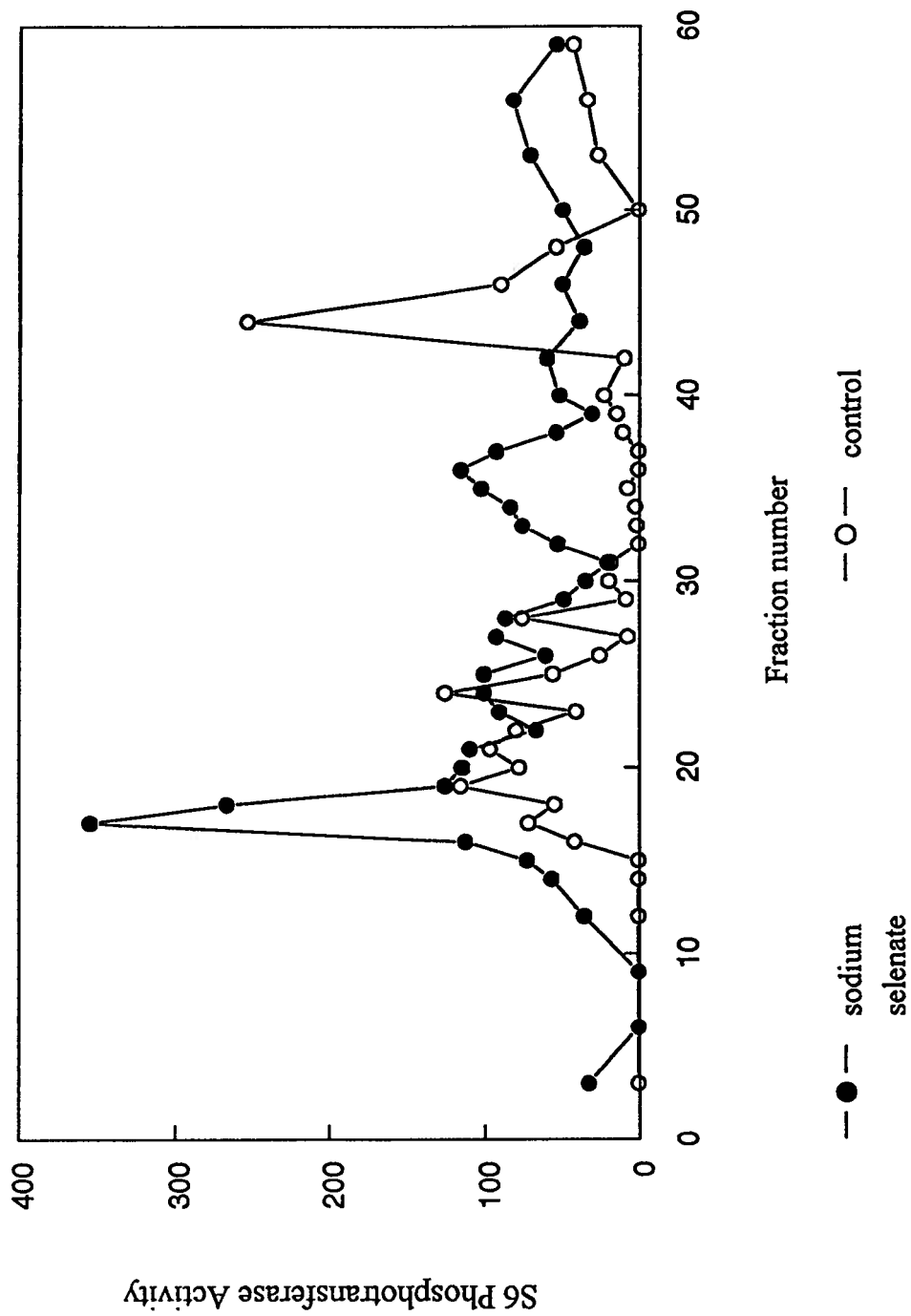


Figure 22

**Mono Q Elution Profile of Vanadyl Sulphate-stimulated
S6-peptide kinase activity Time-Course.**

Mono Q fractions used in Fig. 11 were also assayed for S6-peptide kinase activity as described in Fig. 20. Data points represent a single experiment.

Activation of S6-peptide Kinase by Vanadyl Sulphate

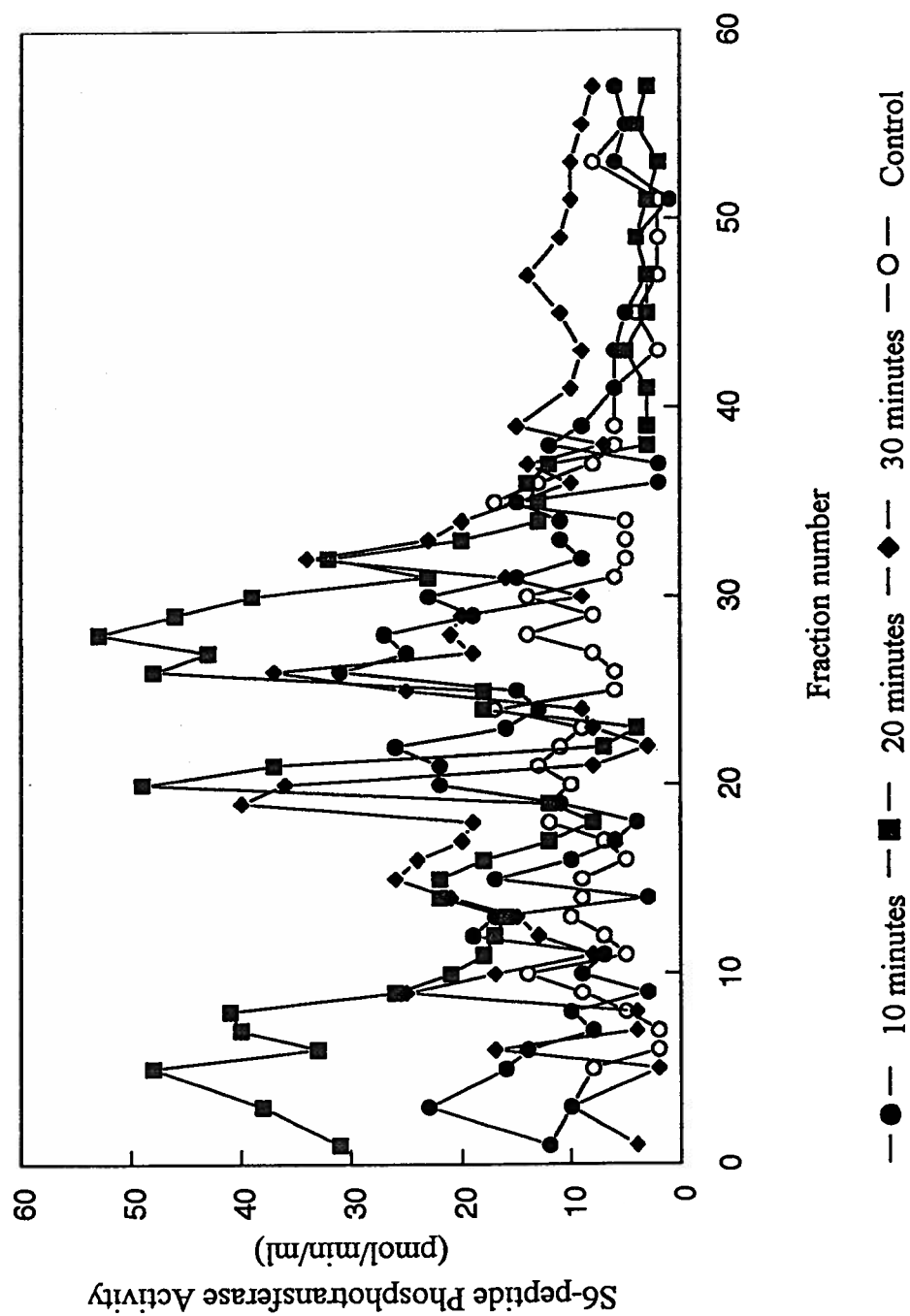


Figure 23**Mono Q Elution Profile of Vanadyl Sulphate-stimulated
Ribosomal Protein S6 kinase Activity Time-Course.**

The fractions from Fig. 22 were assayed for Ribosomal Protein S6 kinase activity using ribosomal 40S subunits. Data points represent a single experiment.

Activation of Ribosomal Protein S6 kinase by Vanadyl Sulphate

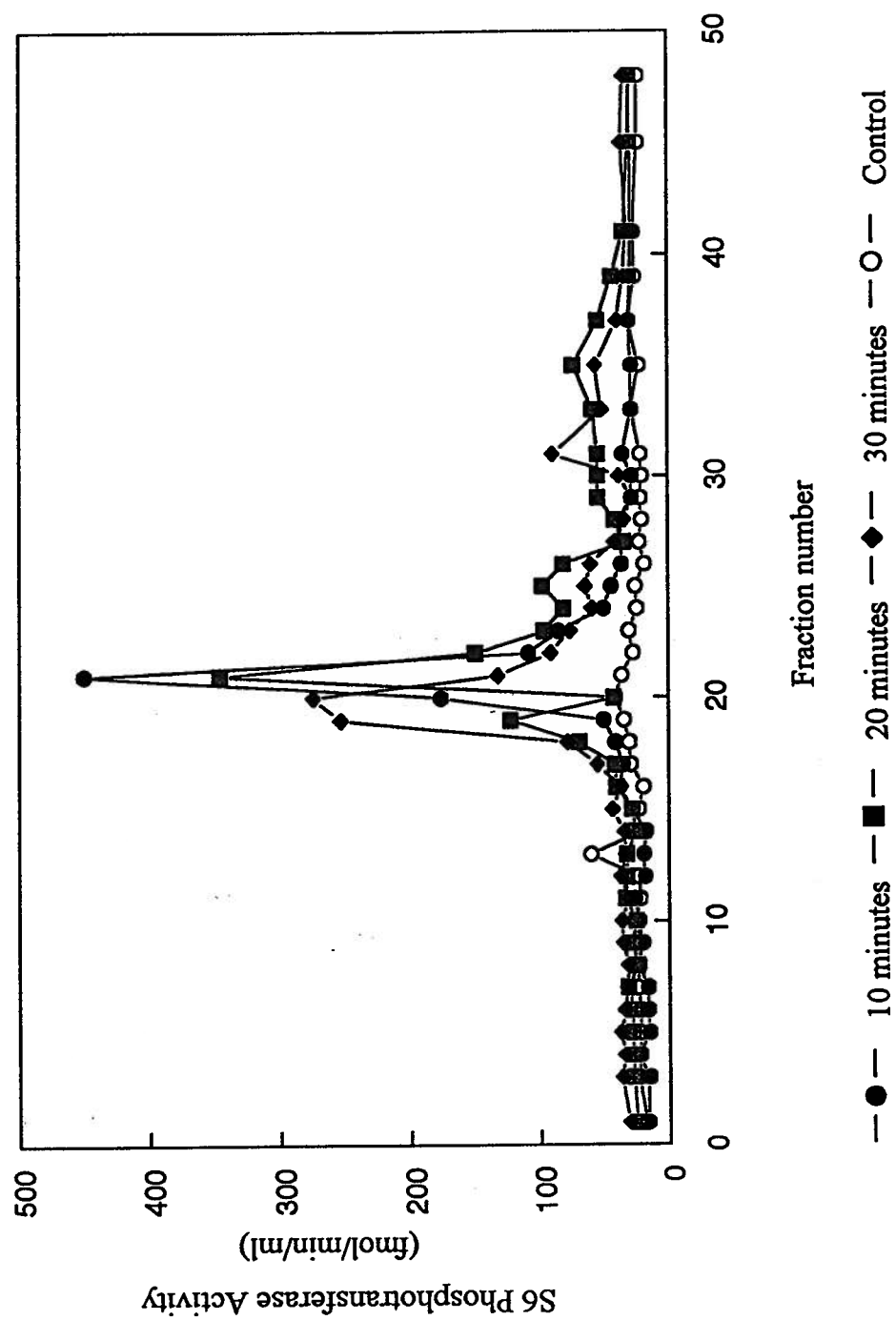


Figure 24

Immunoblotting of Mono Q Fractions from Sodium Selenate and Vanadyl Sulphate-treated samples with S6K-CT Antibody.

