ACTIVATION OF CELL FUNCTION; PHARMACOLOGICAL AGENTS WHICH DEGRANULATE MAST CELLS AND CAUSE SKELETAL MUSCLE TO CONTRACT

Thesis by

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ABSTRACTS

Chapter I

This chapter presents a general introduction to the cellular metabolism of calcium with particular reference to the concept of calcium pools. The importance of the ionized calcium pool of the cytosol in determining the specific function of cells and tissues is discussed.

A model for stimulus-release coupling in mast cells is presented. Excitation-contraction (EC) coupling in skeletal muscle is defined and the fundamental premises required for a theory on EC coupling are given. The two basic theories of excitation-contraction coupling are briefly reviewed.

Chapter II

We have discovered a new class of drugs which induce the release of ^{3}H-serotonin in mast cells. Verapamil, previously described as a "specific calcium antagonist", was found to release ^{3}H-serotonin from mast cells. Our study investigated and compared verapamil and compound 48/80, a drug previously known to be a potent degranulator of mast cells.

This investigation revealed that low concentrations of verapamil inhibited the spontaneous release of ^{3}H-

serotonin and efflux of $^{86}Rb^{+}$ in mast cells. High concentration of verapamil caused an increase in the rate of the release of ^{3}H-serotonin and $^{86}Rb^{+}$ from mast cells. Compound 48/80 causes only an increase in the release of ^{3}H-serotonin and $^{86}Rb^{+}$-efflux. The effect of compound 48/80 and verapamil on the rate of $^{86}Rb^{+}$-efflux in mast cells was investigated.

The significance of these results is discussed. Verapamil, at low concentrations, is thought to act as a "specific Ca channel blocker". The increase in $^{86}Rb^{+}$ efflux from mast cells induced by high concentration of verapamil suggests an increase in concentration of cytosolic Ca^{++}. The mobilization of calcium into the cytosolic pool is believed also to trigger the release of ^{3}H-serotonin from the mast cell. We propose that verapamil is not simply a calcium antagonist in mast cells but also has the ability at higher concentrations to cause an increase in ionic or cytosolic Ca^{2+}.

Chapter III

A comparative study of the effects of compound 48/80, verapamil, and newly synthesized derivative of verapamil called "crosslinked verapamil" on mast cells has been undertaken with the goal to identify the cellular site of action of the drugs. We have studied the role of crosslinking in the mechanism of action of compound 48/80 and verapamil. Analogies are drawn to our study of the interaction of crosslinked antigen with immunoglobulin E bringing about the degranulation of mast cells. Crosslinked verapamil was found to be more potent in causing the release of ^{3}H-serotonin from mast cells.

Pretreatment of mast cells with neuraminidase was found to inhibit the release of ^{3}H-serotonin caused by compound 48/80 but does not change the release of ^{3}H-serotonin caused by verapamil and crosslinked verapamil. We propose that compound 48/80 interacts with components of the cell surface that contain negatively charged sialic acids. In contrast, verapamil and crosslinked verapamil could operate intracellularly or at cell membrane sites that are not affected by pretreatment of the mast cell with neuraminidase.

We also examined possible mechanisms for the degranulation of mast cells by verapamil and discussed the kinetics of ^{3}H-serotonin release from mast cells caused by compound 48/80, crosslinked verapamil and verapamil.

Chapter IV

The nature of a contracture in skeletal muscle caused by compound 48/80, a potent agent of degranulation of mast cells, was investigated. The observation that the contracture was inhibited by pretreatment of the muscle indicated that compound 48/80 interacts with cell surface structures that contain neuraminidase-sensitive sialic acids essential for activity. An increase in myoplasmic [Ca^{++}] during contracture caused by compound 48/80 was suggested by the observation that the rate of $^{86}Rb^{+}$-efflux and the rate of protein degradation are increased when the muscle is challenged by the drug. Measurements of ^{45}Ca uptake during pharmacological challenge of the muscle indicated that the increase in the influx of extracellular Ca^{++} caused by compound 48/80 was not correlated with contracture, i.e., the increase in ^{45}Ca uptake still occurred in muscle pretreated with neuraminidase but unable to undergo contracture when challenged with compound 48/80. This finding and the observation that contracture could occur in the absence of extracellular calcium indicated that the extracellular medium was <u>not</u> the source of Ca^{++} required to cause the increase in myoplasmic [Ca^{++}] sufficient to activate the contractile apparatus.

A contracture of skeletal muscle caused by verapamil was also investigated by examining dose-dependent $^{86}Rb^{+}$-efflux. This study revealed that verapamil could be mobilizing intramuscular pools of calcium in analogy to its effects on mast cells to cause contracture.

A mechanistic model consistent with the experimental results is proposed to explain contracture and increased ^{45}Ca uptake caused by compound 48/80 and tests of the model are offered. Parallels between stimulus-secretion coupling in mast cells and stimulus-contracture coupling in skeletal muscle are summarized.

ABBREVIATIONS

ATP	adenosine triphosphate
DMEM	Dulbecco's modified Eagles' medium
EC	excitation-contraction
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglyco-bis(β-aminoethyl ether) N,N'-tetraacetic acid
IgE	immunoglobulin E
MCB	mast cell buffer (Materials and Methods, Chapter II)
PAB	protein anabolism buffer (Materials and Methods, Chapter IV)
PBS	phosphate buffered saline
PC	phosphatidyl choline
PCA	passive cutaneous anaphylaxis
PE	phosphatidyl ethanolamine
PS	phosphatidyl serine
SAM	S-adenosyl methionine
SR	sarcoplasmic reticulum
t-tubule	transverse tubule
TCA	trichloracetic acid

TABLE OF CONTENTS

LIST OF FIGURES

INDEX OF TABLES

CHAPTER I

BACKGROUND

A multicellular organism requires specialization of cellular function which in turn mandates communication between the various types of cells such that the organism can generate an integrated response to environmental change. A common mechanism of cellular communication involves the interaction of an extracellular messenger with specific cellular receptors causing the release of intracellular secondary messengers, thereby triggering the cellular response. An example of an integrated response that illustrates these points is the immediate hypersensitivity reaction to antigen. An antigen which binds to a complex of immunoglobulin E and a specific IgE-receptor on the mast cell membrane (Metzger, 1977) triggers a change in calcium permeability in the mast cell membrane. This causes a transient rise in the concentration of calcium, a secondary messenger, in the cytosol of the mast cell. The cytosolic Ca^{++} triggers a series of biochemical events that lead to the degranulation and subsequent release of biogenic amines and chemotactic factors from the cell (Hirata and Axelrod, 1980). In turn the biogenic amines and chemotactic factors continue the integrated response of immediate hypersensitivity by specifically interacting with other target cells and tissues. For example, histamine interacts with target cells of the peripheral

vasculature causing vasodilation and increased fluid permeability across the vascular epithelium (Middleton, 1980). Again, these final events of immediate hypersensitivity depend on the interaction of an extracellular messenger, histamine, with a specific cellular receptor which in turn leads to the tissue or cellular response.

The interaction of extracellular messengers with specific cell surface receptors causing the generation of intracellular secondary messengers has two basic mechanisms. The receptor, such as the IgE receptor (Metzger, 1979), could be at the cell surface and serve via induction of changes in structure and enzymatic activity in the membrane as a generator of intracellular secondary messenger or the intracellular messenger could be transported directly into the cytoplasm, such as the steroid-receptor complex (Bischoff and Bryson, 1977), to interact with target structures to cause the cellular response. Regardless of the site of action of the extracellular messenger the induction of intracellular secondary messengers or even tertiary messengers are central features which, depending on the differentiated cell type, initiate or modulate the particular cellular response.

Calcium ions have been implicated as secondary and tertiary messengers in the cellular response to the extra cellular signals in a large number of systems. The messenger function of calcium occurs within the framework of the highly complex cellular metabolism of calcium.

A useful model for understanding the cellular metabolism of calcium involves the concept of calcium pools. The principal pools of calcium of the "typical" cell are extracellular calcium, calcium bound to the plasma membrane, intramitochondrial calcium, calcium within the endoplasmic reticulum, bound calcium in the cytosol and free ionized calcium in the cytosol. The distribution of calcium in these pools is partially determined by the permeability of calcium across the plasma membrane, the mitochondrial membrane and the membrane of the endoplasmic reticulum. Also the, ATP-dependent calcium pumps in these membranes play an important role in determining the distribution of calcium in the cellular pools. In muscle the highly specialized sarcoplasmic reticulum determines to a large extent the transient distribution of calcium in this tissue.

A cellular response that involves messenger calcium is usually correlated with a change in the concentration of free ionized calcium in the cytosol. For example, a transient increase in the free myoplasmic calcium con-

centration from 0.1 μM to 1-2 μM is seen in the excitation-contraction cycles of skeletal muscle (Caputo, 1978). The principal source of the messenger calcium in muscle is the sarcoplamsic reticulum and the target of the messenger is the regulatory troponin of the actin-myosin contractile apparatus. The contractile response is terminated by the removal of the messenger Ca^{++} from the myoplasm by the ATP-dependent pumps of the sarcoplasmic reticulum (Hasselbach, 1980). A partial list of cellular events that are known to be initiated or modulated by changes in the concentration of calcium in the important cytosolic pool are given in Table I.

Table I, Chapter I - Cellular Events Initiated of Modulated by Changes in Cytosolic [Ca^{++}]

Tissue	Effect	Reference
Red cell	K^+-efflux	Gardos (1958)
Salivary gland	differential ion flux and fluid transport	Douglas and Poisner (1963)
Lacrimal gland	differential ion flux and fluid transport	Parod and Putney (1978)
Exocrive pancreas	enzyme release	Case (1978)
Liver cell	α-adrenergia glucose release	De Wulf and Keppens (1976)
Gastrointestinal epithelium	electrolyte and water exchange	Frizzell (1977)
Adipocytes	α-adrenergie activation	Perry and Hales (1970)
Neutrophils	chemotactic and secretory responses	Naccache et al (1977)
Neurons	transmitter release	Rahamimoff et al (1978)
Mast cells	IgE-induced degranulation	Hirata and Axelrod (1980)
Renal cells	gluconeogenesis	Nagata and Rasmussen (1970)
Protozoa	ciliary motility	Schmidt and Eckert (1976)
Pancreatic β-cells	secretion of insulin	Malaisse et al (1976)
Egg cell	activation	Okamota et al (1976)

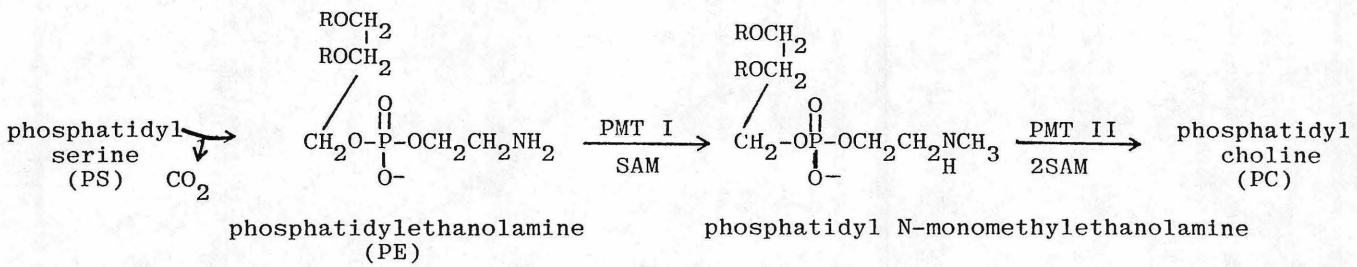
Introduction to Mast Cells

Mast cells are granulocytes found in the connective tissue and are characterized by staining with basic dyes (Ehrlich, 1879). Acid mucopolysaccharides, such as heparin, account for the metachromasia of the granules. The mast cell is closely related to the blood-borne basophil. The basophil is round and regular in cell shape, contains a multilobed nucleus similar to other granulocytes and has relatively few granules. The mast cell has irregular cell shapes, has a round nucleus and has numerous delicate granules. The granules of both cells contain histamine and serotonin stored as an amine-protein heparin complex (Bergendorff and Uvnas, 1972).

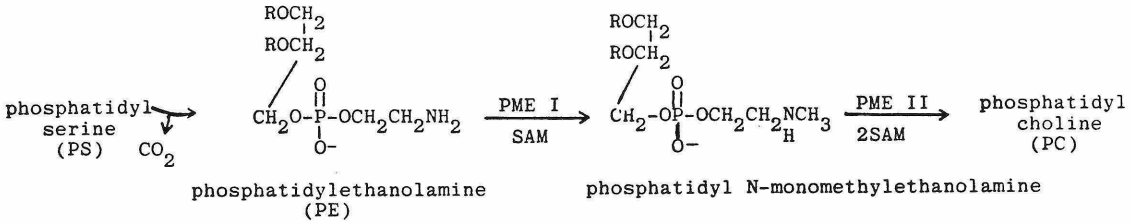
The triggering of the mast cell is the focal point of immediate hypersensitivity. Mast cells when activated release the contents of their granules in a process called degranulation. The granules contain mediators which precipitate either a localized inflammatory reaction or a severe systemic reaction called anaphylactic shock. Mediators, such as histamine, cause the relaxation of smooth muscle of the local vasculature and cause fluid leakage into the surrounding tissue. Also, mast cells release chemotactic factors which attract other leukocytes and enzymes that degrade local tissue, furthering the inflammatory response and possibly laying the groundwork for any subsequent repair.

Allergic responses are triggered by allergens interacting with immunoglobulin E bound to the surface of mast cells. Because allergies serve no obvious function, there is considerable debate on what actually is the evolutionary benefit of the mast cell. Kay (1979) believes that the mast cell plays a key role in protection from parasites. Specifically mast cells were shown to degranulate in the presence of IgE specific for shistosomula. The mast cells release a chemotactic factor (ECFA) which attracts eosinophils. Kay (1979) found the eosinophils could cause the death of the shistosomula if the parasites were precoated with IgG and the C3 component of the complement. Many correlations have been found between parasitic infections and IgE. For example, a role for IgE and the mast cells in protection from parasites was inferred in the study of Grove and Forbes (1975) which showed that individuals with increased levels of blood-borne IgE have an increased resistance to hookworm infestation.