Selected Mono Q fractions from the sodium selenate (A) and vanadyl sulphate (B) treated samples were resolved on 11 % SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with S6K-CT antibodies. For the sodium selenate-treated samples (A) a 52 kDa band was present in fraction 18, associated with peak one of Ribosomal Protein S6 kinase activity, as well as a 55 kDa band in fractions 19 and 20, also associated with peak one. With the vanadyl sulfate-treated samples (B) a 55 kDa band was present in the pooled fractions 17-23. Lane assignments are shown below.

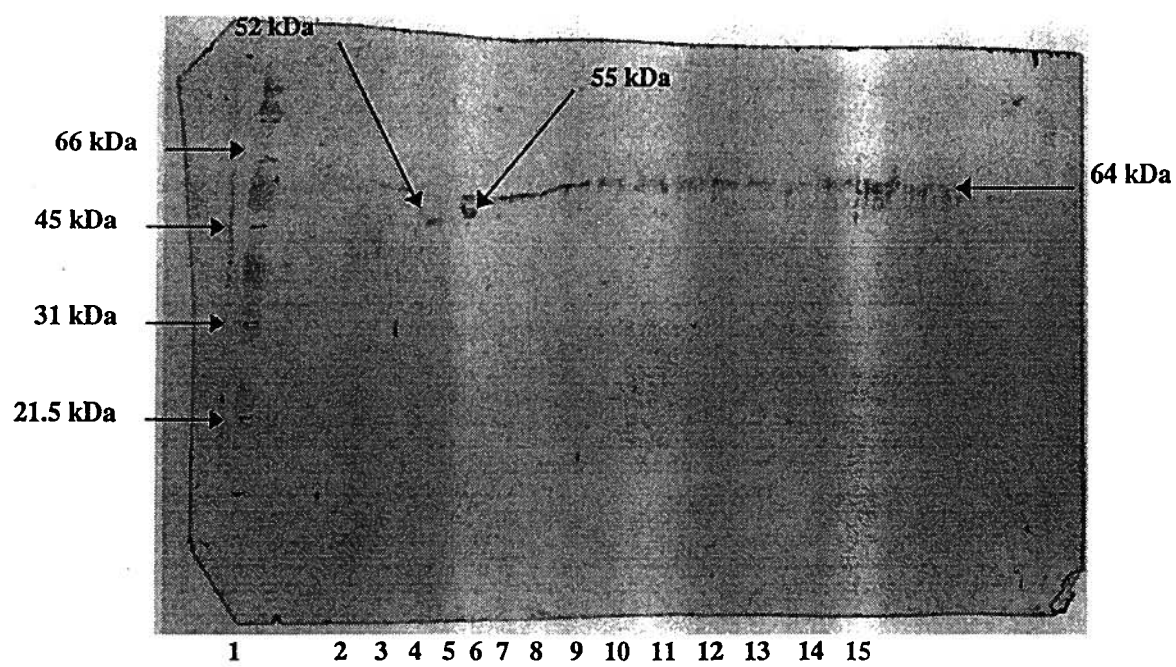
Sodium selenate-treated samples (A)

<u>Lane</u>	<u>Fraction No.</u>
1	molecular weight standards
2	16
3	17
4	18
5	19
6	20
7	21
8	22
9	26
10	27
11	28
12	31
13	37
14	38
15	39

Vanadyl sulphate-treated samples (B)

<u>Lane</u>	<u>Fraction No.</u>
1	molecular weight standards
2	24-29
3	17-23
4	3-5

A)



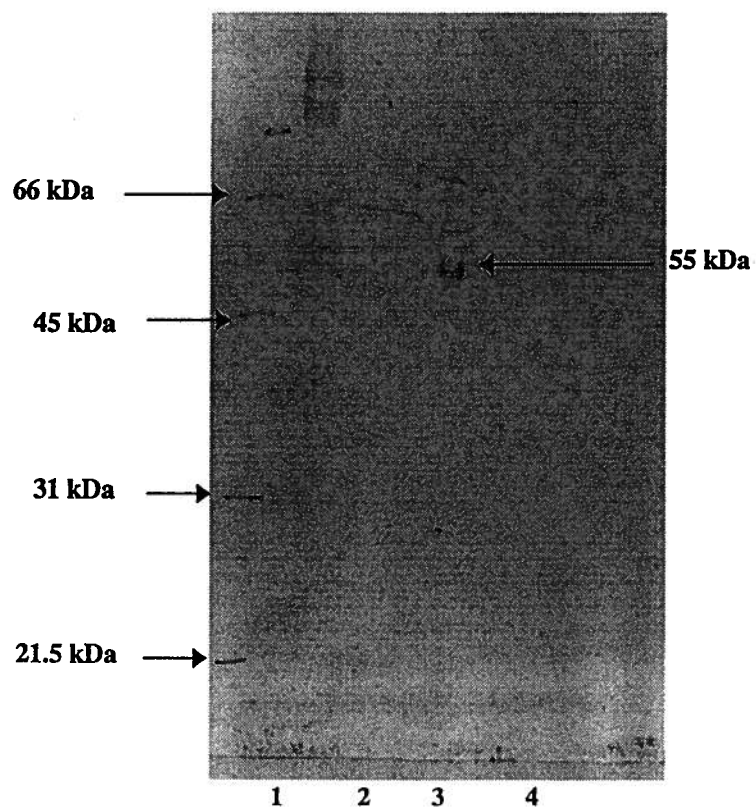
B)

Figure 25**Immunoblotting of Sodium Selenate-treated samples using the ECL detection system.**

Mono Q fractions from sodium selenate-treated samples were resolved as in Fig. 24 but the bands were visualized using the ECL detection system. A) A 68 kDa band was present in fractions 17-20 probed with the S6K-III antibody. B) Using the S6K-CT antibody on the same fractions, a 62 kDa band was present in fractions 17-21. Lane assignments are shown below.

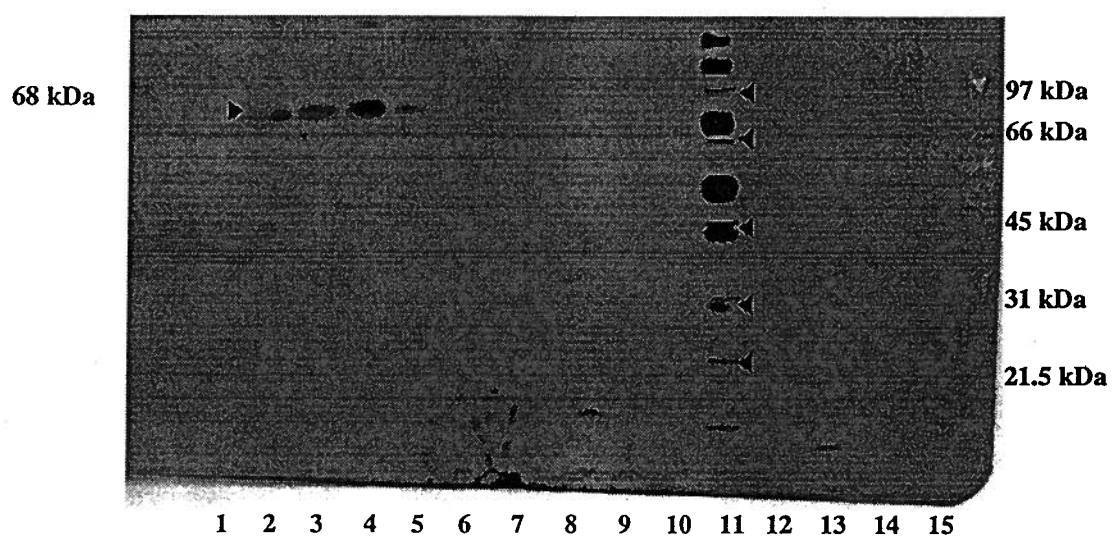
S6K-III Antibody (A)

<u>Lane</u>	<u>Fraction No.</u>
1	16
2	17
3	18
4	19
5	20
6	21
7	22
8	26
9	27
10	28
11	molecular weight standards
12	31
13	37
14	38
15	39

S6K-CT Antibody (B)

<u>Lane</u>	<u>Fraction No.</u>
1	16
2	17
3	18
4	19
5	20
6	21
7	22
8	26
9	27
10	28
11	molecular weight standards
12	31
13	37

A)



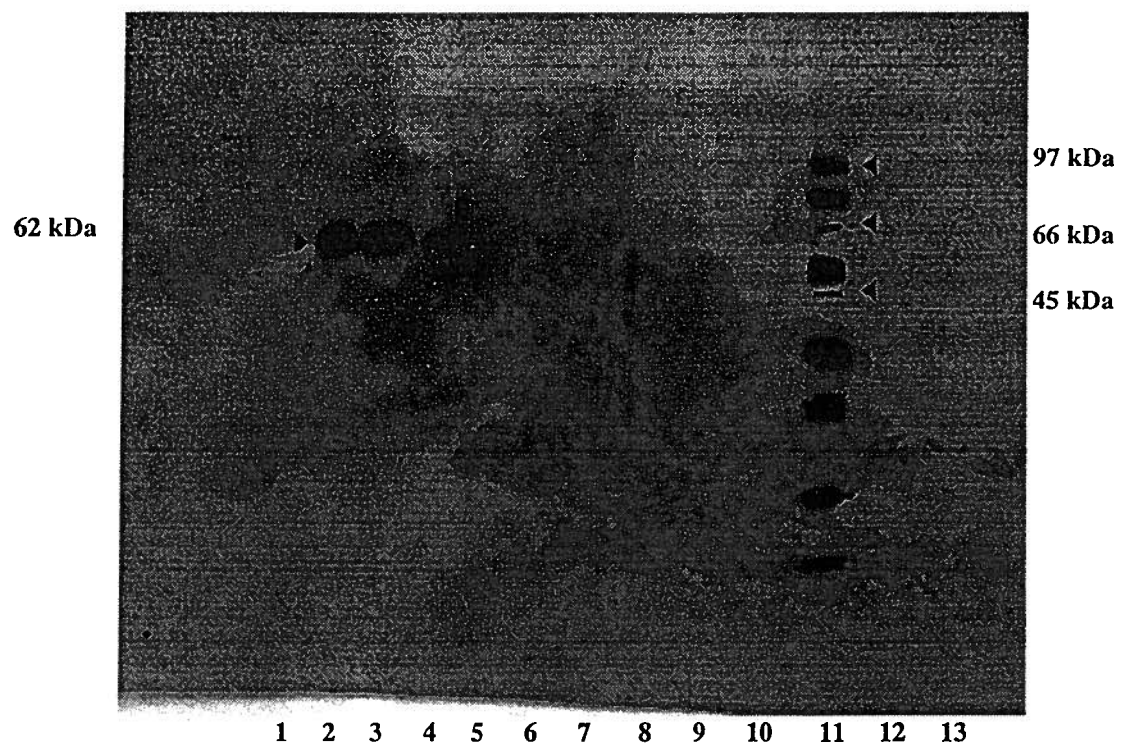
B)