While the function of the mast cell is not clear, much progress has been made on resolving the mechanism of degranulation. Hirata and Axelrod (1980) proposed a mechanism for the IgE-mediated release of histamine from mast cells that tied together many past observations on degranulation. For example, Hirata and Axelrod (1980) found that


phosphatidyl serine (PS)
CO_2
$ROCH_2$
$ROCH_2$
$CH_2O-P-OCH_2CH_2NH_2$
O
O-
PMT I
SAM
$CH_2-OP-OCH_2CH_2NCH_3$
H
PMT II
2SAM
phosphatidyl choline (PC)
phosphatidylethanolamine (PE)
phosphatidyl N-monomethylethanolamine

the central feature in stimulus-secretion coupling in mast cells is the enzymatic transfer of methyl groups to phospholipids. They demonstrated that an early step in the activation of the mast cell was the rapid incorporation of ^{3}H-methyl group from S-adenosyl methionine (SAM) into the phospholipid fraction of the membrane of the mast cell. Degranulation of the mast cells and the methyltransferase were inhibited by a specific methyltransferase inhibitor (Ishizaka et al, 1980). This result explained the long standing observation that



mast cells required the addition of phosphatidylserine to degranulate in the presence of IgE (Keller, 1962). The methylation of the phospholipids was correlated with an increased influx of ^{45}Ca into the mast cells (Ishizaka et al, 1980). The increased permeability to calcium was thought to be caused by the methylation and subsequent flipping of phospholipids of the inner surface of the membrane to outer surface of the membrane which in turn causes a decrease in membrane viscosity. The increase in membrane

fluidity supposedly would allow unknown components of the cell surface to aggregate and thus increase Ca^{++} permeability. Hirata and Axelrod (1980) proposed that the influx of calcium activated the Ca-dependent enzyme phospholipase A_2. The activated phospholipase A_2 converts PC into lysophosphatidylcholine, a substance that promotes calcium-dependent cell fusion (Poole et al, 1970) and enhance degranulation of mast cells (Giacobini et al, 1965). Since the release of histamine involves the fusion of membrane enclosed granules with the outer membrane (Goth and Johnson, 1975) the production of lysophosphatidylcholine and the influx of calcium may be important events in the final stages of degranulation. However, the mechanism of fusion of granules with the cell surface is not known.

The Hirata and Axelrod (1980) model for IgE-mediated release of histamine may not be completely applicable to other agents that degranulate mast cells. For example, the potent degranulating agent, compound 48/80, does not strictly require extracellular calcium for its action (Uvnas and Thon, 1961). However, the Hirata and Axelrod (1980) model which implies that extracellular calcium has a role in degranulation, may be valid if compound 48/80 induces a methylation event that releases calcium

from some yet undetermined calcium pool. The addition of phosphatidylserine is not required for the compound 48/80-mediated degranulation of mast cells (Goth et al, 1971). There is no simple extension of the model of Hirata and Axelrod (1980) that would both explain the requirement for PS in the degranulation of mast cells by IgE and also account for the apparent lack of this requirement in the degranulation of mast cells by compound 48/80. Since degranulation of mast cells by IgE feature utilization of PS in the plasma membrane this particular membrane may have a specific requirment for PS. In contrast, the degranulation of mast cells by compound 48/80 does not necessarily feature changes in phosphilipid methylation in the plasma membrane, but may feature a similar process in the membrane defining the intracellular calcium pools. These intracellular calcium pools are thought to be mobilized in the degranulation of mast cells by compound 48/80 (Douglas and Ueda, 1973) and it is possible that the mast cell does not require PS for degranulation by compound 48/80 because the membrane of the intracellular calcium pools have sufficient PS for this process.

Excitation-Contraction Coupling in Skeletal Muscle

Almost all of the major steps in the contractile activation of skeletal muscle are now well-established. The depolarization of the muscle or muscle fibers at the neuromuscular junction is the first step leading to the contraction of the muscle. The action potential of a cholinergic nerve is correlated with the release of vesicles containing the transmitter acetylcholine. The acetylcholine interacts with specific receptors at the neuromuscular junction (Heidman and Changeaux, 1978) including a uniform depolarization of the transmembrane resting potential of the muscle. The depolarization is basically due to an increased permeability of the muscular membrane to sodium ion. A threshold depolarization occurs when sufficient transmitter is released at the neuromuscular junction. The subsequent action potential spreads throughout the muscle via localized changes of ionic conductance. The muscle action potential features a voltage and time-dependent increase in Na^+ current and a delayed voltage and time-dependent increase in potassium conductance (Adrian et al, 1970). The resting transmembrane potential is re-established after the action potential by the transport of sodium and potassium by Na^+/K^+-ATPases back across the muscle membrane.

The inward spread of the activation of the muscle from the sarcolemma through the transverse tubule system is the second major step in the contractile mechanism (Constantin, 1975). The coupling between the muscular action potential and contraction could be abolished by treatment of the muscle with hypertonic glycerol (Gage and Eisenberg, 1969). Anatomical studies of the muscles treated with glycerol showed that the t-tubule system was physically pinched off from the sarcolemma by the glycerol treatment. These experiments implied that coupling between excitation and contraction in skeletal muscle required a direct communication of the action potential of the sarcolemma into the t-tubule system.

The third basic step in the sequence for the activation of the muscle at the neuromuscular junction to the activation of the contractile apparatus is the coupling between the activation of the t-tubule with the increase in calcium permeability of the membrane of the sarcoplasmic reticulum. Anatomically, the t-tubules and the sarcoplasmic reticulum are close and form distinct junctions called the SR-t-tubule triadic junctions (Huxley, 1971). The t-tubule action potential has been correlated with the radial spread of the activation of skeletal muscle fibers (Constantin, 1975). Despite the correlation

between t-tubule activation and the increase in calcium permeability of the membrane of the sarcoplasmic reticulum, the molecular mechanism of the coupling of these processes is not known. Theories proposed to explain this step in excitation-contraction coupling will be discussed in detail later in this chapter because of their importance to the work reported in this thesis.

The final basic step in the contractile sequence is the release of calcium from the sarcoplasmic reticulum into the myoplasm causing the activation of the contractile apparatus. In skeletal muscle, the myoplasmic calcium interacts with a target protein called troponin-C associated with the actin-myosin contractile apparatus; binding of Ca^{2+} activates an actinomyosin ATPase. The hydrolysis of ATP by the actinomyosin ATPase provides the driving energy for the interdigitation and "sliding" of the actin and myosin filaments during the active shortening of muscle (Katz, 1966). The myoplasmic concentration of free calcium is lowered to the resting level by the action of the ATP-dependent calcium pumps of the sarcaoplasmic reticulum Hasselbach, 1980). This process causes relaxation of the contractile apparatus and reduces the tension of the muscle to the resting state.

The calcium binding protein calmodulin (Cheung, 1971) is known to directly potentiate the reuptake of calcium into cardiosarcoplasmic reticulum (Katz and Reintulla,

1978; Lopaschuk *et al*, 1980). Calmodulin may also indirectly cause a cAMP-dependent increase in sarcoplasmic reticuluar Ca^{++} transport by stimulating cardiac adenylate cyclase activity (Tada and Kirchberger, 1978). However, the role of calmodulin in the relaxation of skeletal muscle is not known.

Excitation-Contraction Coupling Theories: The Coupling Between the t-tubules and the Sarcoplasmic Reticulum

Several theories have been proposed to explain the communication between the t-tubules and the sarcoplasmic reticulum. An early approach was to assume that the currents in the t-tubule system were transmitted directly to the sarcoplasmic reticulum causing a depolarization of the sarcoplasmic reticulum, thus leading to the increase in calcium permeability. Several studies have shown that insufficient ionic current flows through the SR-t-tubule junction to produce a significant depolarization of the sarcoplasmic reticulum (Chandler et al, 1975).

Another early approach featured extracellular calcium ions entering the muscle fiber during the action potential and subsequently initiating the contractile response (Frank, 1957; Bianchi and Shanes, 1959). But it was found that the influx of extracellular calcium during a single contraction was insufficient to account for the increase in myoplasmic concentration of calcium associated with the contractile event. Therefore, to account for this discrepancy it was proposed that the small amounts of calcium entering the muscle triggered an intracellular event that would lead to contraction. This mechanism is called the "trigger Ca^{++}" hypothesis of excitation-contraction coupling (Bianchi, 1968).

Several experiments have shown that the intracellular event that is triggered by calcium was the release of calcium by the sarcoplasmic reticulum. Direct application of calcium buffers to skinned skeletal muscle fibers induces the release of calcium by the SR (Endo et al, 1970). A relatively small increase in the concentration of Ca^{++} can cause a rapid release of calcium from vesicles derived from the sarcoplasmic reticulum (Kupsaw et al, 1980). These two pieces of evidence, repeated in other studies, are essential for support of a mechanism of direct coupling between calcium and calcium release from the sarcoplasmic reticulum of skeletal muscle.

Despite the accumulating evidence that the "trigger-Ca^{++}" hypothesis has value in explaining the coupling between activation of the t-tubules and release of calcium from the SR, the mechanism of the coupling is unknown. For example, the calcium pool that provides the "trigger-Ca^{++}" for the coupling is not known, although extracellular calcium and calcium bound to the membranes of the t-tubules have been suggested (Frank, 1979). There is no conclusive evidence pertaining to the molecular mechanism or even the molecules involved other than Ca^{++} in the coupling between activation of the t-tubules and the release of calcium. The mechanism of excitation-coupling remains one of the major ongoing problems in muscle physiology.

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CHAPTER II

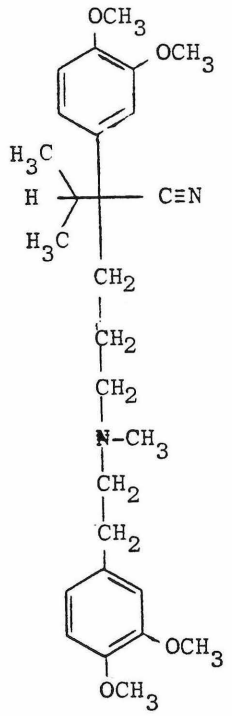
VERAPAMIL; A NEW AGENT FOR ACTIVATION OF MAST CELLS

INTRODUCTION

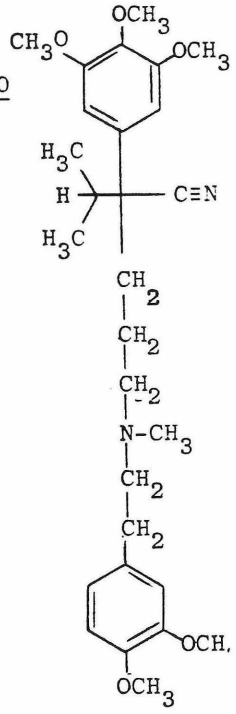
Verapamil

The structure of verapamil and its methoxy-derivative, D-600 are given below.

Verapamil



D-600



Verapamil and D-600 were found to be highly potent inhibitors of excitation-contraction coupling in the mammalian myocardium (Fleckenstein, 1964) and of excitation-contraction coupling in uterine smooth muscle (Fleckenstein and Grun, 1969). The effects on muscle could be overcome by increased extracellular Ca^{++}, therefore these drugs

were designated as calcium antagonists. Since the discovery of verapamil and D-600 as calcium antagonists in heart muscles a large number of systems have been investigated. An incomplete list partially compiled from Rosenberger and Triggle (1978) is given in Table II.

The first general mechanism proposed for the pharmacology of verapamil was by Fleckenstein (Kohlhart et al, 1972), who noted that verapamil did not affect the action potential of mammalian cardiac fibers. The cardiac action potential is primarily due to an influx of Na ions through what is designated as a fast channel. However, verapamil did inhibit cardiac contraction and the transient calcium current associated with depolarizing membrane potentials. Calcium was found to enter the heart through the fast Na-channel and through its own specific Ca-channel (Mascher and Peper, 1969, Reuter, 1967). This flux of calcium through the slow Ca-channel was blocked by verapamil. Fleckenstein (1977) designated verapamil and D-600 as specific Ca-channel antagonists because of these findings. Sympathetic β-receptor stimulating agents such as epinephrine and isoproterenol had an opposite effect by causing an increase in the Ca current across the cardiac membrane (Reuter, 1965).

Verapamil has been shown effective in a variety of therapeutic situations. For example, the drug has been used as

Table II,Chapter II - Effects of Verapamil (or D-600) on Cellular Function

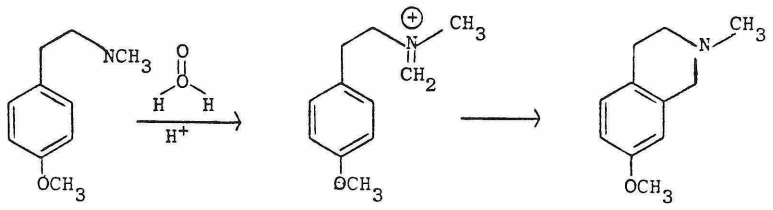
Tissue	Action	Reference
Aorta	relaxation of norepinephrine (NE) effect	Schuman et al (1975)
Portal vein	decreases spontaneous contractions	Golenhofen and Herstein (1975)
Ear artery	relaxation of NE effect	Golenhofen and Weston (1976)
Ileum	relaxation of acetylcholine effect	Triggle et al (1975)
Pancreatic islets	inhibition of insulin release	Malaisse et al, (1976)
Neurohypophysis	inhibition of mytocin release	Dreifus et al, (1975)
Pituitary	inhibition of ACTH release	Eto et al (1974)
Adrenal	inhibition of catacholamine release	Pinto and Trifaro (1976)
Pulminary Artery	relaxation of K^+ stimulation	Haeusler (1972)
Mesenteric vein	relaxation of NE effect	Church and Zsoter (1980)

a depressor of a variety of hyperkinetic cardiac phenomena by virtue of its excitation-contraction decoupling properties on cardiac muscle. Moreover, verapamil has immediate positive effects on cardiac arrhythmias (Shamroth et al, 1972). Verapamil has also been used as a long term therapeutic agent. For example, Sandler et al (1968) in a controlled clinical trial found that verapamil caused a decrease in the frequency of angina pectoris in cardiac patients. Verapamil has also been suggested as a possible drug for the management of hypertension. The hypotensive action of verapamil, at doses below that required for reduction of cardiac output, has been proposed to be due strictly to changes in peripheral vasodilation (Ross and Jorgensen, 1967).

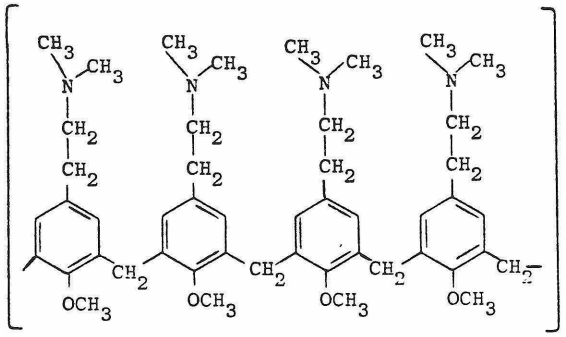
Recently in the popular press, verapamil has been described as a potential "wonder drug" in the treatment of hypertension (Clark and Shapiro, 1981). A possible mechanism for the hypotensive effect of verapamil is given in this study.

Compound 48/80

Compound 48/80 was first synthesized by Baltzby et al (1949). The compound was prepared by an acid catalyzed polymerization of p-methoxyphenethylmethylamine with formalin. Morrison et al (1974) radiolabeled the "compound" with ^{125}I and found a very broad peak in gel filtration of approximately 800 molecular weight. Several factors probably contribute to the complexity of the mixture. One factor is the formation of isoquinolines as a side reaction.



Isoquinolines do not account for the potent ability of compound 48/80 to reduce blood pressure in dogs (Baltzby et al, 1949). When tertiary amines were polymerized the hypotensive activity was of maximum potency. Because tertiary amines do not form isoquinoline derivatives Baltzby et al (1949) proposed that the following structure was the active ingredient in mixtures of polymerized



methoxyphenalkyamines. Another factor possibly contributing to the complexity of the compound 48/80 preparation is the possibility that the isoquinoline units are crosslinked (Evans, 1981, personal communication).

The ability of compound 48/80 to lower blood pressure is probably due to its ability to release histamine from tissue mast cells (Mongar and Schild, 1952).
The release of histamine was due to compound 48/80 causing a degranulation of mast cells and basophils. Compound 48/80 can cause the release of histamines from mast cells in the absence of extracellular calcium (Uvnas and Thon, 1961). Mast cells when treated with 2 mM EDTA for three hours do lose their ability to respond to compound 48/80 (Douglas and Ueda, 1973). Douglas and Ueda (1973) suggested that the EDTA treatment of mast cells caused a depletion of the intracellular pools of calcium that are mobilized when the mast cells are challenged by compound 48/80.
In contrast, the degranulation of mast cells caused by complexes of IgE and antigen have an absolute requirement for extracellular calcium (Ishizaka et al, 1979).

Stimulus-Permeability Coupling

Gardos (1958) found that treatment of red cells with EDTA causes a decrease in the possible leakage of potassium from red cells. Whitman (1968) later proposed that the decrease in potassium permeability was due to a decrease in the concentration of ionized calcium in the red cell. Lew and Ferreira (1978) showed that treatment of red cells with the calcium specific ionophore A23187 caused an increase in potassium permeability. This they ascribed to the activation of a Ca-sensitive potassium channel by the inward movement of extracellular calcium. The mechanism for the activation of such "channels" is unknown (Putney, 1979).

The Ca-dependent changes in potassium efflux have been observed in a wide variety of cells and tissues since the discovery of the "Gardos effect" (Putney, 1979). Putney (1979) has coined the term stimulus-permeability coupling to describe this effect. Examples of systems that exhibit stimulus-permeability coupling are activation of salivary glands, stimulation of lymphocytes, hormone action on liver cells, and contraction of smooth muscle such as the ileum. This study has found that efflux of rubidium-86 (an analogue of potassium (Putney, 1979)) in skeletal muscle and mast cells was sensitive to challenges by verapamil and compound 48/80.

MATERIALS AND METHODS

Isolation of Rat Peritoneal Mast Cells

The house bred ratsused in this study were predominantly of the Fisher-344 breed and occasionally of the Wister-Furth breed. No differences were observed between the breeds in cell yields and cell responses. Rats between 150 and 300 grams were sacrificed by cervical dislocation with a blunt guillotine. The rats were injected in the peritoneal cavity with either phosphate buffer saline (PBS) or sterile mast cell buffer (MCB). MCB contained 137 mM NaCl, 2.6 mM KCl, 1.0 mM $MgCl_2$, 10 mM Tris (hydromethyl)aminomethane, 1 mg/ml bovine-serum albumin and 1 mg/ml glucose. MCB was titrated to pH 7.4 with concentrated HCl.

This buffer was modified from Cochrane and Douglas (1976) but omitted heparin for it was found that heparin precipitated compound 48/80. MCB, unless otherwise designated, was used in all the pharmacological studies on rat peritoneal cells and P815 mastocytoma cells. It is very important to note that $CaCl_2$ is not a component of MCB. The $CaCl_2$ was omitted when it was found that the spontaneous release of ^{3}H-serotonin from mast cells isolated in the presence of a calcium containing solution was approximately two-fold higher than cells isolated in buffer without caclium. Furthermore, rat peritoneal cells

isolated in buffers with no calcium were more reactive to the various drugs used in this study. There is a possibility that the presence of Ca^{++} during the isolation somewhat desensitizes the mast cells to further ^{3}H-serotonin release. If Ca^{++} was required for a drug challenge of the mast cells, a volume of a calcium chloride stock solution was added to the MCB until an appropriate final concentration was obtained (usually 2.0 mM). Often, but not reproducibly, this addition of $CaCl_2$ caused a small release of ^{3}H-serotonin from the mast cells.

The peritoneal cavity of the rats was massaged manually to dislodge cells from the inner peritoneal wall. The peritoneal cavity was then cut open and the fluid was transferred to a plastic centrifuge tube using a Pasteur pipette. B. L. Aalseth improved the yields of mast cells by developing a variation of the standard technique. The rat peritoneal cavity was widely exposed by forceps. A second person then jetted 10 ml of MCB from a syringe (#22 needle) onto the exposed surface of the peritoneal cavity. This wash was transferred to the centrifuge tube by pipette to complete the first stage of the cell isolation.

A typical yield of leukocytes was $5 \times 10^{5} - 10^{6}$ cells per rat of which approximately 50% were identified as mast cells. Two methods were used to crudely identify mast cells.

Under the light microscope, mast cells underwent a profound and quick change ($\sim$ 1 minute) when exposed to relatively high concentrations of compound 48/80. The mast cell visibly degranulated and a characteristic halo of vesicles surrounded the cells. Those cells were assumed to be mast cells because of their sensitivity to the classical mast cell degranulating agent; compound 48/80. A staining procedure differentiated mast cells from the other leukocytes. The rat peritoneal cells were exposed to 1% alcian blue and 3% acetic acid in 0.15 M NaCl. The cells were washed by centrifugation and the stain discarded. Then the cells were immediately exposed to 0.1% safranin O in 1% acetic acid and 0.15 M NaCl. After five minutes the cells were washed and resuspended in saline. This preparation demonstrated the stages in mast cell differentiation. Small lymphocyte-like mast cells contain only calcium blue-stained granules. Large mature mast cells contain safranin-positive heparin granules and thus stain pink. Generally, approximately 50-70% of the leukocytes (cells not obviously red cells) showed this pink staining. Another 5-10% of the leukocytes showed the characteristic blue staining of the immature mast cell.

Purified mast cells used to verify the staining techniques were isolated by the method of Morrison et al (1974). Their method used a 1XG gravity separation of

rat peritoneal cells in a 1.5% BSA solution of buffered saline. The procedure was modified by centrifuging the syringe at 100XG for eight minutes. Even with this extra g-force the separation was not as good as described by Morrison et al (1974) and there was no detectable "mast cell" pellet. Nevertheless, this partial isolation allowed some verification of the above staining procedure.

In general mast cells were not isolated from the rat peritoneal cell mixture for several expedient reasons. Morrison et al (1974) showed that compound 48/80, a degranulating agent used in this study, bound exclusively to the mast cell population. More important, the assay for mast cell amine release involved prepacking of the rat peritoneal cells with ^{3}H-serotonin. Morrison et al (1974) demonstrated that the serotonin was incorporated almost exclusively into mast cells. Furthermore, the ^{3}H-serotonin behaved the same as the endogeneous histamine. The release of ^{3}H-serotoin and histamine was identical when measured as a function of dosage of compound 48/80. Thus, for practical purposes the mast cells were not isolated from the rat peritoneal cell mixture. The terms mast cells and rat peritoneal cells are used interchangeably in this study because of the exact functional similarities with regards to elicited release of biogenic amines.

The second stage of mast cell preparation involved the loading of ^{3}H-serotonin into the cells. The cells from one to four rats were suspended at room temperature in 50 ml of MCB containing approximately 20 μCi of ^{3}H-serotonin (Amersham). After 1-1.5 hours the cells were washed four to five times in 50 ml of PBS or MCB with the final suspension always in MCB. These cells were aliquoted into 1.5 ml microcentrifuge tubes for subsequent preincubations and pharmacological challenges. Double label experiments using ^{86}RbCl (Amersham) were accomplished by simultaneous loading of cells with ∿ 30 μCi of $^{86}Rb^{+}$ along the ^{3}H-serotonin.

Measurement of ^{3}H-Serotonin Release in Rat Mast Cells

In a typical mast cell experiment 1.0 ml of ^{3}H-serotonin packed cells were aliquoted into plastic microcentrifuge tubes. If preincubation was desired a small volume of the stock solution (i.e., 100X $CaCl_2$) was first added to the microcentrifuge tube. After the preincubation period small volumes of drug or hapten stock solutions were added to the tubes. The tubes were then shaken and placed in a 37^{o}C shaker-water bath for the desired length of time. The tubes were then centrifuged at high speed in Eppendorf table top ultracentrifuges for three minutes. Aliquots of the supernate were transferred to scintillation vials. The ^{3}H-serotonin and ^{86}Rb were counted in scintillation fluid with a Beckman counter using standard methods. In a procedure to determine the maximum possible release of ^{3}H-serotonin, several cell samples were not centrifuged. These samples were acidified with $\sim$ 25 $\mu\ell$ of concentrated hydrochloric acid and placed in a steam bath for 10-20 minutes. Aliquots of the product were counted and the results served as the 100% potential release level for ^{3}H-serotonin in the rat peritoneal cell population.

P815 Mastocytoma: Origin and Maintenance

The P815 mastocytoma was induced in a male DBA/2 mouse by repeated exposure to methylcholanthrene (Dunn and Potter, 1957). The distinguishing feature of the tumor cells were their high degree of granular metachromasia when stained by 0.5% toluidine blue. The number of stained granules per cell was greatly reduced when compared to the normal mast cell. The tumor was first introduced to tissue culture by Lundak and Raidt (1973). The cell line was obtained from the Salk Institute of Biological Studies.

The P815 mastocytoma cell line was maintained in incubators kept at 37°C and 5% CO_2/100% H_2O. The medium was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum (GIBCO). Further additions were 1.2 mg/ml glutamine, 0.3 mM non-essential amino acids (GIBCO), 100 µg/ml streptomycin and 100 units/ml penicillin G. Cell counts were determined with standard hemocytometers.

Verapamil

The verapamil used in this study was a generous gift from Knoll Pharmaceutical Company, Whippany, New Jersey.

RESULTS

Results Establishing the General Nature of the Release of ^{3}H-Serotonin from Mast Cells

Four basic criteria were established to show that the release of ^{3}H-serotonin from mast cells caused by verapamil involved degranulation of mast cells; 1) dose-dependent release of amines from the cell; 2) morphological changes similar to those caused by the "classical" degranulating agent, compound 48/80; 3) dependence of the process on metabolic energy; 4) demonstration that the drug did not lyse or kill the cell. The results establishing these criteria are described below.

Verapamil causes a dose-dependent release of ^{3}H-serotonin from rat mast cells (Figure 1). The rat cells were loaded with ^{3}H-serotonin in a method very similar to that described by Morrison et al (1974). They concluded that the release of ^{3}H-serotonin was exactly correlated with the release of histamine, an indication that both of these biogenic amines were released by the same mechanism upon degranulation of the mast cell. Morrison et al (1974) also showed that the ^{3}H-serotonin was taken up almost exclusively by the mast cells in a mixture of rat peritoneal cells and suggested that the ^{3}H-serotonin was bound in the granules

of the mast cell to the amine-protein-heparin complex described by Bergendorff and Uvnas (1972).

Mast cells exposed for one hour to high concentrations of verapamil (2 mg/ml) were observed under the light microscope to undergo a degranulation reaction similar to what is seen for mast cells exposed to the "classical" degranulation agent, compound 48/80. The degranulation of mast cells is dependent on cellular levels of ATP (Johanson, 1980). Figure 1 demonstrates that release of ^{3}H-serotonin from mast cells by verapamil was inhibited by preincubation of the cells in a mixture of the metabolic poisons, KCN and 2-deoxyglucose. As a positive control, the "classical" degranulating agent compound 48/80 was also shown to require metabolic energy for the degranulation of mast cells (Figure 2).

Mast cells exposed for one hour to concentrations of verapamil that could induce maximal release of ^{3}H-serotonin in five minutes were found to exclude the trypan blue viability stain. Less than 2% of the mast cells showed staining by the dye indicating that the cells were viable and the drug was not releasing ^{3}H-serotonin through non-specific lysis.

Figure 1

The role of energy metabolism in the release of ^{3}H-serotonin from rat peritoneal cells challenged by verapamil. The metabolically inhibited cells (-o-o-) were preincubated in 10 mM 2-deoxyglucose and 0.3 mM KCN for one hour and 20 minutes at 25°C in glucose-free MCB. Normal cells (-●-●-) were preincubated in regular MCB in parallel with the metabolically inhibited cells. The challenge with verapamil was for one hour at 37°C. The statistical error was estimated to be 10%.