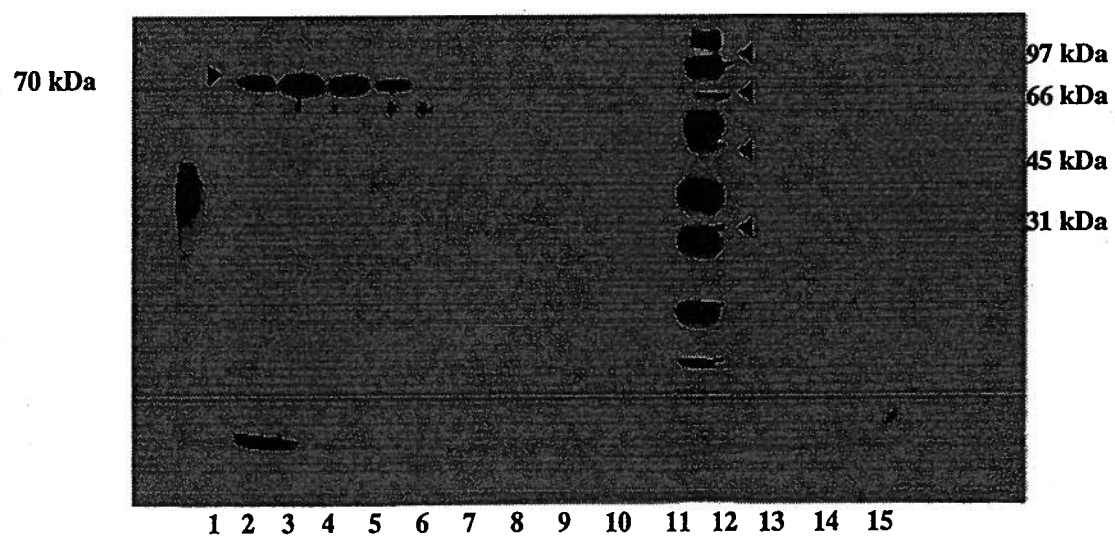
Figure 26

Immunoblotting of Vanadyl Sulphate-treated samples **using the ECL Detection System.**

Mono Q fractions from vanadyl sulphate-treated samples were resolved as in Fig. 24 but the bands were visualized using the ECL detection system. A) Samples probed with S6K-III antibody had a 70 kDa band in fractions 17-21. B) Using the S6K-CT antibody, a 69 kDa band was present in fractions 17-20. Lane assignments are shown below.

<u>Lane</u>	<u>Fraction No.</u>
1	16
2	17
3	18
4	19
5	20
6	21
7	22
8	26
9	27
10	28
11	molecular weight standards
12	31
13	37
14	38
15	39

A)



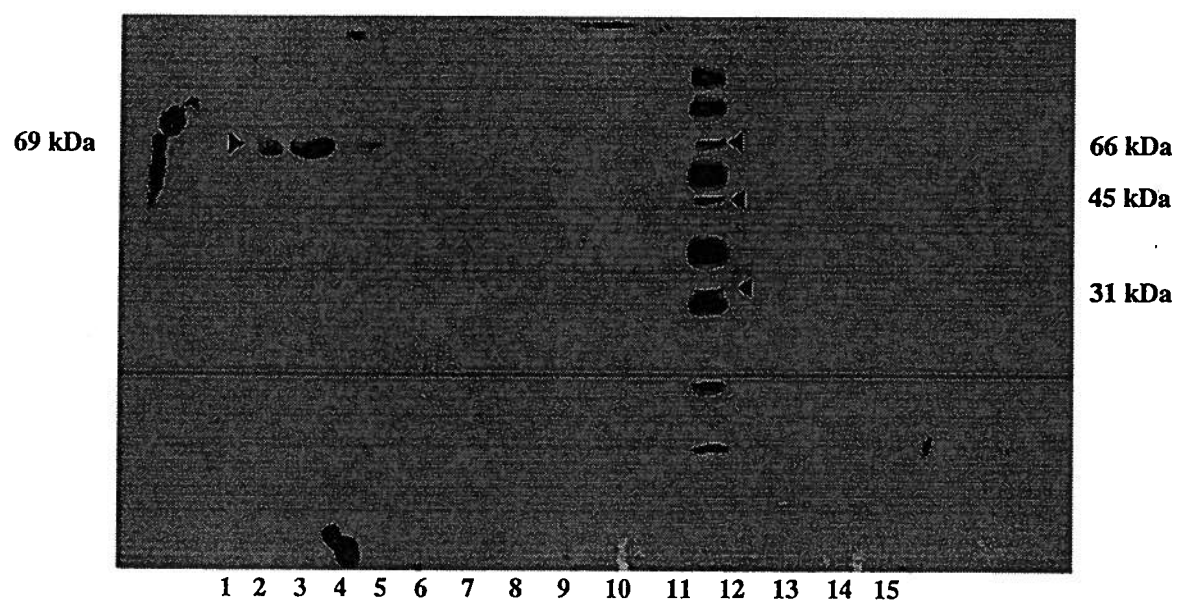
B)

Figure 27**Effects of Genistein on MBP kinase Activity.**

Extracts from adipocytes incubated with 10 nM insulin (5 minutes), 1 mM sodium selenate (10 minutes), 1 mM vanadyl sulphate (20 minutes), or untreated controls in the presence (diagonal stripes) or absence (solid) of 200 nM genistein were assayed for MBP kinase activity.

Effects of Genistein on MBP kinase Activity

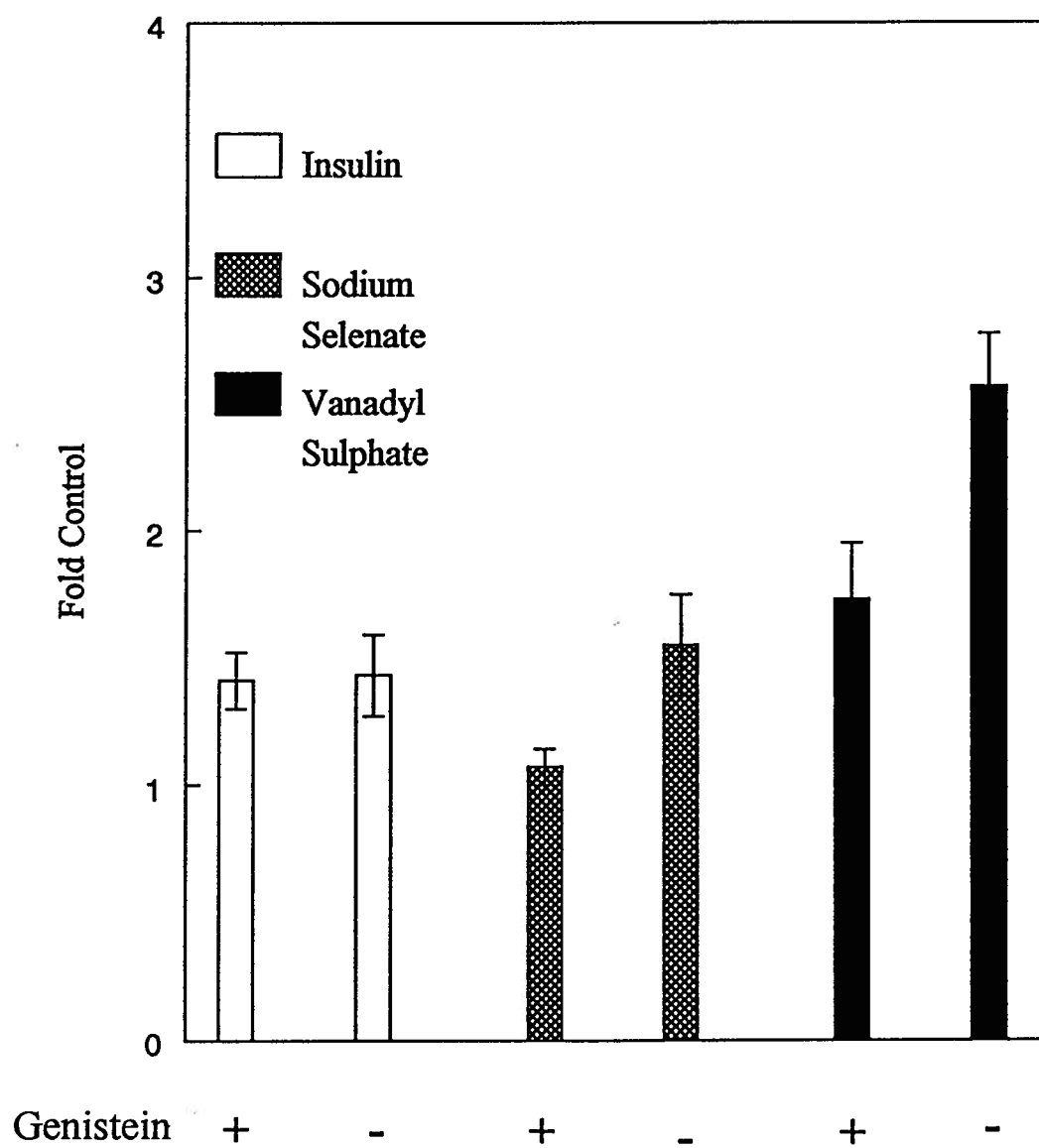


Figure 28**Effects of Genistein on Ribosomal Protein S6 kinase Activity.**

The extracts prepared in figure 27 were assayed for Ribosomal Protein S6 kinase activity.