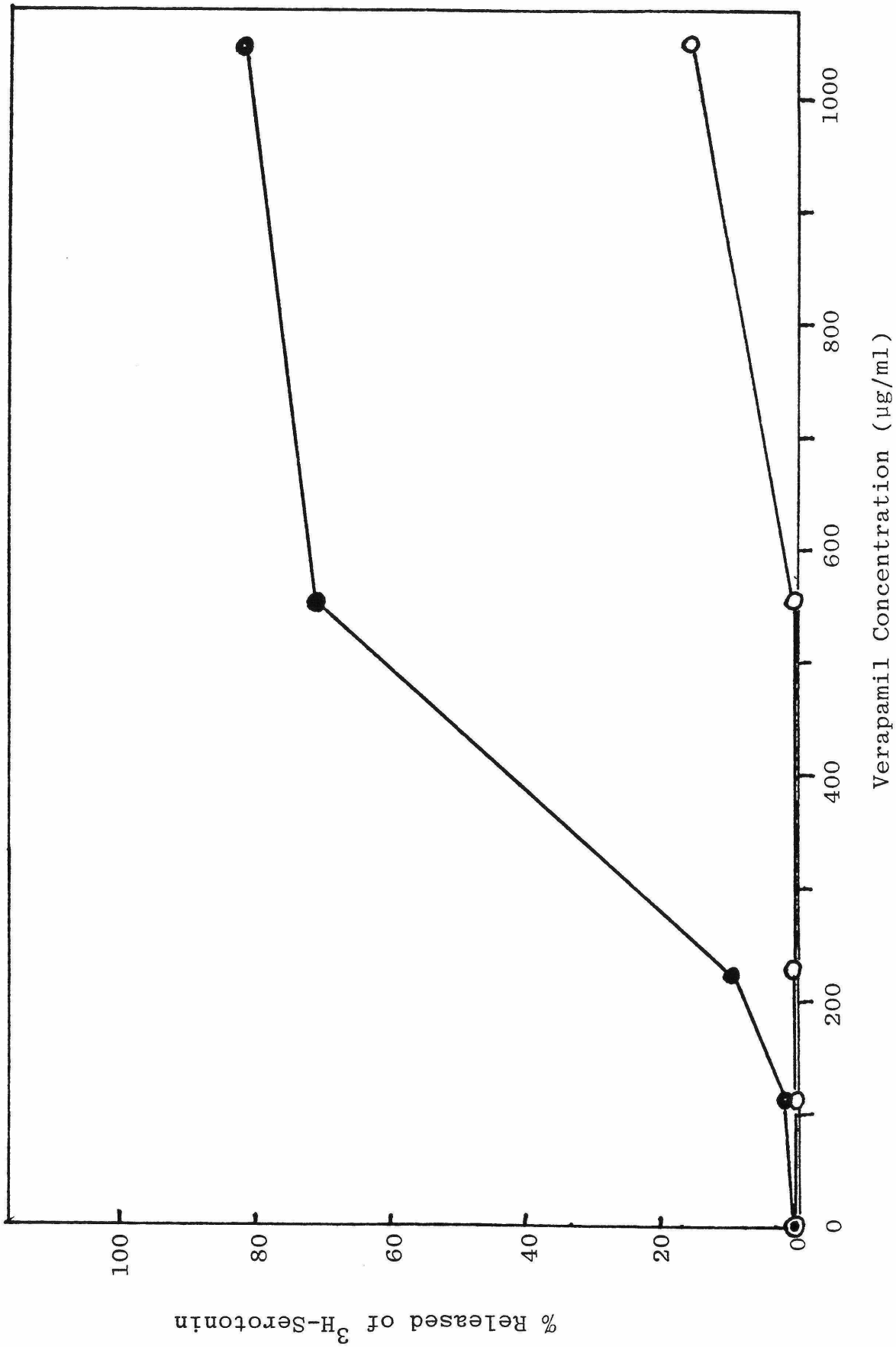
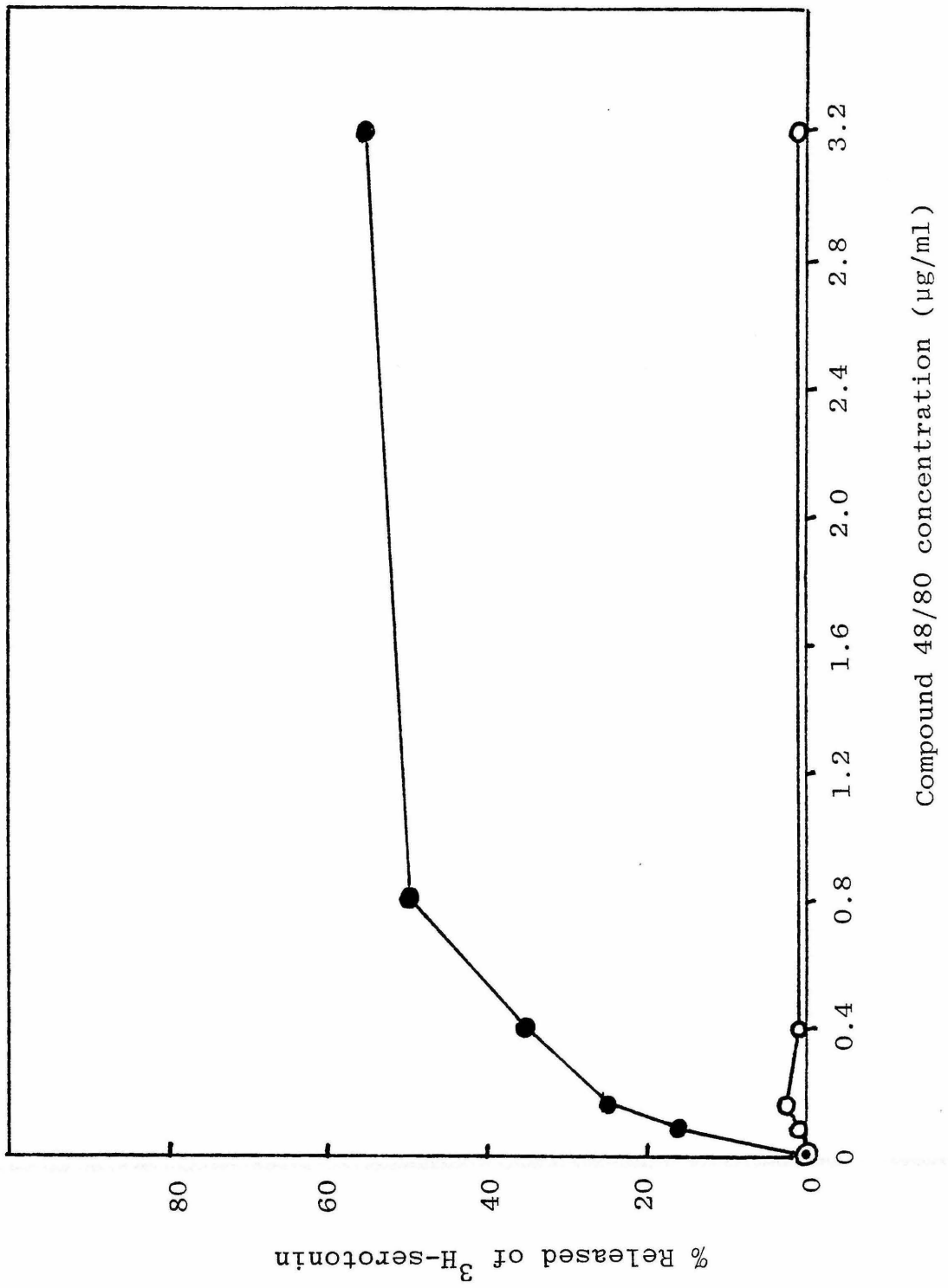

% Released of ^{3}H-Serotonin
0
20
40
60
80
100
0
200
400
600
800
1000
Verapamil Concentration (µg/ml)

Figure 2

The role of energy metabolism in the release of ^{3}H-serotonin from rat peritoneal cells challenged with compound 48/80. The metabolically inhibited cells (-o-o-) were preincubated in 10 mM 2-deoxy-glucose and 0.3 mM KCN for 40 minutes at 25°C in glucose-free MCB. Normal cells (-●-●-) were pre-incubated in regular MCB in parallel with the meta-bolically inhibited cells. The challenge with com-pound 48/80 was for one hour at 37°C. The statis-tical error was estimated to be 10%.


% Released of ^{3}H-serotonin
0
20
40
60
80
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
Compound 48/80 concentration (µg/ml)

$^{86}Rb^{+}$-^{3}H-Serotonin Double Label Experiments in Mast Cells

Loading of $^{86}Rb^{+}$ and ^{3}H-serotonin into mast cells and then subsequently monitoring the release of these radioactive substances from the mast cells represents a novel experimental method (Figures 3 and 4). The results of the double label experiment indicate that compound 48/80 causes a dose-dependent increase in the initial rate (15 minute challenge) of $^{86}Rb^{+}$-efflux and release of ^{3}H-serotonin from rat peritoneal cells. (Figure 3) The results for rat peritoneal cells challenged with verapamil were somewhat different (Figure 4). Low concentrations of verapamil cause a small decrease in the spontaneous release of ^{3}H-serotonin and $^{86}Rb^{+}$. Whereas high concentrations of verapamil caused an increase in the release of ^{3}H-serotonin and $^{86}Rb^{+}$ from the mast cells.

The release of $^{86}Rb^{+}$ is thought to be proportional to the release of K^{+} from cells (Putney, 1979). However, the exact relationship between K^{+}-efflux and $^{86}Rb^{+}$-efflux has not been established for the mast cell system.

Compound 48/80 caused a concentration dependent increase in the rate of $^{86}Rb^{+}$-efflux in P815 mastocytoma cells (Figure 5). Unlike normal mast cells (Figure 2), relatively high concentrations of compound 48/80 were required to cause a significant increase in the rate of $^{86}Rb^{+}$-efflux from the P815 cells. Figure 5 showed

there was no difference between the dose-response curves for $^{86}Rb^{+}$-efflux in P815 cells challenged with compound 48/80 in the presence of extracellular calcium (2 mM Ca^{++}) and in the absence of extracellular calcium (short preincubation in MCB containing 0.1 M EGTA).* It has been shown that $^{86}Rb^{+}$-efflux is proportional to the concentration of cytosolic Ca^{++} in other cell systems (Putney, 1979), accordingly we assume that the increase in $^{86}Rb^{+}$-efflux in P815 cells challenged with compound 48/80 likewise reflects an increase in the concentration of calcium in the cytosolic pool.

Figure 6 shows a dose-dependent increase in $^{86}Rb^{+}$-efflux in P815 cells challenged by verapamil. This result is very similar to the data from $^{86}Rb^{+}$-efflux in P815 cells challenged with compound 48/80.

*This short preincubation in 0.1 M EDTA was of short enough duration and low enough concentration to not significantly deplete the intracellular pools of calcium but insured there was complete removal of all extracellular calcium. In contrast, preincubation of mast cells in 2 mM EDTA for three hours caused mast cells to lose their ability to respond to compound 48/80 (Douglas and Ueda, 1973).

Figure 3

Double label experiment measuring the initial rates of release of $^{86}Rb^{+}$ and ^{3}H-serotonin from rat peritoneal cells challenged with compound 48/80. The cells were isolated and loaded with $^{86}Rb^{+}$ and ^{3}H-serotonin by the methods described in Materials and Methods, Chapter II. The challenge with compound 48/80 was for 15 minutes at 37°C in MCB. The spontaneous release of ^{3}H-serotonin (---o---o---) was 795 $\pm$ 70 cpm and the maximum possible release was 17,100 $\pm$ 500 cpm. The spontaneous release of $^{86}Rb^{+}$ (-●-●-●-●-) was 404 $\pm$ 18 cpm and the maximum possible release was 795 $\pm$ 25 cpm. The standard deviation for the blanks and the maximum possible release determine the ranges of the statistical error in this experiment.

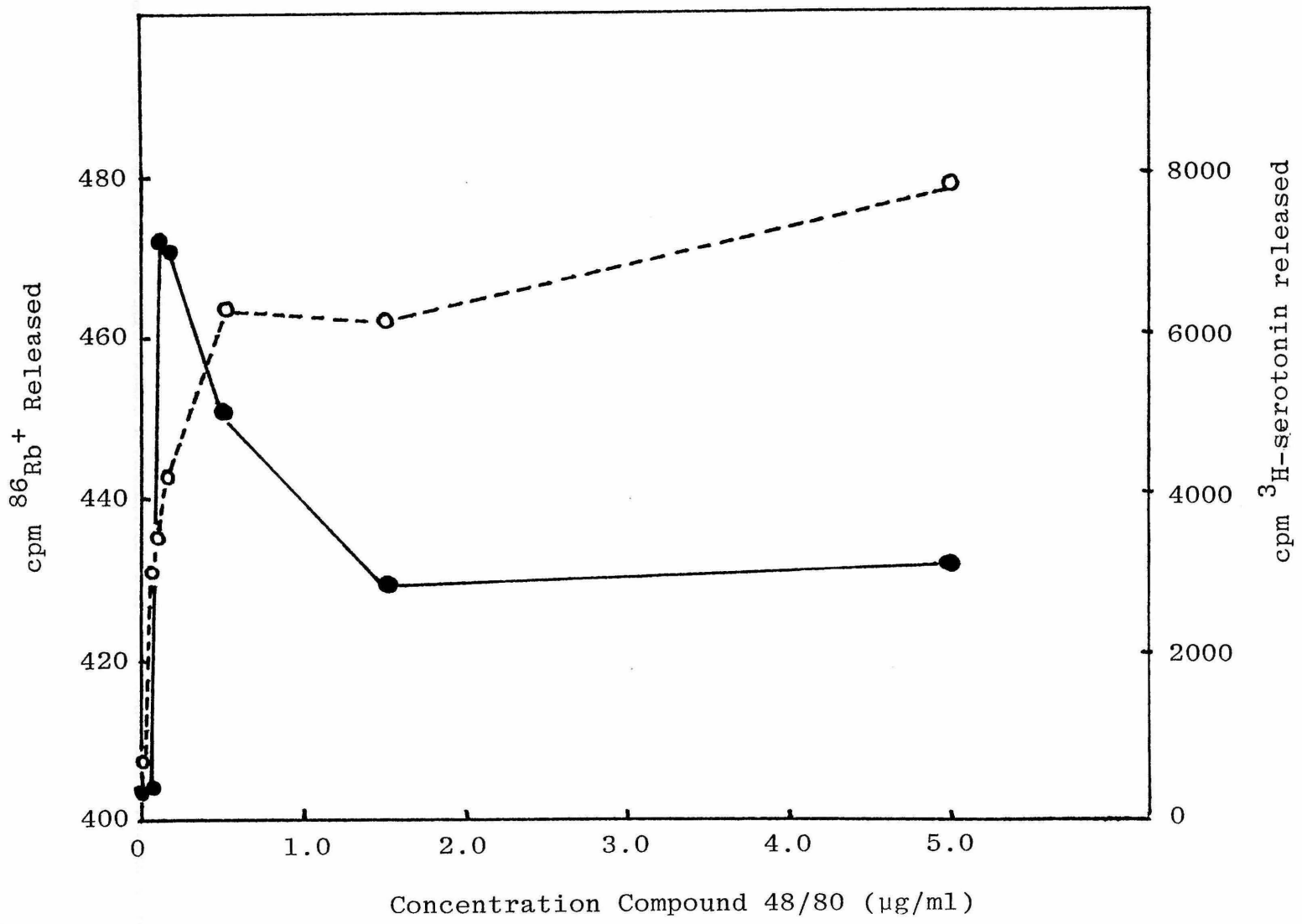

480
460
440
420
400
cpm $^{86}Rb^{+}$ Released
8000
6000
4000
2000
0
cpm ^{3}H-serotonin released
0
1.0
2.0
3.0
4.0
5.0
Concentration Compound 48/80 (µg/ml)

Figure 4

Double label experiment measuring the initial rates of release of $^{86}Rb^{+}$ and ^{3}H-serotonin from rat peritoneal cells challenged with verapamil. The details of this experiment are described in Figure 3. The table below includes standard deviations for the counts of the blank and maximum possible release.

	Spontaneous Release	Maximum Possible Release
^{3}H-serotonin (---o---o---o---)	795 ± 70 cpm	17,100 ± 500 cpm
$^{86}Rb^{+}$ (-●-●-●-●-)	404 ± 18 cpm	795 ± 25 cpm

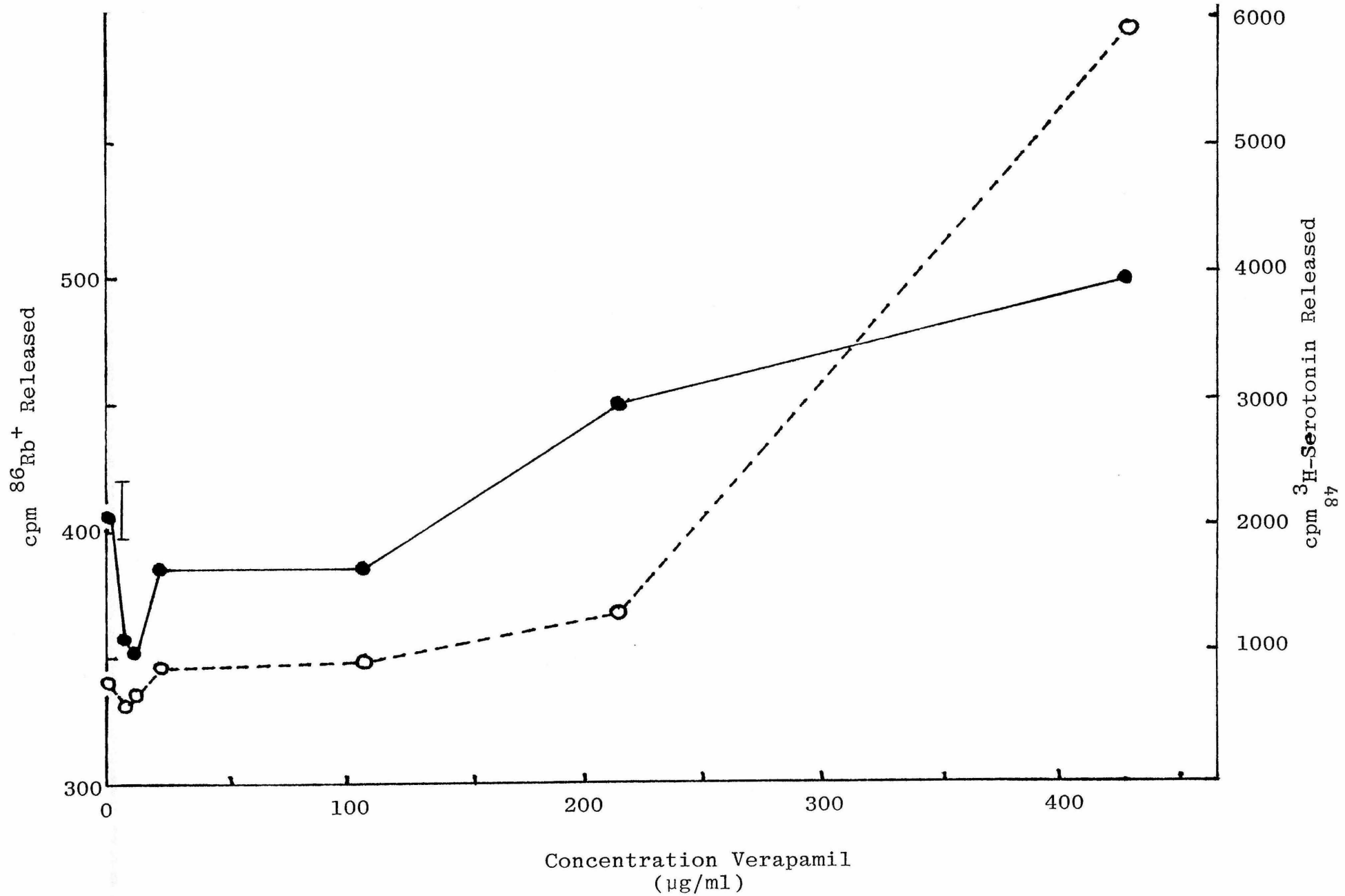

cpm $^{86}Rb^{+}$ Released
cpm ^{3}H-Serotonin Released
500
400
300
6000
5000
4000
3000
2000
1000
0
100
200
300
400
Concentration Verapamil
(µg/ml)

Figure 5

The release of $^{86}Rb^{+}$ from P815 Mastocytoma cells challenged by compound 48/80. The mastocytoma cells were suspended in a 2 μCi/ml $^{86}RbCl_2$ of MCB for 37°C for two hours. The cells were washed five times in Ca-free MCB and divided in two. One aliquot was incubated for 30 minutes in 2 mM Ca^{++} MCB (-●-●-) and the second aliquot was incubated 30 minutes in 0.1 mM EGTA MCB (--o---o---). The cells were aliquoted into small portions and challenged with compound 48/80 for 10 minutes. The maximum $^{86}Rb^{+}$ count for the mastocytoma cells was 2320 cpm. The estimated error of each sample was 10%.

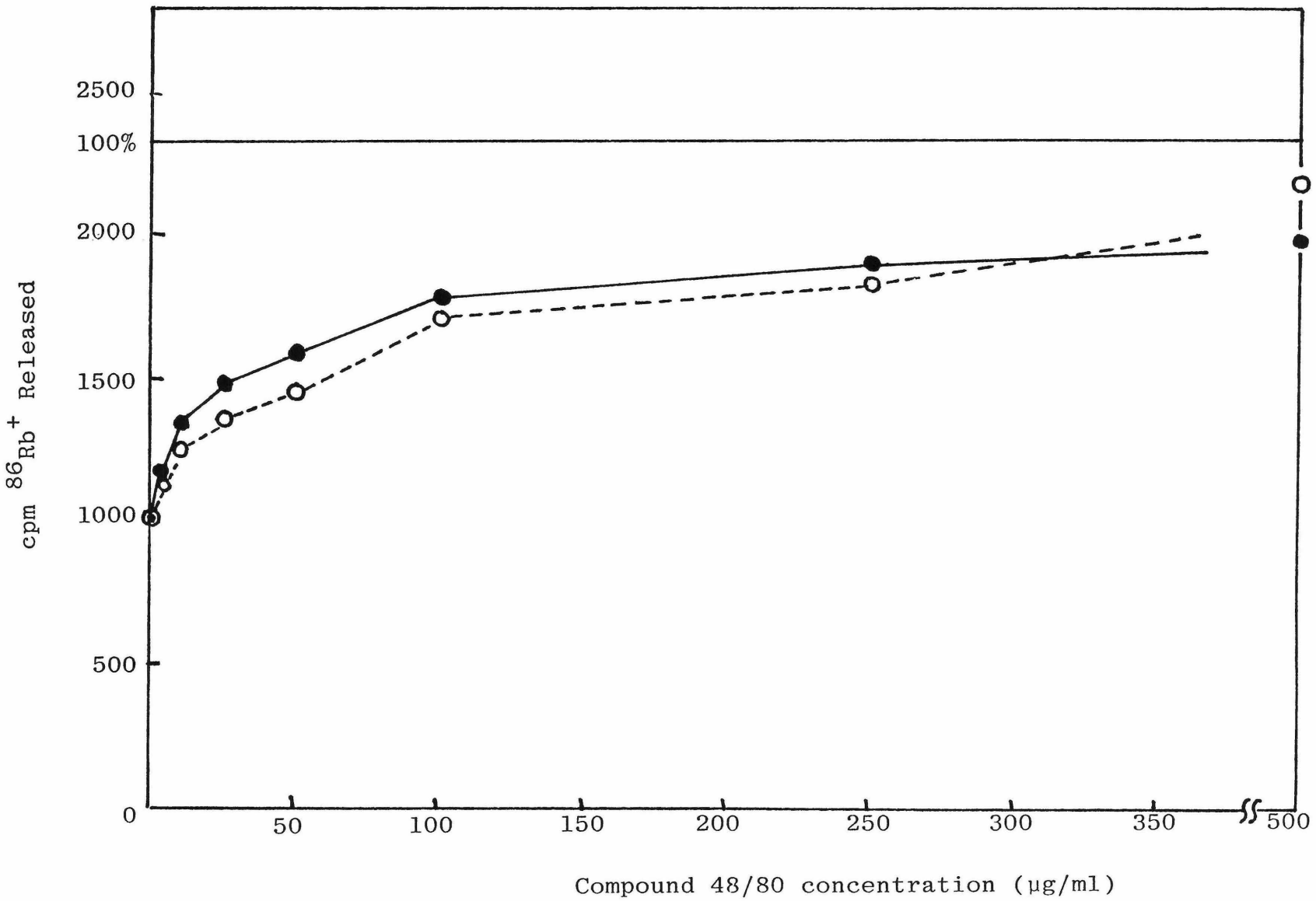
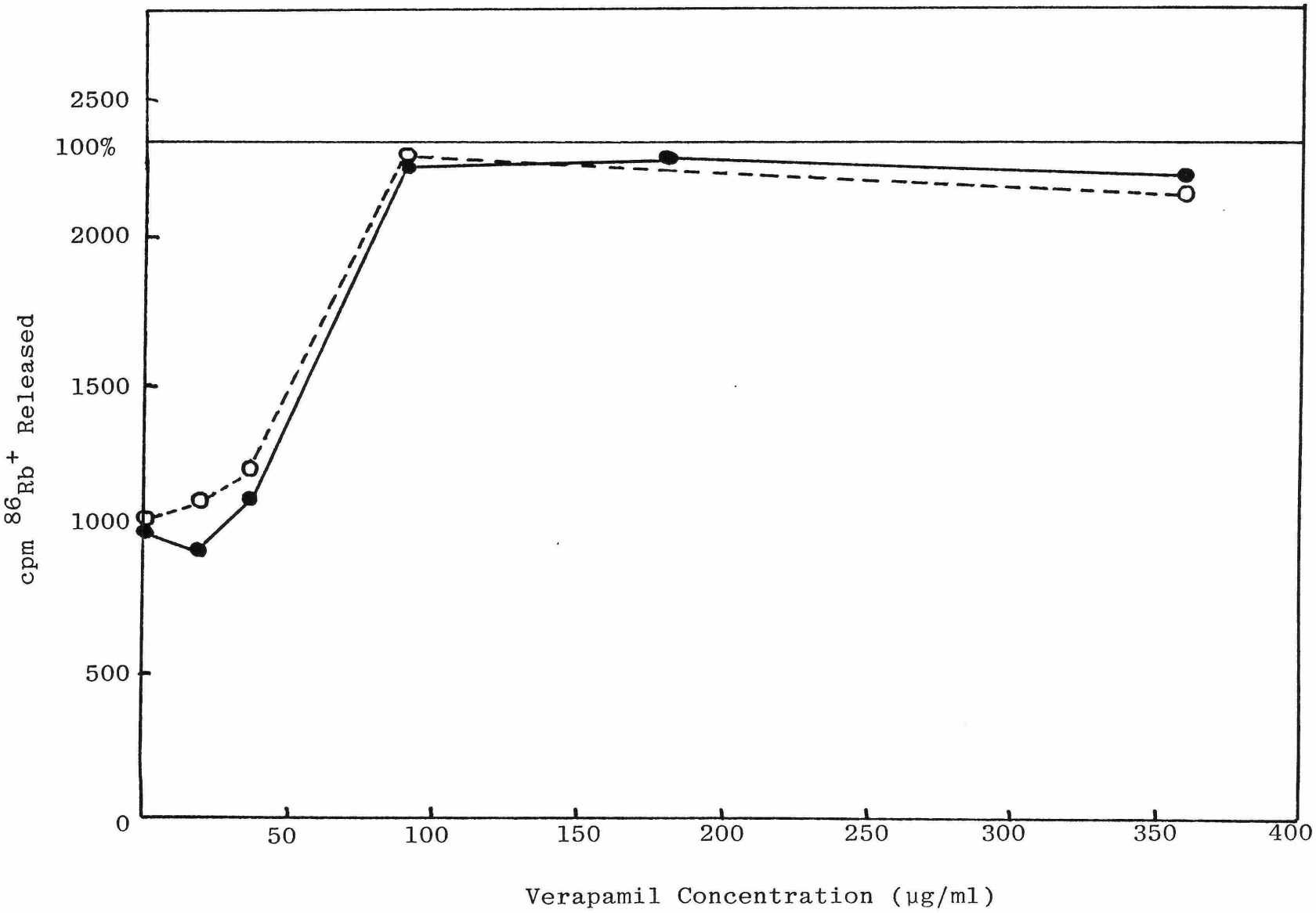

2500
100%
2000
1500
1000
500
0
cpm $^{86}Rb^{+}$ Released
50
100
150
200
250
300
350
500
Compound 48/80 concentration (µg/ml)

Figure 6

The release of $^{86}Rb^{+}$ from P815 mastocytoma cells by verapamil. The detailed procedure for challenging the mastocytoma cells with verapamil is described in Figure 5. The cells were pre-incubated in EGTA (---o---o---) and 2 mM Ca^{++} (-●-●-●-) then challenged with verapamil for 10 minutes.


2500
100%
2000
1500
1000
500
0
cpm $^{86}Rb^{+}$ Released
50
100
150
200
250
300
350
400
Verapamil Concentration (µg/ml)

DISCUSSION

The Effect of Verapamil on Mast Cells

The data in Figures 1 and 5 illustrate that verapamil caused the release of ^{3}H-serotonin from rat mast cells. This is the first demonstration that verapamil could cause the release of biogenic amines from mast cells.

The discovery that verapamil causes the release of ^{3}H-serotonin from mast cells is surprising considering the previously proposed mechanism of action of the drug and what is known about the mechanism of degranulation of mast cells. Fleckenstein (1977) proposed that verapamil was a member of the class of drugs that were specific "calcium-channel" antagonists. The release of histamine from mast cells caused by the classic degranulation agent compound 48/80 is thought to occur by a mechanism involving the release of calcium from intracellular pools into the cytosolic calcium pool (Ueda and Douglas, 1973). If the release of ^{3}H-serotonin from mast cells caused by verapamil is dependent on the release of calcium from membrane-bound intracellular pools and if the drug can function by blocking intracellular "calcium-channels" of these pools, then verapamil, itself, would not be expected to cause a release of ^{3}H-

serotonin from mast cells. The degranulation of mast cells caused by the interaction of antigen and membrane-bound IgE requires the influx of extracellular calcium (Hirata and Axelrod, 1980). If verapmil, functions by blocking "calcium-channels" and therefore inhibits the influx of extracellular calcium it would not be expected to cause the degranulation of mast cells. The surprising result that verapamil, itself, causes the release of ^{3}H-serotonin from mast cells, therefore, casts serious doubt on the proposition that the drug is acting strictly as a specific "calcium-channel" antagonist as described by Fleckenstein (1977).

The measurement of $^{86}Rb^{+}$-efflux from mast cells was an attempt to define a role for cytosolic calcium in the mechanism of action of verapamil and compound 48/80. Figures 3 and 4 showed that the release of ^{3}H-serotonin from mast cells by compound 48/80 and high concentration of verapamil occurred with an increase in $^{86}Rb^{+}$-efflux from the mast cells. In a wide variety of cells and tissues an increase in the efflux of $^{86}Rb^{+}$ (an analogue of K^{+}) was correlated with an increase in cytosolic Ca^{++} concentrations (Putney, 1979). If this correlation could be extended to the mast cell, the release of ^{3}H-serotonin by compound 48/80 and high concentrations of verapamil would feature an increase in the concentration of cytosolic

Ca^{++}. If indeed the degranulation of the mast cell caused by compound 48/80 and high concentrations of verapamil features an increase in the concentration of cytosolic Ca^{++} and because the processes occur in the absence of extracellular calcium then the drugs could be mobilizing calcium from an intracellular pool into the cytosol.