* denotes significant difference from samples not treated with genistein ($p < 0.05$)

Effects of Genistein on Ribosomal Protein S6 kinase Activity

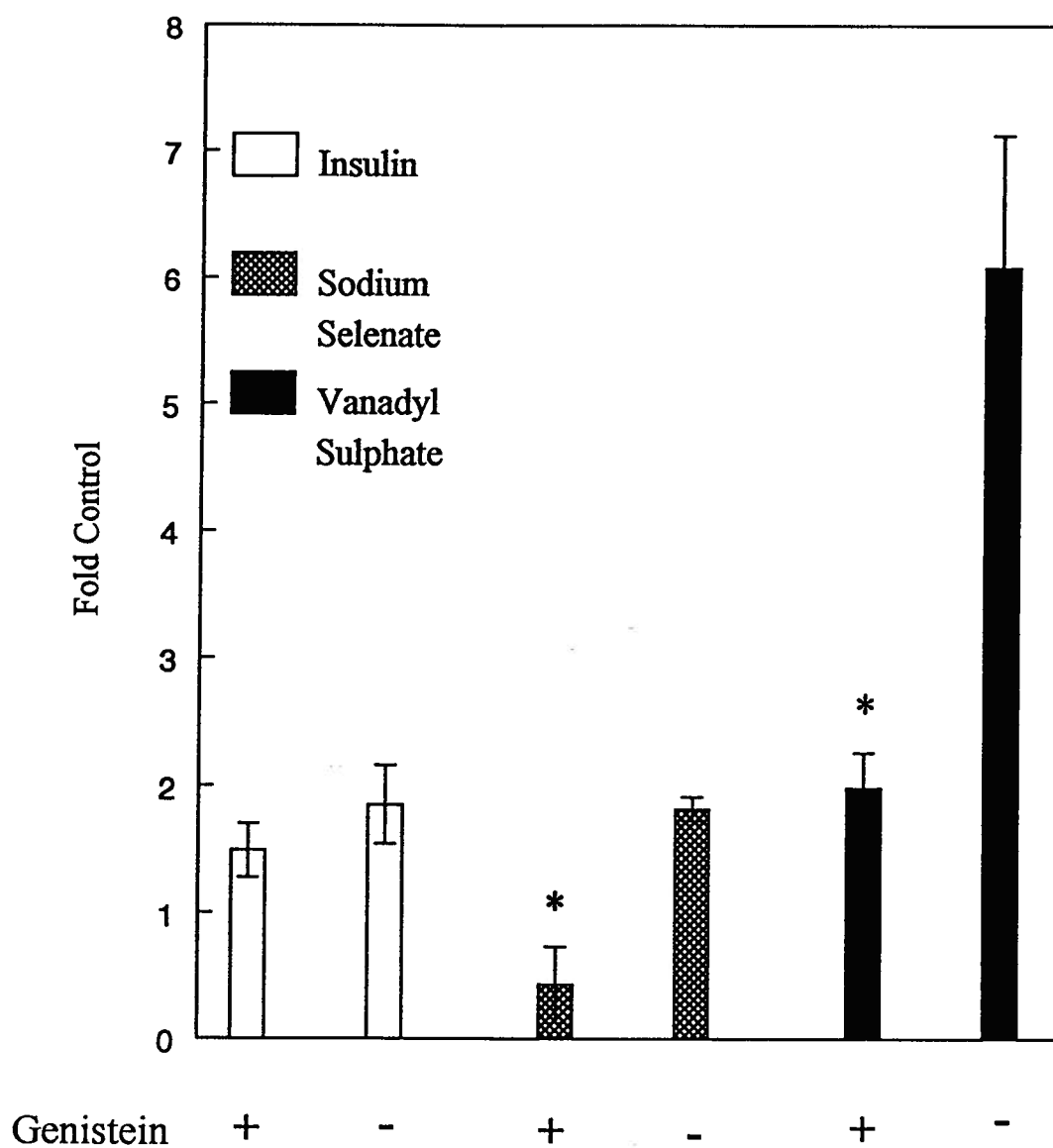


Figure 29**Effects of Rapamycin on MBP kinase activity of isolated rat adipocytes
stimulated with 1 mM sodium selenate or 1 mM vanadyl sulphate.**

Isolated rat adipocytes pre-incubated for 30 minutes with 50 ng/ml rapamycin or vehicle were stimulated with sodium selenate or vanadyl sulphate for 20 minutes. Cellular extracts from these cells were then measured for MBP kinase activity.

Effects of Rapamycin on MBP kinase Activity

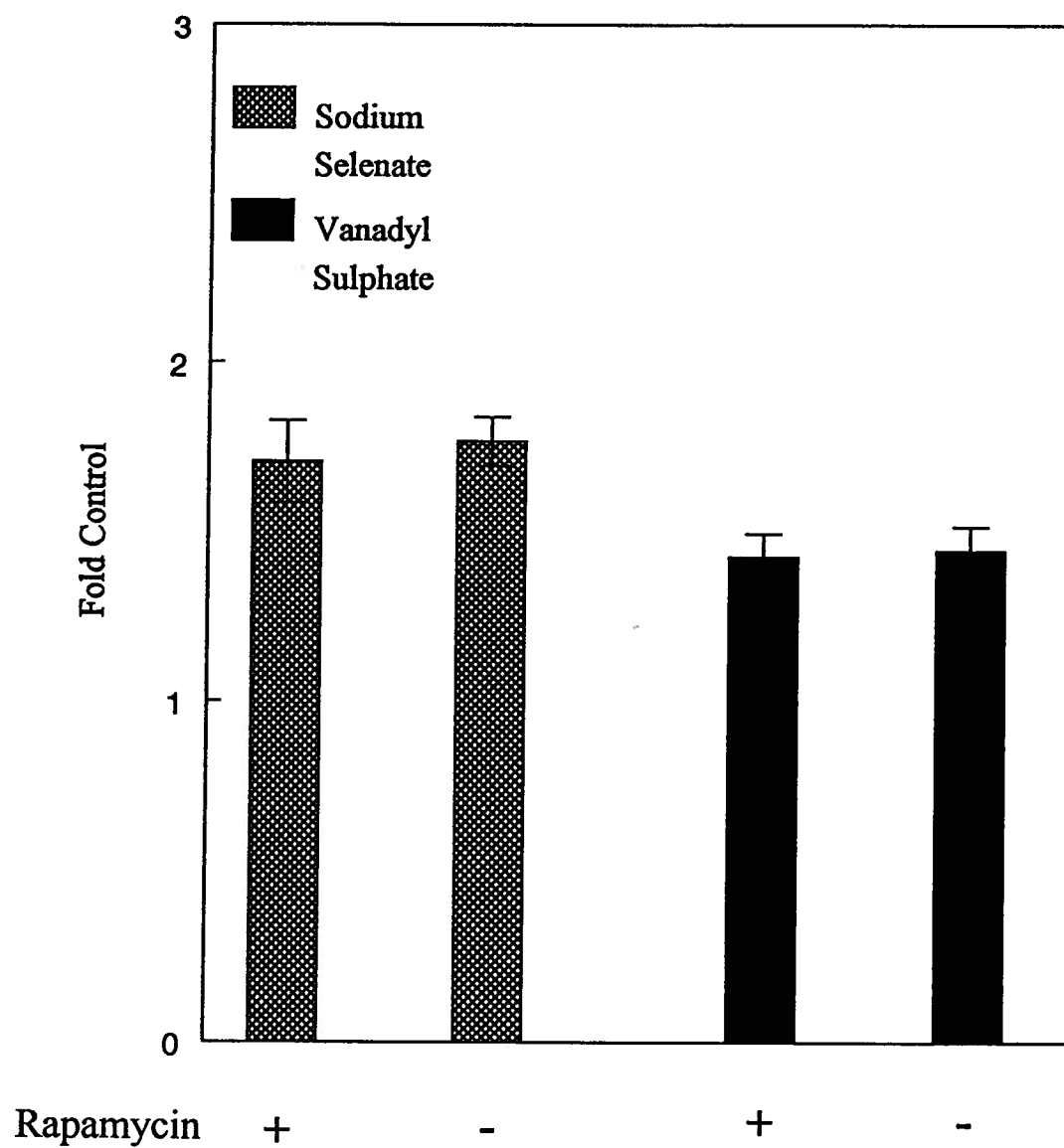
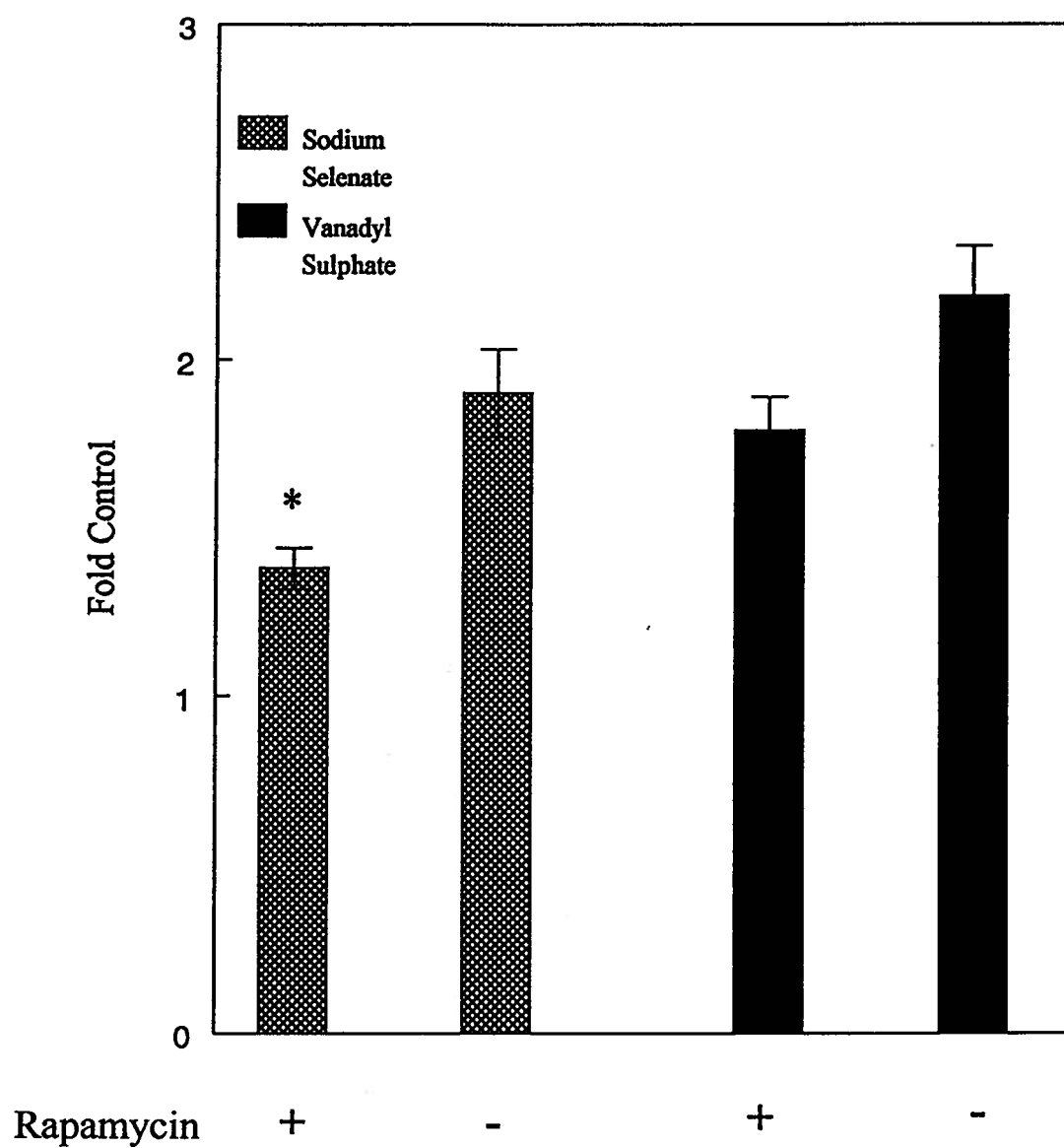


Figure 30

Effects of Rapamycin on Ribosomal Protein S6 kinase activity
of isolated rat adipocytes stimulated
with 1 mM sodium selenate or 1 mM vanadyl sulphate.

Cellular extracts prepared in figure 29 were also assayed for Ribosomal Protein S6 kinase activity. * denotes significant difference from samples not treated with rapamycin ($p < 0.05$)

Effects of Rapamycin on Ribosomal Protein S6 kinase Activity



DISCUSSION

In this study, we have used the isolated rat adipocyte as the system in which the insulin-mimetic properties of vanadium and selenium were investigated. Rodbell (1964) was the first to establish a consistent protocol for the isolation of rat adipocytes using collagenase digestion. The isolated rat adipocyte has since been used as a valuable tool in numerous studies involving the investigation of the biological effects of insulin and insulin-mimetic compounds. Adipocytes are sensitive to a wide variety of insulin-mimetic effects which include stimulation of glucose uptake, increased lipogenesis, and inhibition of lipolysis. Furthermore, the effects observed in isolated cell studies are not subject to the interference which might be present in the intact animal. In addition, the observations are made in a relatively uniform group of cells and are not compounded by the response of other cell types and systems to insulin or the insulin-mimetic agents being investigated. However, this system is not without its limitations. The observations made in isolated cells may not be fully representative of the physiological responses, which in the whole animal are brought through the concerted effects of neuronal, hormonal, and chemical factors that cannot be duplicated in isolated cells. In addition, the isolated cell may not be identical to its intact counterpart as it exists *in situ*. For example, the collagenase treatment of adipocytes may cause desensitization to insulin through the partial digestion of their insulin receptors. Although isolated adipocyte studies would be greatly complimented by intact animal studies, they but still remain a valuable tool in the initial investigation of the mechanism of action of compounds affecting complex systems provided that only a limited number of parameters are investigated.

I. MBP KINASES

Isolated rat adipocytes were incubated with relatively high concentrations of insulin (10 nM), sodium selenate (1 mM), and vanadyl sulphate (1 mM), for increasing amounts of time to 30 minutes in order to establish a time-course of activation for MBP kinase. In subsequent experiments we also established a dose-response for the above agents. MAP kinases have been shown to incorporate ^{32}P into MBP in a specific and linear fashion under controlled experimental conditions, thus making the phosphorylation of MBP an effective means of measuring MAP kinase activity (Erikson *et al.*, 1990; Boulton *et al.*, 1991). In all of our MBP kinase experiments we have used the phosphorylation of MBP as a means of quantitation of MAP kinase activity. This was done by scintillation counting of p-81 phosphocellulose which can bind phosphorylated MBP and other phosphoproteins through ionic interactions.

We observed maximal MBP kinase stimulation after 5 minutes of incubation with insulin, similar to that reported by Haystead *et al.* (1990) although in work done with 3T3-L1 adipocytes maximal MBP kinase activation occurred after 10 minutes (Ray and Sturgill, 1987). Other investigators working with Chinese Hamster Ovary (CHO) cells and rat 1 HIRc B fibroblasts have also shown a 5 minute maximal activation time (Dickens *et al.*, 1992; Boulton *et al.*, 1990). Tobe *et al.* (1992) injected insulin into the liver of the intact rat directly via the portal vein. They reported maximal stimulation of MAP kinase activity 4.5 minutes after injection. In our own laboratory, Hei *et al.* (1993) observed maximal MAP kinase activity in rat skeletal muscle 10 minutes after an i.v. injection of insulin into the intact rat. In a similar recent study, Zhou *et al.* (1993) have suggested the sequential activation of MAP kinase isoforms in rat skeletal muscle with one isoform, believed to be ERK1, being maximally stimulated 3 minutes after insulin injection while the other, believed to be ERK2, being activated after 10 minutes. The magnitude of the maximal MBP kinase stimulation in our study was ~1.7-fold-control which again was similar to that reported by

Haystead *et al.* (1990) who also used isolated rat adipocytes. In addition, in both our study and that of Haystead *et al.* (1990) the response of the cells was of a transient nature whereas the effect of insulin on cultured cells seemed to be of longer duration. Ray and Sturgill (1987) reported a 3.4-fold increase in 3T3-L1 adipocytes while in rat fibroblasts and CHO cells, both of which were induced to overexpress human insulin receptors, maximal MAP kinase stimulation was over 5-fold above control (Boulton *et al.*, 1990; Dickens *et al.*, 1992). The higher response of cultured cells could perhaps be due to a higher number of intact insulin receptors, which in the case of isolated adipocytes may have been damaged due to their exposure to collagenase and have therefore made the cells somewhat resistant to insulin. We used insulin concentrations ranging from 1 pM to 100 nM but only saw stimulation of MBP kinase activity at the 10 and 100 nM concentrations. Other investigators working with cultured cells, which are not affected by collagenase treatment, have also observed optimal effects on MBP kinase activity at 10 nM insulin with no effects present at concentrations below 1 nM (Boulton *et al.*, 1990; Dickens *et al.*, 1992). Therefore, we are within the insulin concentration range and time-course necessary to stimulate MAP kinase activity as reported previously in the literature for both isolated rat adipocytes and cultured cells making our isolated rat adipocytes a suitable tool to investigate the effects of sodium selenate and vanadyl sulphate on the insulin signal transduction pathway.

It is our understanding that the effects of selenium on MAP kinases have not been previously reported in the literature and that our study represents novel findings. We have demonstrated that incubation of isolated rat adipocytes with sodium selenate results in increases in MBP kinase activity in a time and dose-dependent manner up to a maximum of ~2-fold-control at 10 minutes of incubation with 1 mM sodium selenate. Ezaki (1990) was the first to report the *in vitro* insulin-mimetic effects of selenium. He demonstrated significant increases in glucose-transport, glucose-transporter translocation, cAMP phosphodiesterase activity, ribosomal S6 protein phosphorylation, and the tyrosyl phosphorylation of a number of cellular proteins in isolated adipocytes following their incubation with 1 mM sodium

selenate. Both our study and that of Ezaki report the presence of the insulin-mimetic effects of selenium on adipocytes after nearly 30 minutes of incubation which is in contrast to the transient nature of effects seen with insulin. In addition, maximal MBP kinase stimulation in our study occurred after 10 minutes which corresponds to maximal glucose transport activity reported by Ezaki although these two events have not been linked. The possibility that the insulin-mimetic effects of sodium selenate are mediated via the IR through conformational changes caused by interactions with receptor sulfhydryl groups has been suggested. Although selenium compounds have been reported to interact with glucocorticoid receptors through their sulfhydryl groups, they were shown to play an inhibitory role. Also these receptors are structurally and functionally different from insulin receptors (Tashima *et al.*, 1988). In addition, although Ezaki (1990) reported the tyrosine phosphorylation of a number of unidentified proteins in adipocyte cellular extracts following sodium selenate stimulation no stimulation of IRTK activity or inhibition of tyrosine phosphatases was observed. Pillay and Makgoba (1992) who reported the phosphorylation of the EGF receptor in A431 cells following sodium selenate treatment saw no tyrosine phosphorylation of IRS-1 in NIH 3T3-HIR-3.5 cells, although they also observed tyrosine phosphorylation of a number of proteins in response to selenium. The above two studies suggest that the insulin-mimetic properties of selenium are perhaps mediated through a post insulin-receptor mechanism and are most likely not due to inhibitory effects of sodium selenate on tyrosine phosphatases. The above investigators have suggested that, based on the lack of tyrosine phosphatase inhibition by sodium selenate and the large number of tyrosine-phosphorylated proteins observed in these studies, sodium selenate may be activating an unidentified tyrosine kinase(s) which, based on our study, may result in the activation of MBP kinases and other downstream ser/thr-protein kinases.

We constructed a dose-response curve for the stimulation of MBP kinase activity with selenium by incubating isolated adipocytes for 10 minutes with concentrations of sodium selenate ranging from 100 nM to 10 mM. The optimal concentration of sodium selenate in

our study was 1 mM, the same as that reported by Ezaki (1990) for both glucose uptake and cAMP phosphodiesterase activity, although Ezaki reported effects at lower concentrations (100 μ M) than we observed. We found no stimulation at lower concentrations and no significant increases in MBP kinase activity at 10 mM. Our results are also consistent with those of Pillay and Makgoba (1992) who constructed a dose-response curve for tyrosine phosphorylation of the EGF receptor by incubating A431 cells with sodium selenate (concentrations ranging from 10 μ M to 10 mM). They also saw effects only at 1 and 10 mM sodium selenate with no stimulation of phosphorylation at lower concentrations. Interestingly, a recent report has shown the inhibition of the EGF receptor in A431 cells by MAP kinases through the activation of an unidentified phosphatase (Griswold-Prenner *et al.*, 1993). If sodium selenate also activates MAP kinases in A431 cells as we have seen in the adipocytes, the results of Pillay and Makgoba (1992) might have been somewhat attenuated. The sodium selenate concentrations used in both our study and the others mentioned would be considered high in a physiological setting. However, there are a number of compounds in the cell incubation buffer, including large amounts of BSA, which may bind to selenate and significantly decrease the free amounts available. Furthermore, unless sodium selenate is working through the IR or some other cell surface interaction, it must pass through the hydrophobic outer membrane of the adipocyte in order to interact with the intracellular machinery. This may require high levels of sodium selenate to drive the reaction and may also explain the longer incubation, as compared to insulin, necessary for the manifestation of maximal MAP kinase stimulation.

We also investigated the effects of vanadyl sulphate on MBP kinases in the isolated rat adipocyte. We found more than double the maximal MBP kinase stimulation observed in either the insulin or the sodium selenate samples with vanadyl sulphate maximal activation exceeding 5-fold control. The initial activation of MBP kinase activity by vanadyl sulphate occurred at 2.5 minutes of incubation, similar to that observed for insulin and sodium selenate, but like sodium selenate and unlike insulin, the stimulation was long-lasting.

Vanadium has been studied extensively both *in vivo* and *in vitro* as an insulin-mimetic agent. Numerous studies have shown its stimulation of glucose-transport and other insulin-mimetic effects on the isolated rat adipocyte. The stimulation of MBP kinase activity by vanadyl sulphate has not previously been shown in the rat adipocyte, although Scimeca *et al.* (1991) have conducted some work complementary to our study in the NIH-3T3-HIR 3.5 cells with sodium orthovanadate. They immunoprecipitated, using ERK-1-specific antibodies, a 44 kDa protein which was phosphorylated *in vivo* in response to a 5 minute incubation with 10 mM sodium orthovanadate, as opposed to the 1 mM concentration we used in our study with vanadyl sulphate. This protein was also shown to have significant MBP kinase activity following its immunoprecipitation from cells exposed to insulin or sodium orthovanadate. Therefore in agreement with the work of Scimeca *et al.* (1991) in the NIH-3T3-HIR 3.5 cells we also report the presence of a vanadyl sulphate-stimulated MAP kinase in the cytoplasmic extracts of isolated rat adipocytes. In our study we constructed a time-course of MBP kinase activation with vanadyl sulphate with 20 minutes as the optimal incubation period whereas Scimeca *et al.* (1991) presented a time-course for phosphorylation and activation only for immunoprecipitated protein with a longer incubation leading to increased phosphorylation and activation. In addition, they only used two concentrations of sodium orthovanadate, 10 mM for the intact cell incubations and 200 μ M for their time-course experiment. We constructed a dose-response curve ranging from 100 μ M to 10 mM vanadyl sulphate but did not observe any stimulation of MBP kinase activity at concentrations less than 1 mM. As with sodium selenate the presence of various substances within the media could be interacting with the vanadyl sulphate thus making it unavailable to the cell. Other investigators working with the insulin-mimetic effects of vanadium compounds on isolated rat adipocytes have also reported optimal insulin-mimetic effects with the use of high concentrations of vanadium compounds whereas low concentrations have been shown to prolong insulin-binding and action possibly through their inhibition of tyrosine phosphatase activity (Green, 1986; Heffez *et al.*, 1990; Fantus *et al.*, 1990). Interestingly, as we increased

the vanadyl sulphate concentration above 1 mM, we saw corresponding decreases in MBP kinase activity. There are a number of possibilities that can help explain this trend. One is that vanadyl sulphate is toxic at higher doses leading to cell death which would decrease the number of viable cells responsive to the effects of vanadyl sulphate. Another possibility may be that higher concentrations of vanadyl sulphate could potentially be forming polymeric forms of vanadium compounds (Wilsky, 1990) which may not be able to enter the cell in order to manifest their effects or may not have the biological effects that might be present in the monomeric forms. A third possibility may involve the non-specific stimulation or inhibition of unknown factors involved in the regulation of the signal transduction pathway with higher concentrations of vanadyl sulphate which may lead to a decrease in the overall response.