The data of Figure 4 show a dose-dependent effect of verapamil that may be due to the drug acting as a specific "calcium-channel" antagonist (Fleckenstein, 1977). Low concentrations of verapamil (< 50 µg/l) caused a small decrease in both the spontaneous release of ^{3}H-serotonin and the spontaneous efflux of $^{86}Rb^{+}$ from mast cells. If the correlation described by Putney (1979) between $^{86}Rb^{+}$-efflux and the concentration of cytosolic Ca^{++} is valid for mast cells, this decrease in $^{86}Rb^{+}$-efflux caused by verapamil at low concentrations implies a decrease in the concentration of cytosolic Ca^{++}. This slight decrease in the spontaneous rate of $^{86}Rb^{+}$-efflux is the only evidence that could be a consequence of the blocking of "calcium-channels" in mast cells. Other effects caused by high concentrations of verapamil, such as the increased release of ^{3}H-serotonin from mast cells, must occur while overriding the effects of the proposed blockage of "calcium-channels" in the mast cells.

Compound 48/80 (Figure 5) and verapamil (Figure 6) cause a dose-dependent increase in $^{86}Rb^{+}$-efflux from P815 mastocytoma cells. The P815 cells are thought to be similar to immature mast cells and contain fewer granules relative to the normal mature mast cell (Dunn and Potter, 1957). The P815 cells do not undergo the morphological changes associated with degranulation when exposed to the "classical" degranulation agent compound 48/80. Therefore, the results of Figures 5 and 6 seem to indicate that the increase in $^{86}Rb^{+}$-efflux caused by compound 48/80 and verapamil could occur in "mast cells" that are unable to degranulate. If one assumes $^{86}Rb^{+}$-efflux is correlated with the concentration of cytosolic Ca^{++} (Putney, 1979), this result suggests that verapamil can, in fact, mobilize intracellular calcium into the cytosolic pool. In P815 cells, this early step in degranulation does not lead to degranulation itself because of a functional defect in the cells at some later step in the degranulation pathway.

Possible Indirect Effects of Verapamil on Blood Pressure

Verapamil has been useful in controlling acute and chronic hypertension (Zsoter, 1980). We propose that a component of the hypotensive effect of verapamil is due to the release of vasoactive substances from mast cells and basophils. For example, histamine and the slow reacting substance of anaphylaxis (SRS-A) are vasoactive substances released from mast cells that could lower blood pressure. Histamine causes an increase in the leakage of fluids from the peripheral vasculature into the connective tissue and relaxation of the smooth muscles of the peri-perhal vasculature both of which can lower blood pressure (Middleton, 1980). Also, SRS-A causes an increase in peripheral vascular permeability (Lewis and Austen, 1977). Interestingly, Ross and Jorgensen (1967) found that the hypotensive effect of verapamil in cats was totally accounted for by changes in the peripheral circulation, a principal site of action for both histamine and SRS-A.

Drug-induced hypotension caused by the release of vasoactive substances from mast cells is not without precedent, for Baltzby et al (1949) found that compound 48/80, the "classical" agent of degranulation of mast cells, caused a large decrease in the blood pressure of dogs. Vasoactive substances released from mast cells

probably do not account for all of the hypotensive effect of verapamil as the ability of verapamil to cause the *in vitro* relaxation of smooth muscles of the vasculature (Rosenberger and Triggle, 1978) could also account for a component of the *in vivo* hypotensive effect of the drug.

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CHAPTER III

A COMPARATIVE STUDY OF THE ACTIVATION OF MAST CELLS BY COMPOUND 48/80 AND VERAPAMIL

INTRODUCTION

Crosslinking and Degranulation of Mast Cells; IgE

Chapter II of this thesis examined two classes of drugs that caused stimulus-secretion coupling in mast cells. The bifunctionality of verapamil and the polymeric nature of compound 48/80 suggest that the drugs can act as crosslinking agents. This introduction briefly reviews the role of crosslinking and aggregation in the degranulation of mast cells.

Many of the structures involved in the degranulation of mast cells by antigen and IgE are understood on a molecular basis. For example, a specific receptor for IgE on rat basophilic leukemic cells has been characterized as a glycoprotein of molecular weight 77,000 (Metzger, 1978). IgE binds reversibly to the receptor with a high affinity ($Ka \geq 10^{10}\ M^{-1}$) and there are approximately 10^6 receptors per tumor cell (Metzger *et al*, 1976).

IgE has the Y shape characteristic of immunoglobulins. The molecule is composed of two heavy and two light chains which form seven distinct globular domains. Each arm of the Y is composed of two such domains, (V_H; V_L and C_H1; C_L); the V_H; V_L domain contains the antigen combining site. The three remaining regions of the heavy chain (C_H2, C_H3, C_H4) form three domains which together constitute the Fc

tail of the antibody; this tail contains the site which binds to the IgE receptor (Metzger, 1979). The primary structures of the ε heavy chain of a human IgE-myeloma protein has been determined (Bennich and von Bahr-Lindstrom, 1974) as has the partial nucleotide sequence and the genomic location of the gene coding for the constant region of a mouse ε heavy chain (Nishida et al, 1981).

IgE binds with high affinity to its receptor in the absence of antigen. Such binding does not itself activate the mast cell nor will the binding of monomeric antigen to the cell-bound IgE activate the mast cell. For example, Siraganian et al (1975) demonstrated that bivalent antigen was the minimal unit required to degranulate mast cells. Moreover, Ishizaka and Ishizaka (1969) found that crosslinking IgE bound to mast cells with anti-IgE antibodies caused the mast cell to relase histamine. Monomeric Fab' fragments of the anti-IgE antibodies were ineffective in degranulating the mast cells. Furthermore, antibodies that bound directly to IgE-receptors were found to trigger the release of histamine from mast cells (Ishizaka and Ishizaka, 1978). Monomeric Fab' fragments of the anti-receptor antibody were ineffective in degranulating the mast cells. This finding demonstrated that crosslinking of the receptors for IgE, regardless of IgE and antigen, was sufficient to degranulate the mast cell. In the same system, crosslinking of

the IgE receptors with antibody caused an increase in Ca^{++} permeability in the mast cells (Ishizaka *et al*, 1979) and stimulated phospholipid methylation in the mast cells (Ishizaka *et al*, 1980). Phospholipid methylation and increased permeability to Ca^{++} in the plasma membrane are early steps in the degranulation of mast cells caused by IgE (Hirata and Axelrod, 1980. Segal *et al* (1977) demonstrated that dimerized IgE, as opposed to monomeric IgE, was able to trigger the release of histamine from mast cells. The dimeric IgE was prepared by chemical crosslinking with a bifunctional reagent and did not require antigen for its activity.

In summary, the evidence is overwhelming that the triggering of mast cells by IgE involves receptor aggregation via a crosslinking event.

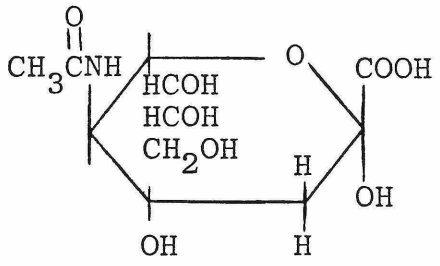
Crosslinking and Degranulation of Mast Cells; Non-Antibody Reagents

A large variety of agents besides IgE are capable of releasing histamine from mast cells. Some of these agents clearly do not involve crosslinking in their mechanism. For example, A23187, a calcium ionophore activates mast cells by transporting calcium across the cell membrane (Cochrane and Douglas, 1974). Others, such as dextran with their extensive polymeric structure may trigger the release of histamine by the relatively non-specific aggregation of cell surface receptors (Baxter and Adamik, 1977).

Compound 48/80 is one of a large number of polycationic molecules that cause the release of histamine from mast cells. Among the polycationic agents that affect mast cells are polylysine, protamine, somatostatin, bee venom mellitin, polymixin B, and bradykinin. The anaphylatoxins, C3a and C5a, have a large percentage of basic amino acids in their primary sequences (Hugli, 1975; Fernandez and Hugli, 1976) including a carboxy-terminal arginine that is essential for activity (Dias da Silva and Lepow, 1967; Cochrane and Muller-Eberhard, 1968).

Sialic Acids and Neuraminidase

Sialic acids are ubiquitous components of all vertebrate cell membranes (Gottschalk, 1972). The negatively charged sialic acids which occur as the terminal residues of complex heteropolysaccharides of glycoproteins are almost exclusively linked from carbon atom 2 of the sialic acid to either D-galastose (at positions 3 or 6) or to N-acetyl-D-galactosamine (at position 3) or to other sialic acids (at position 8) (Tuppy and Gottschalk, 1972). In addition to glycoproteins, glycolipids have been found to have sialic acid components. For example, Hakomori and Saito (1969) found a glycosphingolipid on red cells that contained N-glycolylneuraminic acid and Kaufman et al (1968) isolated a similar glycosphingolipid from bird brains. On the red cell membrane, sialic acids account for 80% of the surface charge density at physiological pH as measured by microelectrophoresis (Herndier and Brooks, 1975). The high content of sialic acid on plasma membranes and their negative charge at physiological pH make sialic acids likely candidates for binding with the polycationic agents that cause degranulation of mast cells (Baxter and Adamik, 1977).



N-acetylneuraminic acid
(a sialic acid)

Neuraminidase is a specific enzyme which cleaves the terminal sialic acids off the heteropolysaccharide units of glycoproteins. The specificity of the enzyme is relatively independent of the preceding saccharide unit but is sensitive to the pattern of oxygen substitution on the sialic acid itself (Schauer, 1973). Esterification of the carboxyl group of neuraminic acid blocks the action of neuraminidase completely (Karkas and Chargaff, 1964). This highly specific action of neuraminidase insures that the treatment of cells with the enzyme removes only sialic acids and does not cause non-specific damage to the cell membrane.

RESULTS

Molecular Weight Determination of Crosslinked Verapamil

The G-15 column was used to separate the iodinated crosslinked verapamil from the unreacted iodine. This column was previously calibrated by tritiated sugars. The tetrasaccharide glucose α(1 → 4) glucose α(1 → 3) glucose α(1 → 4) glucose isolated from Aspergillus niger was obtained via a generous gift by Dr. Nordin (Tung and Nordin, 1970). This tetrasaccharide was reduced with NaBT4 (Amersham) for 16 hours at 5°C. The reaction was terminated by cold $NaBH_4$ ml and the addition of glacial acetic acid. Similarly ^{3}H-glucose α(1 → 4) glucitol and ^{3}H-glucitol were synthesized by borohydride reduction. The elution volumes (V) of these sugars on the G-15 column served as molecular weight markers for the crosslinked verapamil. A plot of $V-V_0/V_o$, where V_0 is the void volume, versus logarithm of molecular weight for the sugars and crosslinked verapamil is shown in Figure 7.

A portion of the lyophilized ^{125}I-crosslinked verapamil was resuspended in water. Also dissolved in this solution were the molecular weight markers; reduced glutathione, vitamin B_{12}, blue dextran, and bovine trypsin inhibitor. The mixture was separated by gel filtration on a Sephadex G-25 column. The ^{125}I-crosslinked verapamil was monitored by gamma counting aliquots of the column

effluent. The column profiles of the molecular weight markers were determined by UV-visible spectrometry. A plot of $V-V_0/V_0$, were V is the elution volume and V_0 is the void volume, versus log of the molecular weight for the G-25 column is given in Figure 8.

Extrapolation from Figures 7 and 8 gives a molecular weight of 1290 $\pm$ 170 for crosslinked verapamil on the G-15 column and 1260 $\pm$ 180 for the G-25 column. The theoretical molecular weight for a monoiodinated dimer with a methylene bridge is 1173. The theoretical molecular weights of monoiodinated monomer and monoiodinated trimer with two methylene bridges are 570.5 and 1514, respectively. A dimeric structure for iodinated cross-linked verapamil is proposed since only one peak was found in the ^{125}I-gamma count profile and there were no significant counts where a trimer or a monomer would theoretically occur in the profile.

Figure 7

Molecular weight determination of crosslinked verapamil gel filtration on a G-15 Sephadex column. The molecular weight standards were tritiated glucitol (182), glucose α(1 → 3) glucitol (344) and glucose α(1 → 4) glucose α(1 → 3) glucose α(1 → 4) glucitol (668).