Although not yet fully proven, vanadium compounds are believed to exert their insulin-mimetic effects through a post-receptor mechanism (Green, 1986). Vanadium compounds have been well established as potent phosphatase inhibitors (Swarup *et al.*, 1982; Lau *et al.*, 1989), which has been suggested as a potential mechanism for the phosphorylation and activation of cellular proteins. There is increasing evidence for a more active role for protein phosphatases in cellular functioning (Cohen and Cohen, 1989). Vanadium compounds may be mediating their effects in part via the inhibition of certain protein phosphatases. In a recent paper, Peraldi and Van Obberghen (1993) have provided evidence for the existence of a insulin-regulated tyrosine phosphatase acting as an inhibitor of MAP kinases in NIH-3T3-HIR fibroblasts. They have suggested that this phosphatase is constitutively active in unstimulated cells and is subject to ser/thr phosphorylation and deactivation following insulin or vanadate treatment. The stimulation of MAP kinases by okadaic acid (Haystead *et al.*, 1990; Gotoh *et al.*, 1990), an inhibitor of phosphatases 2A and 1 which are more specific towards phosphorylated serine and threonine residues, suggests a role for other potential phosphatases in the signal transduction cascade of insulin and growth factors. It would be very difficult to speculate the exact mechanism through which vanadyl

sulphate stimulated MBP kinase activity in the adipocytes in our study. The large magnitude of MBP kinase stimulation and the long duration of MBP kinase activation by vanadyl sulphate resemble that seen with okadaic acid, although vanadium compounds are considered primarily to be phosphotyrosine phosphatase inhibitors whereas okadaic acid is more specific towards ser/thr phosphatases. Nevertheless, the contribution of the phosphatase inhibitory effects of vanadium compounds should be taken into account with regard to the overall effects of vanadium. Based on the numerous *in vitro* and *in vivo* studies conducted on vanadium compounds, it seems likely that the insulin-mimetic effects of vanadium are brought about by more than one route. We have shown that MBP kinase which are involved in the ser/thr phosphorylation cascade of insulin are stimulated in a dose and time-dependent manner by vanadyl sulphate. Whether the activation of these MBP kinases is related to the metabolic effects of vanadium on the adipocyte remains to be shown.

II. S6 KINASES

The cellular extracts obtained for the MBP kinase time-course and dose-response experiments for insulin, sodium selenate, and vanadyl sulphate were also assayed for S6 kinase activity. Two different methods of measuring S6 kinase activity, both involving the phosphorylation of the substrate with ^{32}P from radio-labeled ATP, were used in our experiments. The first was a paper assay similar to that used for the measurement of MBP kinase activity except for the substrate used which was a synthetic peptide resembling parts of the ribosomal S6 protein (see methods for details). The major disadvantage with the use of this substrate is the possibility that it might be phosphorylated non-specifically by other kinases recognizing the ser/thr phosphorylation motif that it contains. In addition, the p70^{S6K} kinase family, one of the two major families of S6 kinases, are highly specific towards the S6 protein contained within ribosomal 40S subunits and may not phosphorylate the S6-peptide to the same extent as they would the 40S subunit. This, however, may also be

of benefit since it would allow us to somewhat distinguish the contribution of the two S6 kinase families in the overall S6 kinase activity of the samples. The S6-peptide also offers the advantage of allowing us to perform assays on a large number of samples in a short period of time. The S6-peptide substrate was only used to assay S6 kinase activity of the Mono Q fraction (to be discussed later). The second assay technique involved the use 40S ribosomal subunits purified from rat liver as substrate which are then isolated using gel electrophoresis and counted using liquid scintillation counting(see methods). This method is more specific towards S6 kinase activity than the first due to the presence of a physiological substrate whose phosphorylation is more specifically measured. It, however, requires considerable time and effort in both substrate preparation and the actual assay. All crude cellular extracts and Mono Q fractions were assayed for Ribosomal Protein S6 kinase activity using this technique.

In our study, we have demonstrated the time and dose-dependent stimulation of Ribosomal Protein S6 kinase activity following treatment of intact adipocytes with insulin, sodium selenate, and vanadyl sulphate.

The phosphorylation of ribosomal S6 protein through the activation of S6 kinases has been established as one of the most consistent biological effects of insulin (Smith *et al.*, 1980; Rosin *et al.*, 1981; Stefanovic *et al.*, 1986). We therefore felt it necessary to show and compare with others the effects of insulin in our system prior to the investigation of sodium selenate and vanadyl sulphate. With the time-course, we observed maximal stimulation after 10 minutes of incubation with more than a 2-fold increase over control. Our results were again similar to those of Haystead *et al.* (1990) who also reported a maximal stimulation of ~2-fold over control between 10-20 minutes. They reported increased activity over control even after 60 minutes of incubation whereas the Ribosomal Protein S6 kinase activity of our insulin-stimulated samples returned to basal levels after 30 minutes. We, however, used ribosomal 40S subunits as substrate in our assays, making them highly specific for Ribosomal Protein S6 kinase activity. The synthetic S6-peptide used by Haystead *et al.*

(1990) could potentially be phosphorylated by other ser/thr-protein kinases which may have longer duration of action than S6 kinases. The intact liver shows maximal S6 kinase activity 7.5 minutes after insulin injection which is also similar to our results (Tobe *et al.*, 1992). In rat skeletal muscle, S6-peptide kinase activity is maximally stimulated after 10 minutes whereas S6 kinase activity reaches its highest levels after 30 minutes. This could perhaps be due to the differential activation of the S6 kinase families present. In 3T3-L1 adipocytes, maximal p90^{rsk} activation was shown after 5 minutes of incubation with insulin which returned to basal levels after 15 minutes, whereas p70^{S6K} activation was highest after 15 minutes and remained high for 60 minutes (Fingar *et al.*, 1993). It is possible, therefore, that the two S6 kinases would have a different time-course of activation in other systems as well. The p90^{rsk} family has been shown to be downstream of the MAP kinases (Sturgill *et al.*, 1988). This is consistent with our time-course data in which the Ribosomal Protein S6 kinase activity detected was maximal at a later time-point than MBP kinase activity therefore suggesting the possibility that MBP kinase activation may be necessary for Ribosomal Protein S6 kinase stimulation, which may indicate a role for the p90^{rsk} family. However, in our dose-response experiments with insulin, Ribosomal Protein S6 kinase activation occurred at a 10 times lower concentration, 1 pM, than that necessary to stimulate MBP kinase activity, 10 pM. Therefore, at least some component of our observed Ribosomal Protein S6 kinase activity is stimulated in a MBP kinase-independent manner. The p70^{S6K} is believed to be the major S6 kinase family stimulated in mammalian cells and has been shown not to be downstream of MAP Kinases, making it a possible source of some of the Ribosomal Protein S6 kinase activity that we have observed (Blenis *et al.*, 1991; Chung *et al.*, 1991). Our Ribosomal Protein S6 kinase dose response is consistent with others showing detectable S6 kinase activity and S6 protein phosphorylation at 1 nM and maximal activation at 100 nM (Stefanovic *et al.*, 1986; Ezaki, 1990). Based on the similarity of our insulin time-course and dose-response to other investigators, our isolated rat adipocytes seem to be a suitable system

with which to investigate the effects of sodium selenate and vanadyl sulphate on Ribosomal Protein S6 kinase activity.

We have shown the stimulatory effects of sodium selenate on the Ribosomal Protein S6 kinase activity of isolated rat adipocytes in a time and dose-dependent manner. Most studies with selenium compounds are centred around their antioxidant role with only a few reports available on their insulin-mimetic properties. Ezaki (1990) has shown the phosphorylation of ribosomal S6 protein following the treatment of intact cells with 1 mM sodium selenate for 10 minutes in the presence of ^{32}P . We have shown an initial stimulation of Ribosomal Protein S6 kinase activity of approximately 3-fold control after 10 minutes of incubation with sodium selenate. Maximal stimulation of over 8-fold control occurred after 19 minutes of incubation. Ezaki (1990) did not construct a time-course or dose-response curve for S6 protein phosphorylation and did not measure Ribosomal Protein S6 kinase activity. In addition to our time-course, we also established a dose-response by measuring the Ribosomal Protein S6 kinase activity of the same extracts used for the MBP kinase dose-response. As with insulin, we observed Ribosomal Protein S6 kinase stimulation, ~2-fold control, at a 10 times lower concentration, 100 μM , than that needed for MBP kinase activation, 1 mM. This again suggests a MBP kinase-independent component to our total Ribosomal Protein S6 kinase activity which may in part be attributed to p70^{S6K} kinase activity based on chromatographic and immunological evidence as well as our studies with rapamycin, a specific blocker of the p70^{S6K} kinase family.

As mentioned in the discussion of MBP kinases, the insulin-mimetic effects of sodium selenate on glucose-transport and glucose-transporter translocation in isolated rat adipocyte have already been demonstrated (Ezaki, 1990). In our study we have shown the effects of sodium selenate on the signal transduction cascade of insulin in terms of Ribosomal Protein S6 kinase activation. The connection between the two effects remains to be elucidated. Fingar *et al.* (1993) have shown a dissociation of glucose transport and p70^{S6K} activation in 3T3-L1 adipocytes treated with insulin. It would therefore be

reasonable to assume that the two systems may also not be linked in isolated rat adipocytes stimulated with sodium selenate or other insulin-mimetic agents. We, however, cannot attribute our observed Ribosomal Protein S6 kinase activity solely to $p70^{S6K}$. We observed a decrease in the stimulation of Ribosomal Protein S6 kinase activity by sodium selenate in adipocytes pre-incubated with rapamycin, a specific inhibitor of the $p70^{S6K}$ kinase family, but enough activity remained to suggest the presence of other Ribosomal Protein S6 kinase(s) within our system. The link between of the *rsk* S6 kinase family, already known to be present in rat adipocytes, and glucose transport has not yet been elucidated. In addition, Hei *et al.* (1994) have identified an insulin-stimulated S6 kinase in the skeletal muscle of the rat which is distinct from the two main families of S6 kinases identified so far. The presence of this new kinase in adipocytes and its potential role in the mediation of the biological effects of insulin also need to be investigated. The possibility also exists that the stimulation of glucose-transport in adipocytes by sodium selenate is brought about through a signaling pathway other than the ser/thr-phosphorylation cascade.