Figure

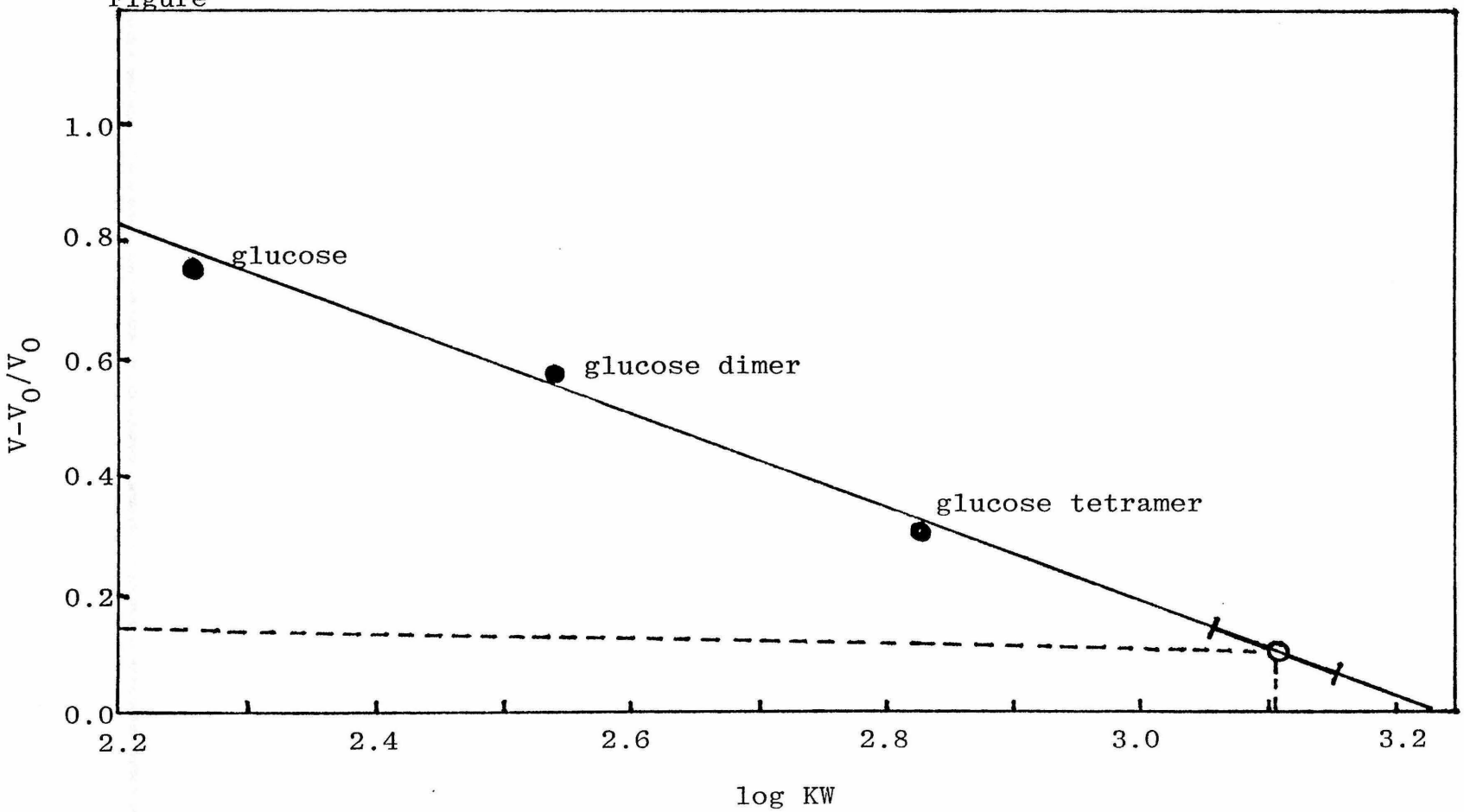
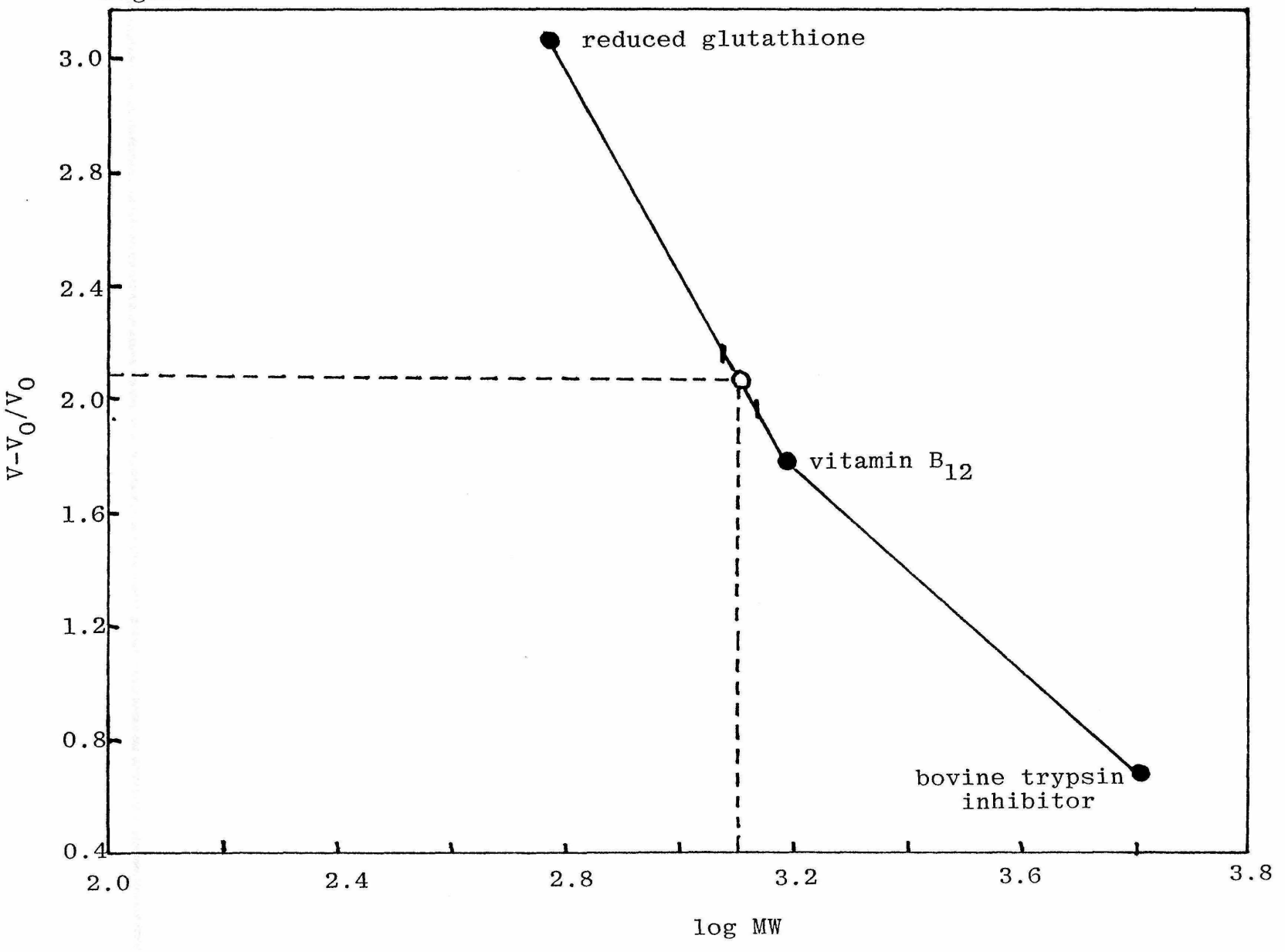

1.0
0.8
0.6
0.4
0.2
0.0
V-V0/V0
glucose
glucose dimer
glucose tetramer
2.2
2.4
2.6
2.8
3.0
3.2
log KW

Figure 8

Molecular weight determination of crosslinked verapamil: gel filtration of a G-25 Sephadex column. The molecular weight standards were blue dextran (2×10^6), bovine tryspin inhibitor (6518), vitamin B_{12} (1355) and reduced glutathione (307).

Figure


reduced glutathione
vitamin B_{12}
bovine trypsin inhibitor
$V-V_0/V_0$
3.0
2.8
2.4
2.0
1.6
1.2
0.8
0.4
2.0
2.4
2.8
3.2
3.6
3.8
log MW

NMR Characterization of Crosslinked Verapamil

In order to elucidate the structure of the methylene bridged verapamil dimer, a 500.13 MHz proton nuclear magnetic resonance (nmr) study was undertaken on both the monomer and dimer in D_2O. A summary of the results follows.

Figure 9 is the 500.13 MHz proton nmr spectrum for verapamil in D_2O. The assignments of the resonances were obtained via homonuclear decoupling experiments. One interesting feature is the asymmetry of the resonances for the isopropyl methyl groups ($\delta \sim 0.8$, 1.2 ppm). This is due to the fact that, because of the sterically hindered nature of the isopropyl group's position in the molecule, rotation of the group is severely hindered. Thus, the chemical shifts of the two methyl groups are affected differently by the ring currents of the adjacent phenyl group.

Figure 10 shows the assigned spectrum and proposed structure for the methylene-bridged dimer. Most of the assignments were obtained using homonuclear decoupling. Confirmation of the existence of the bridge ($\delta \sim 3.8$ ppm) was obtained via a T_1 experiment. Because of the immobility of the $-CH_2-$bridge relative to the rapidly rotating $-OCH_3$ groups, the T_1 of the bridge should be shorter than that for the $-OCH_3$ groups was observed. The signal assigned the protons of the methylene bridge ($\delta \sim 3.8$ ppm) had an integrated intensity of two protons per proposed dimer.

The asymmetric nature of the dimer was inferred from the loss of symmetry for the downfield $-CH_3$ groups attached to the isopropyl groups. If the dimer were symmetric, this resonance would be a clean doublet; in fact, it is two overlapping doublets (which appear as a triplet). This in conjunction with the observation of two $N-CH_3$ resonances ($\delta \sim 2.8$ ppm), supports the proposed structure.

The relative order of reactivity of O-dimethoxybenzene to electrophilic substitution (para > ortho) and steric effects due to the bulky isopropyl and cyano groups (Evans, 1981, personal communication) were also considered in determining the position of the bridging methylene.

In order to detect the amount of terminal aromatic CH_2OH groups within the sample, the nmr spectrum of the dimer was obtained in d_6-DMSO. Two resonances at $\delta \sim 4.4$ and 4.7 ppm were found which could be assigned to such groups. Their total concentration in the sample is less than 10%, as determined by comparing their integral with that for both the aromatic protons and the methylene bridge. Thus, the predominant species has the structure shown in Figure

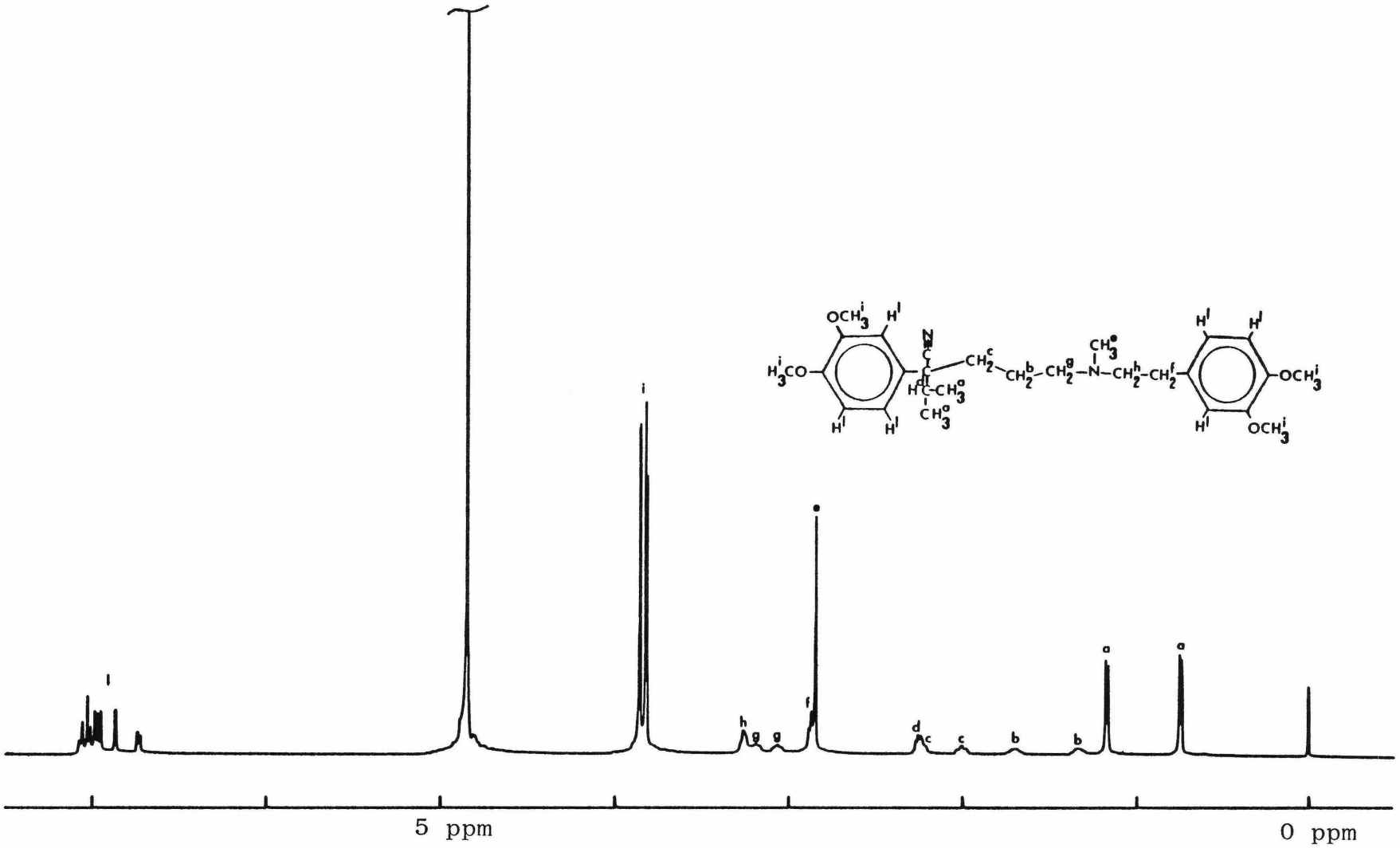


Figure 9. Proton magnetic resonance (500.13 MHz) spectrum and peak assignments of monomeric verapamil in D_2O.

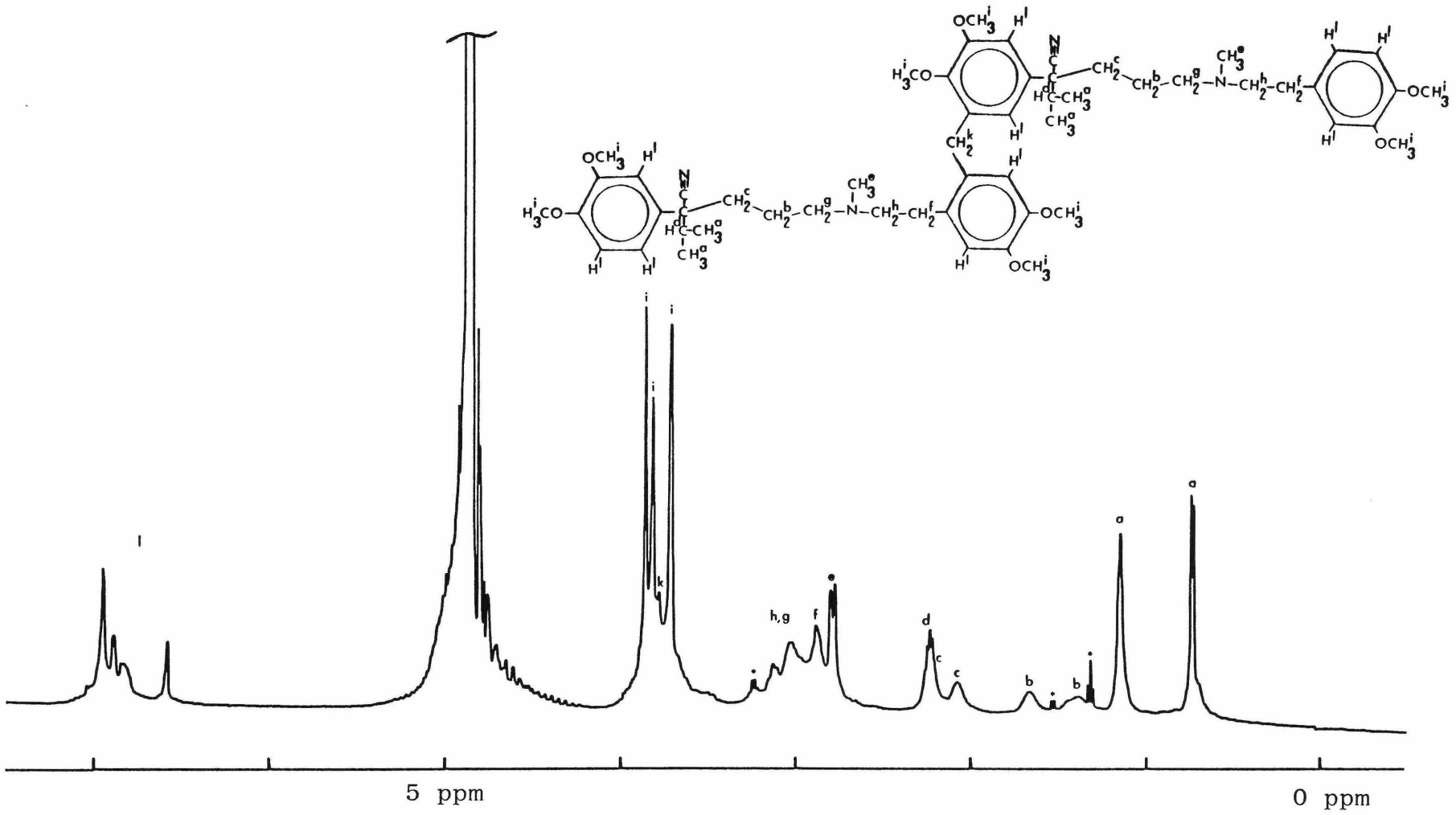


Figure 10. Proton magnetic resonance (500.13 MHz) spectrum and peak assignments of crosslinked verapamil in D_2O.