Perhaps the most dramatic results in our study are those of the stimulation of Ribosomal Protein S6 kinase activity with vanadyl sulphate. We observed a 15-fold control stimulation of Ribosomal Protein S6 kinases at the maximal points for both our time-course and dose-response experiments. Unexpectedly, the optimal time-point and concentration for Ribosomal Protein S6 kinase activation were the same as that for MBP kinases. The effects of vanadyl sulphate on Ribosomal Protein S6 kinases were of sustained duration with over 11-fold control stimulation remaining after 30 minutes of incubation. The dose-response of Ribosomal Protein S6 kinase stimulation was also similar to that of MBP kinases in that concentrations above the optimal 1 mM actually led to a decrease in activity which we feel can partly be explained by similar reasons to those mentioned in the MBP kinase discussion. Unlike the MBP kinase dose-response curve however, initial activation occurred at 313 μ M, less than one-third the 1 mM concentration necessary for MBP kinase activation, and Ribosomal Protein S6 kinase activity returned to normal levels at the 10 mM concentration at

which MBP kinase activity remained 2-fold above control. These observations as with those for insulin and sodium selenate suggest that not all of the Ribosomal Protein S6 kinase activity we measured is downstream of MBP kinases. This again suggests a role for the p70^{S6K} kinases or other S6 kinase(s) which are not downstream of MAP kinases. In fact, an early study in the identification and characterization of the p70^{S6K} kinase family in the Swiss mouse 3T3 cells involved the use of vanadium compounds as activators (Jeno *et al.*, 1988). Although a time-course or dose-response curve were not constructed in this study, they chose 1 mM as the optimal concentration and 30 minute as the optimal time-point, both of which would be consistent with conditions in which we observed high Ribosomal Protein S6 kinase activity. In a later study by the same group, vanadium compounds, at a concentration of 3.8 mM, were shown to induce a 10-fold stimulation of p70^{S6K} kinase activity in Swiss mouse 3T3 cells (Ballou *et al.*, 1988). Vanadium compounds have since been shown to stimulate S6 kinase activity even in the intact animal. Vanadate injected directly via the portal vein into intact liver was shown to increase S6 kinase activity 3-fold over control, although this could not necessarily be attributed to the stimulation of any one S6 kinase family. We also conducted studies with rapamycin to determine the role of the p70^{S6K} family in the overall stimulation observed in cellular extracts from vanadyl sulphate-treated cells. Contrary to our expectations, we did not see a significant decrease in Ribosomal Protein S6 kinase activity of cells pretreated with rapamycin following vanadyl sulphate treatment. However, it must be noted that even the samples which were not treated with rapamycin had a low level of activity compared to our previous results suggesting potential problems with the experiment. These results must therefore be examined carefully with only a limited emphasis being placed on them. We also conducted some experiments with genistein, an inhibitor of tyrosine-kinases in order to gain some insight into the route through which MBP kinases and Ribosomal Protein S6 kinases in our study are activated. Due to the relative non-specificity of genistein and the potential effects that it may have in addition to its tyrosine-kinase inhibition, we must be careful of our interpretation of the data.

We therefore did not discuss the effects of genistein on MBP kinases because of the low magnitude of its effects. As expected, genistein prevented the stimulation of Ribosomal Protein S6 kinases by insulin with some minor effects on sodium selenate effects. The effects of genistein were most prominent with the vanadyl sulphate-treated cells with Ribosomal Protein S6 kinase activity decreasing from 6-fold control to less than 2-fold control. This suggests that tyrosine kinases play an important role in the activation of Ribosomal Protein S6 kinases by vanadyl sulphate but are not the only route through which the effects of vanadium are mediated. The suggestion that the effects of vanadium compounds are mediated through the insulin-receptor tyrosine kinase has been made but remains an area of dispute because of contrary evidence provided by other investigators (Fantus *et al.*, 1991; Green, 1986). Shisheva and Schecter (1993) have suggested that the insulin-mimetic effects of vanadium compounds in isolated adipocytes are mediated in part through the inhibition of a constitutively active cytosolic phosphotyrosine phosphatase. Based on this reasoning, the inhibition of phosphotyrosine phosphatases would have little effect if the tyrosine kinases were already inhibited. It seems highly likely however, that the actions of vanadyl sulphate are brought about through more than one mechanism. As with sodium selenate, we cannot link the many other insulin-mimetic actions of vanadium compounds with the activation of Ribosomal Protein S6 kinases which we have observed. However this remains a potential route through which these events may be mediated.

III. CHROMATOGRAPHY AND IMMUNOBLOTTING

To further analyze the MBP kinase and S6 kinase activities in our crude cellular extracts, we subjected the sodium selenate, vanadyl sulphate, and control extracts to Mono Q anion-exchange chromatography and assayed the obtained fractions for MBP kinase and S6 kinase activity. In addition, we probed selective fractions with MAP kinase and S6 kinase selective antibodies. Mono Q columns are often used in the purification of MAP kinases and

S6 kinases from crude cellular extracts. The column has a high affinity for these enzymes allowing extraneous material to be washed away. The kinases are then eluted using increasing concentrations of NaCl.

A. **MBP kinase Mono Q and Immunoblot Analysis**

We observed five peaks of MBP kinase activity in the Mono Q fractions of the sodium selenate-treated samples and four peaks for our control and vanadyl sulphate-treated samples. All of these peaks are not necessarily due to specific MAP kinase activity since other ser/thr-protein kinases can also potentially phosphorylate MBP. The two most prominent peaks for the sodium selenate-treated and vanadyl sulphate-treated samples, eluted at 346 and 386 mM NaCl. We will limit our discussion to these two peaks which will be referred to as peak 1 (eluted at 346 mM NaCl) and peak 2 (eluted at 386 mM NaCl). For the sodium selenate samples, peak 1 had the highest MBP kinase activity with 45 pmol/min/ml while peak 2 had 27 pmol/min/ml of activity. For the vanadyl sulphate samples, both peaks had ~56 pmol/min/ml of activity. The control samples did not contain the two above-mentioned peaks but had a small peak (10 pmol/min/ml) at 359 mM NaCl. They also contained a large peak of 31 pmol/min/ml eluting at 638 mM NaCl which was not present in the other samples. This peak most likely represents an experimental anomaly since it was not present in the control sample for the vanadyl sulphate time-course control samples and eluted at very high NaCl concentration, nearly double the NaCl concentration necessary to elute peak 1 and peak 2. Previous reports of MAP kinase purification using Mono Q chromatography mainly report the presence of two peaks attributable generally to the 42 kDa ERK2 and the 44 kDa ERK1 eluting in the order mentioned although at least six different MAP kinases have been identified (Ahn *et al.*, 1991; Cobb *et al.*, 1991; Pelech and Sanghera, 1992). Our peaks of MBP kinase activity appear to elute later than those reported by Haystead *et al.* (1990), who observed two peaks of MBP kinase activity eluting at ~240

mM and ~270 mM NaCl, from extracts of isolated adipocytes stimulated with okadaic acid. Other reports, using different systems, also show two peaks of MBP kinase activity which again elute at lower NaCl concentrations than we have observed such as ~150 mM and ~200 mM in PC12 cells and ~100 mM and ~200 mM in rat skeletal muscle (Boulton *et al.*, 1991; Zhou *et al.*, 1993). In a review of MAP kinases by Pelech and Sanghera (1992) ERK1 and ERK2 are listed as eluting at ~330 mM NaCl and ~320 mM NaCl, respectively, which are closer to the value we have obtained. The elution profile of ERK1 and ERK2 is slightly different for each particular report based on the various conditions inherent to the particular study. Even in our own study, the Mono Q profile of the samples from the vanadyl sulphate time-course was slightly different from our other profiles. In all three time-points of vanadyl sulphate incubation, only one prominent peak of MBP kinase activity eluting at 345 mM NaCl was present. This peak most likely encompasses the two peaks of MBP kinase activity seen in other vanadyl sulphate Mono Q profile and the sodium selenate Mono Q profile and again elutes at a NaCl concentration similar to what has been reported in the literature (Pelech and Sanghera, 1992). In the control Mono Q sample profile from the time-course experiment an early eluting peak broad peak was present which was not observed in the vanadyl sulphate-treated time-course profile or any of the other MBP kinase Mono Q profiles including the mentioned control profile. It is unknown whether this peak represents a MAP kinase or another ser/thr-protein kinase active in unstimulated adipocytes.

We probed the fractions containing the peaks of MBP kinase activity from the vanadyl sulphate time-course and sodium selenate samples and probed them with antibodies specific towards MAP kinases. We used both R1 and R2 antibodies with the vanadyl sulphate-treated samples but decided to only use the R1 antibody for the sodium selenate fractions, which were probed later, based on our results with the vanadyl sulphate fractions. The R2 antibody detected a 42 and a 38 kDa band in the fractions immediately preceding the main peak of MBP kinase activity for the vanadyl sulphate samples but failed to detect anything for the actual peak fractions. With the R1 antibody we detected a 42 kDa band for

both the peak fractions and those preceding them which also contained a 44 kDa band. The phosphorylation of the MAP kinases will increase their affinity for the Mono Q column making activated MAP kinases elute slightly later than inactivated MAP kinases. The peaks of activity could therefore be due to a small amount of activated MAP kinases with the remaining unstimulated MAP kinases eluting earlier. The antibodies would not necessarily discriminate between stimulated or unstimulated MAP kinases and could therefore show a stronger reaction with the fractions before the peaks because of the greater amount of MAP kinases they could contain. We have observed bands within the molecular weight range expected for MAP kinases from fractions near peaks of MBP kinase activity using specific antibodies against MAP kinases. We therefore feel that there is a strong possibility that the MBP-kinase activity we have seen both in our crude samples and in the Mono Q profile of vanadyl sulphate-stimulated samples could potentially be due to the presence of MAP kinases. Because we have seen the presence of two bands and based on previous reports reporting the presence of both ERK1 and ERK2 in similar tissues, it seems likely that more than one family of MAP kinases would be present in these samples. We felt that we obtained better results with the R1 antibody because it allowed us to detect a 42 kDa band in the peak fractions which was not present with the R2 antibody. We therefore only used the R1 antibody for probing the fractions from the sodium selenate Mono Q samples. With sodium selenate samples, we observed a 52 kDa band in fractions encompassing both peaks whereas a 50 kDa band was present in two fractions from peak 1. The molecular weights of the band we observed are not consistent with that reported for ERK1, 44 kDa, and ERK2, 42 kDa, although the bands are present in peak regions of MBP kinase activity. Based on our immunoblot data alone, we cannot make any assumptions on the presence of MAP kinases. We did however, observe consistent MBP-kinase activity both in our crude samples and in our Mono Q fraction which corresponded to the fractions from the vanadyl sulphate samples. We therefore feel that the activity we have observed with our sodium selenate samples is due to MAP kinases, although this was not immunologically verified. We originally tried

probing the crude extracts (data not shown) but found interference from the large amounts of BSA present. We therefore decided to probe the fractions from the Mono Q column which would contain lesser amounts of interfering proteins. The main difficulty with these immunoblot experiments including those involving S6 kinases, to be discussed below, was the low amounts present of the kinases in Mono Q fractions. To increase the amounts of kinase present, we increased the amount of samples which again led to difficulties. Firstly, we overloaded our polyacrylamide gels used in the SDS-PAGE electrophoresis which led to decreased resolution of our proteins making our molecular weight determinations inaccurate. In addition, increased samples again led to increased presence of extraneous proteins which directly interfered with our immunoblots. We also used the more sensitive ECL detection but did not detect any bands possibly due to the an incompatibility of this system to our antibodies (data not shown). Logistical considerations prevented clarification of experimental anomalies potentially due to technical difficulties or the further explorations of other options which might potentially have improved our results such as the immunoprecipitation of the MAP kinases in the crude extracts.

B. S6 kinase Mono Q and Immunoblot Analysis

The Mono Q fractions from the sodium selenate-treated, vanadyl sulphate-treated, and control samples were assayed for S6 kinase activity using both a synthetic S6-peptide and purified ribosomal 40S subunits as discussed previously for the crude samples. A significant difference in the substrate specificity of the p70^{S6K} and the p90^{rsk} S6 kinase families has been an apparent low affinity for the synthetic S6-peptide RRLSSLRA, patterned after the phosphorylation site of the S6-protein, by the p70^{S6K} S6 kinase family (Avruch *et al.*, 1991). Previous studies have used the phosphorylation of this peptide as a measure of S6 kinase activity (Haystead *et al.*, 1990). This could potentially have led to a selection against the contribution of the p70^{S6K} S6 kinase family in the overall S6 kinase activity. We,

however, used the synthetic peptide AKRRRLSSLRASTSKSESSQK which may serve as a better substrate for p70^{S6K} S6 kinases based on the increased number of upstream residues to the palindrome sequence (Price *et al.*, 1989). This corresponds to our results in which the peaks observed using ribosomal 40S subunits were also present using our synthetic peptide. More peaks of S6 kinase activity were apparent with the S6-peptide substrate than the ribosomal 40S subunits which could in part be attributed to the action of other ser/thr-protein kinases. We will therefore limit our discussion to only some of the peaks present which were of interest to us.

The most prominent S6-peptide kinase peak of activity for the sodium selenate samples, eluted at ~226 mM NaCl, corresponded to a peak in the vanadyl sulphate time-course samples as well as a small peak in the control samples. This peak elutes at a similar NaCl concentration to that reported by Haystead *et al.* (1990) in the S6-peptide-kinase Mono Q profile of insulin-stimulated isolated rat adipocytes and that of Erikson and Maller (1986) in their early work on the purification of p90^{rsk} from *Xenopus* eggs. This peak was also present in the Ribosomal Protein S6 kinase profile of the sodium selenate samples. We therefore feel that this peak likely represents an important Ribosomal Protein S6 kinase in the overall activity observed in the cellular extracts. Peak four of the sodium selenate S6-peptide-kinase profile was also present in the control S6-peptide-kinase Mono Q profile. The peak for the control samples however, was nearly double the magnitude than the sodium selenate samples. This peak was also present in the control Ribosomal Protein S6 kinase profile. As with the MBP kinase Mono Q profile, this peak seems to represent a kinase that is inactivated following sodium selenate treatment although this remains to be shown.

In further analysis of our sodium selenate Mono Q fractions, we used the antibodies S6K-III, recognizing sub domain III of p70^{S6K} S6 kinase family, and S6K-CT or *rsk*-CT which recognizes the carboxyterminal of the p90^{rsk} S6 kinase family. The S6K-III peptide used to make the antibody has a homologous region to the N-terminal catalytic region of the p90^{rsk}, the S6K-III antibody could therefore potentially cross-react with p90^{rsk} family

whereas the S6K-CT would not be expected to react with the p70^{S6K} S6 kinase family based on the significant differences that exist between the carboxyterminals of the two S6 kinase families (Banerjee, 1990). We, however, found bands which were recognized by both antibodies in some of our immunoblot experiments. As with the immunoblot experiments for the MAP kinases, both BCIP/NBT colouring reagents and the ECL detection system were used. We did not obtain satisfactory results with the BCIP/NBT colouring reagents. When using the S6K-III antibody, no bands were evident and the bands present using the S6K-CT antibody were difficult to interpret. The low amounts of kinase versus total protein once again forced us to use high concentrations of protein which again led to overloading of the gel and poor resolution. Bands with an apparent molecular mass range of ~52-55 kDa were present in the major Ribosomal Protein S6 kinase peak region while a ~64 kDa band was present in all the fractions probed. No clear conclusions can be made with these results since the molecular weights are most likely inaccurate due to gel overloading and the antibody reacts with other proteins present. With the ECL system, we were able to use much lower concentrations of proteins which prevented the overloading of the gels. However, bands corresponding to our molecular weight standards were also evident possibly due to a non-specific reaction of these proteins with the secondary antibody. A 68 kDa band was present in the major Ribosomal Protein S6 kinase peak fractions using the S6K-III antibody suggesting the potential presence of p70^{S6K} kinases. With S6K-CT antibody we were able to detect two bands within these fractions, a relatively dark ~62 kDa band and a light ~54 kDa band. The 62 kDa band could again represent a p70^{S6K} kinase cross-reacting with the S6-CT antibody with the ~54 kDa bands representing the same enzyme in a lower phosphorylation state. Both, however, could represent other kinases or proteins reacting non-specifically to our antibody as could the 68 kDa band detected using the S6-III antibody. One possibility could be that the bands present are due to the non-specific reaction of the secondary antibody to BSA. Our crude samples contain large amounts of BSA some of which could still be present in the Mono Q fractions. The molecular weight of BSA is ~66

kDa and ECL has been shown to visualize the bands for the molecular weight standards. We therefore cannot make any definite conclusions using our immunoblot data on its own. But based on the fact that these bands correspond to peak fractions of Ribosomal Protein S6 kinase activity, we feel that it would be valid to view them as potential S6 kinases. The possibility that the observed Ribosomal Protein S6 kinase activity present both in these and the crude samples is due to a different Ribosomal Protein S6 kinase stimulated by sodium selenate but not recognized by these antibodies also remains such as an adipocyte homologue of the recently reported insulin-stimulated S6 kinase present in rat skeletal muscle (Hei *et al.*, 1993).

The S6-peptide kinase Mono Q profile of the vanadyl sulphate time-course samples contained a number of peaks some of which are likely due to the phosphorylation of our peptide by kinases other than S6 kinases since corresponding peaks in the Ribosomal Protein S6 kinase Mono Q profile are completely absent. The major peaks of Ribosomal Protein S6 kinase activity were present for all vanadyl sulphate-stimulated samples but varied in magnitude based on the incubation time. The 20 minutes samples generally had the highest peaks in accordance to that observed for the crude samples in which the 20 minutes incubation time resulted in the highest Ribosomal Protein S6 kinase activity. Peak 3 of the S6-peptide kinase profile corresponds to the major peak discussed for the sodium selenate samples and is also the present as the major peak of the Ribosomal Protein S6 kinase Mono Q profile. This peak elutes at a NaCl concentration range of 235-290 mM which differs slightly for each time-point. The levels of activity are also different for each time-point possibly indicating different phosphorylation states of the kinases which could be in part be responsible for the difference in the elution for the different time-points. Peaks 4 and 5, eluting at ~345 mM and ~375 mM NaCl, respectively, were also major peaks present in the S6-peptide kinase Mono Q profile but corresponded to a minor peak in the Ribosomal Protein S6 kinase profile. Insulin-stimulated p90^{rsk} in rat skeletal muscle has also been shown to elute at similar concentrations to that seen for peak 4 (Hei *et al.*, 1993). These

peaks also correspond to the major MBP kinase peak of these samples discussed earlier and could therefore represent non-specific phosphorylation of the S6-peptide by MAP kinases. They could also potentially be due to the activity of the p70^{S6K} kinase family. The p70^{S6K} kinases are believed to elute later than the p90^{rsk} kinases in the Mono Q column and studies using their specific inhibitor rapamycin have shown them to be the more important S6 kinases in mammalian tissue although this is the subject of some controversy based on more recent evidence (Dickens et al., 1992; Chung et al., 1991; Hei et al., 1994). These peaks were not, however, present in the Ribosomal Protein S6 kinase Mono Q profile of the insulin-stimulated adipocytes reported by Haystead *et al.* (1990). But as discussed earlier, the S6-peptide used in that study was not a good substrate for the p70^{S6K} kinase family which could have led to the absence of a p70^{S6K} S6 kinase peak.

Selected fractions from the vanadyl sulphate samples were subjected to immunological analysis as described for the sodium selenate samples. Once again high levels of protein necessary for band detection using BCIP/NBT colouring reagents led to gel overloading and poor resolution. A 55 kDa band was evident for the major peak of Ribosomal Protein S6 kinase activity observed for the Ribosomal Protein S6 kinase and the S6-peptide-kinase profile using the S6K-CT antibody. Using the ECL development system, a 69 kDa and a 70 kDa band were present in the peak fractions using S6K-CT and S6K-III antibodies respectively. As with the sodium selenate samples the presence of this band may suggest a role for the p70^{S6K} kinase family in our overall Ribosomal Protein S6 kinase activity. But again the possibility exists that these bands are unrelated to the observed Ribosomal Protein S6 kinase activity and are a result of non-specific interactions since both antibodies show similar bands for both sodium selenate and vanadyl sulphate in the same fractions. However, these are the fractions in which Ribosomal Protein S6 kinase activity was observed for both agents. Although based on our results, we cannot make any major conclusions on the identity of the S6 kinase family observed in our experiments.

CONCLUSIONS

- 1) Our isolated rat adipocyte preparation contained MBP kinases and Ribosomal Protein S6 kinases which were stimulated by insulin in a time and dose-dependent manner and were thus a suitable system in which to investigate the insulin-mimetic compounds sodium selenate and vanadyl sulphate.
- 2) Sodium selenate was able to stimulate MBP kinases and Ribosomal Protein S6 kinases to a degree equal to or higher than insulin in a time and dose-dependent manner. This suggests that sodium selenate has effects on the signal transduction pathway of insulin although the precise link to the metabolic effects is yet to be made.
- 3) Vanadyl sulphate stimulates both MBP kinases and Ribosomal Protein S6 kinases to a higher degree than either insulin or sodium selenate in isolated rat adipocytes. The mechanism of this stimulation is unknown but may involve tyrosine kinases in the case of the Ribosomal Protein S6 kinases based on our genistein experiments.
- 4) Both ERK1 and ERK2 might contribute to the overall MBP kinase activity seen with sodium selenate and vanadyl sulphate, although their immunological identifications remains inconclusive.
- 5) A ~70 kDa band in peak Mono Q fractions of Ribosomal Protein S6 kinase activity suggests a role for the p70^{S6K} kinase family although these results are somewhat equivocal in nature. Other S6 kinases are also believed to play a role in the overall observed Ribosomal Protein S6 kinase activity based on our rapamycin experiments.

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