Figure 11 illustrates the relative ability of compound 48/80, crosslinked verapamil and verapamil to cause the release of ^{3}H-serotonin from mast cells. Several experiments were pooled and the mean concentrations of drug required to cause 50% of the maximum response was determined. The results for the release of ^{3}H-serotonin from mast cells are summarized below.

Drug	Concentration at 50% Release
Compound 48/80 (6)	0.08 ± 0.04 µg/ml
Crosslinked verapamil (3)	6.2 ± 0.2 µg/ml
Verapamil (3)	180 ± 20 µg/ml

The effect of the treatment of mast cells with neuraminidase on the ability of compound 48/80, crosslinked verapamil and verapamil to release ^{3}H-serotonin from mast cells was determined in the experiment depicted in Figure 11. The data show that the release of ^{3}H-serotonin from mast cells by compound 48/80 was inhibited by treatment of the cells with neuraminidase while in contrast neuraminidase treatment of the cells had an insignificant effect on the release of ^{3}H-serotonin from mast cells caused by verapamil and crosslinked verapamil.

Figure 11

The release of ^{3}H-serotonin from rat peritoneal cells challenged by compound 48/80, crosslinked verapamil and verapamil. The cells were isolated, loaded with ^{3}H-serotonin, and preincubated by the methods described in Materials and Methods, Chapter II. Subsequent experiments (Figure 16) have shown that the release of ^{3}H-serotonin by crosslinked verapamil was as high as 95 $\pm$ 5%. The challenges by compound 48/80 (-●-●-●-●-) crosslinked verapamil (-■-■-■-) and verapamil (-▲-▲-▲-▲-) were for one hour at 37^{o}C in MCB. The spontaneous release of ^{3}H-serotonin (Δ) was 12%.

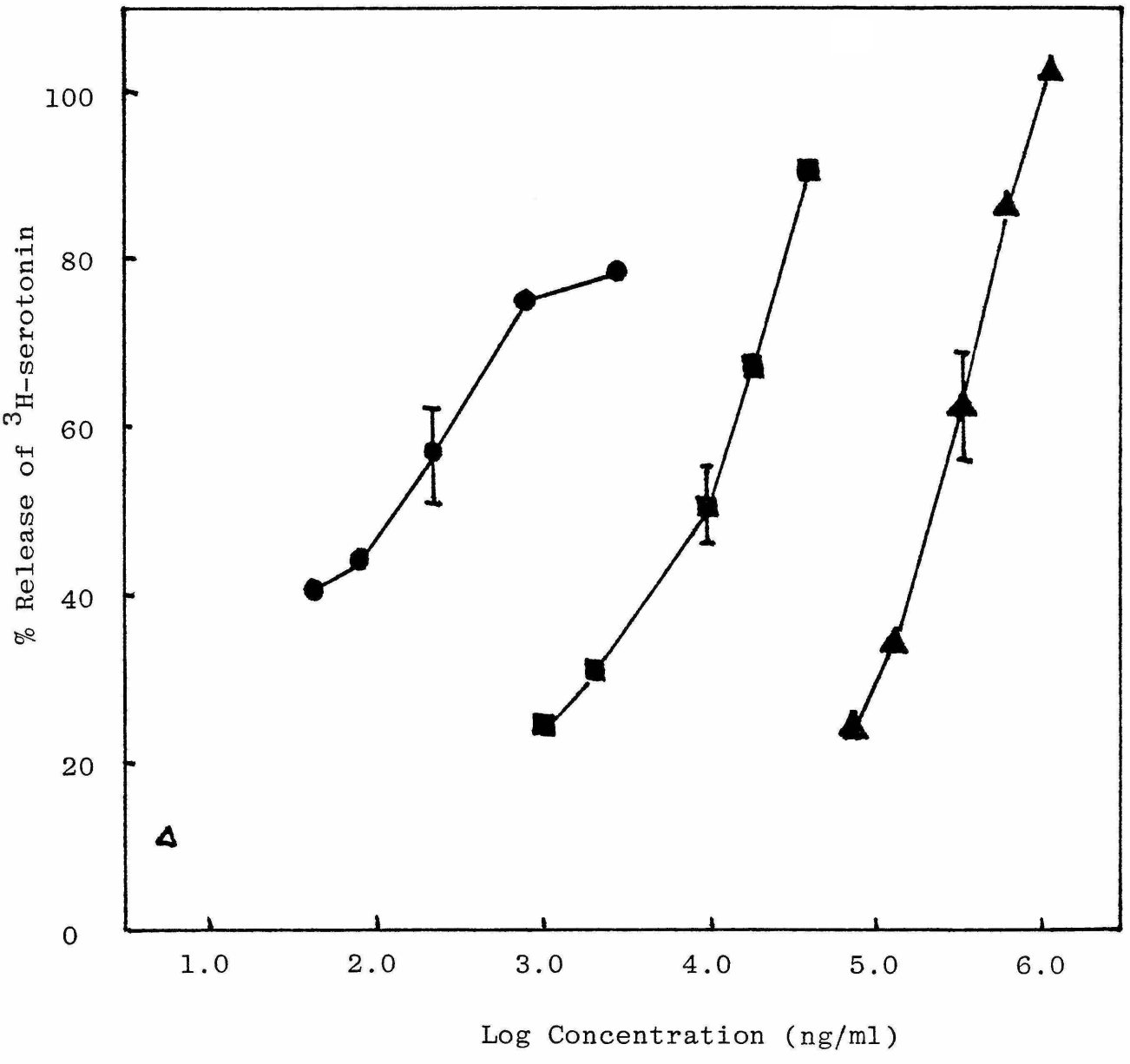

100
80
60
40
20
0
% Release of ^{3}H-serotonin
1.0
2.0
3.0
4.0
5.0
6.0
Log Concentration (ng/ml)

Figure 12

Effect of neuramindase on the release of ^{3}H-serotonin from rat peritoneal cells challenged with compound 48/80, crosslinked verapamil and verapamil. The cells were pre-incubated with neuraminidase exactly as described in Materials and Methods and challenged with the various drugs for one hour at 37^{o}C. The values given for release of ^{3}H-serotonin (cpm) were calculated by subtracting a blank of 1050 cpm for control cells (-●-●-●-●-) and 1350 cpm for cells pretreated with neuraminidase (----o----o----o----). The molar concentrations of the drugs were calculated assuming that crosslinked verapamil is a dimer and compound 48/80 has a molecular weight of 800 as determined by Morrison et al (1974). The curves at the left are compound 48/80, the curves in the middle are crosslinked verapamil and the curves on the right are verapamil. The estimated error on the cpm of the released ^{3}H-serotonin was 5%.

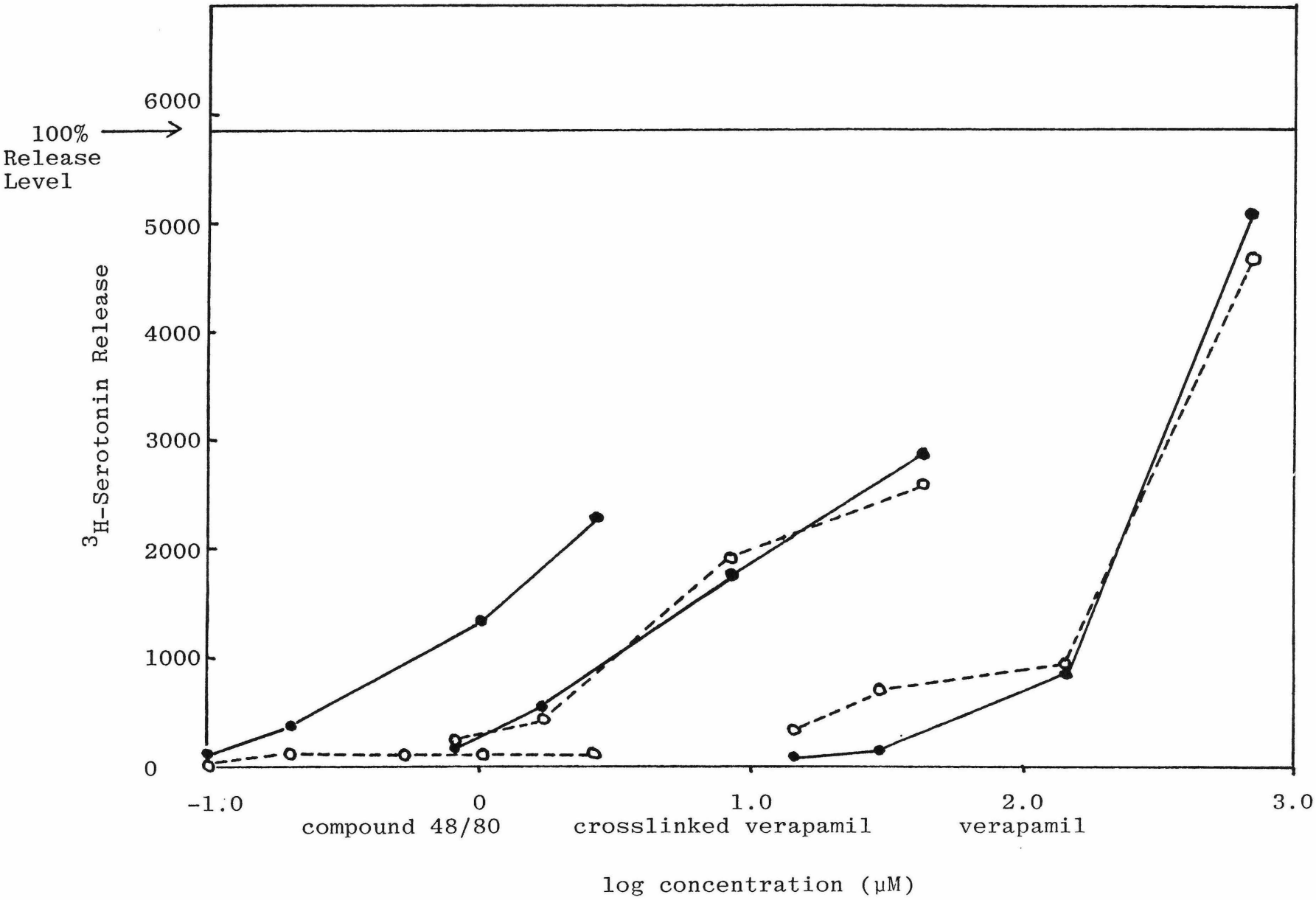

6000
100%
Release
Level
5000
4000
3000
2000
1000
0
^{3}H-Serotonin Release
-1.0
0
1.0
2.0
3.0
compound 48/80
crosslinked verapamil
verapamil
log concentration (μM)

Table III shows the results of two experiments examining the role of crosslinking of antigen in the release of ^{3}H-serotonin from mast cells by the IgE-14-205 protein. The crosslinking of ovalbumin by Woodward's reagents K and the relatively complex protocol of this experiment are described in detail in the Materials and Methods section. The addition of only crosslinked ovalbumin or only ovalbumin to the mast cells did not cause a significant release of ^{3}H-serotonin (not shown). The results of Table III are seen to indicate that crosslinked ovalbumin cause a greater release of ^{3}H-serotonin from IgE-treated mast cells than monomeric ovalbumin.

Figure 13represents dose-response curves for the release of ^{3}H-serotonin from mast cells challenged with compounf 48/80 at various concentrations of extracellular calcium. The results show that increases in the concentration of extracellular calcium causes a decrease in the ability of compound 48/80 to induce the release of ^{3}H-serotonin from mast cells. This result agrees qualitatively with the preliminary finding of Kagayama and Douglas (1974), that 110 mM $CaCl_2$ in the extracellular abolished the degranulation of mast cells caused by compound 48/80.

Figure 14 shows the effect of extracellular calcium concentration on the release of ^{3}H-serotonin from mast cells challenged with verapamil. The release of ^{3}H-serotonin

at the submaximal dose of verapamil (179 μg/ml) was relatively uneffected by the concentration of extracellular calcium.

Figure 15 shows the effect of the concentration of $LaCl_3$ on the uptake of ^{45}Ca into P815 mastocytoma cells. At concentrations of $LaCl_3$ greater than approximately 1mM, the La^{+++} caused a dose-dependent increase in the uptake of ^{45}Ca into the mastocytoma cells.

Figures 16 and 17 illustrate timed experiments examining the kinetics of the release of ^{3}H-serotonin from rat mast cells by compound 48/80, crosslinked verapamil and verapamil. Figure 16 shows that relatively high concentrations of verapamil and crosslinked verapamil cause the release of ^{3}H-serotonin from mast cells. This experiment illustrates that a component of the release of ^{3}H-serotonin by verapamil and crosslinked verapamil is fast (< 5 minutes). Similarly, the data of Figure 17 show that relatively low concentrations of these drugs could cause this same fast component (< 5 minutes) of release of ^{3}H-serotonin from the mast cells.

Table III Release and Retention of ^{3}H-Serotonin in Mast Cells Preincubated in IgE 14-205 Protein Then Challenged by Crosslinked Ovalbumin or Ovalbumin
Chapter III

[IgE]	[Ovalbumin]	% Release	% Retention
0.50 µM	18.0 µM X	80	20
0.50 µM	18.0 µM	45	55
0.50 µM	---	0	100
0.14 µM	6.0 µM X	22	78
0.14 µM	6.0 µM	19	81
0.14 µM	---	0	103
0.14 µM	2.0 µM X	46	54
0.14 µM	2.0 µM	28	72
0.14 µM	---	2	98
0.27 µM	5.8 µM X	59	41
0.27 µM	5.8 µM	36	64
0.27 µM	---	9	91
1.08 µM	20.7 µM X	46	54
1.08 µM	20.7 µM	48	52
1.08 µM	---	9	91
0.27 µM	12.4 µM X	46	54
0.27 µM	12.4 µM	0	101
0.27 µM	---	0	115

X refers to crosslinked ovalbumin

Figure 13

Effect of calcium concentration on ^{3}H-serotonin release from rat peritoneal cells challenged by compound 48/80. The rat peritoneal cells were isolated in regular MCB which contains no calcium. The cells were preincubated in various concentrations of $CaCl_2$ for 15 minutes at 37°C. The cells were then challenged by compound 48/80 for one hour at 37°C. The (-o-o-o-o-) curve represents cells preincubated in MCB containing no calcium. The (-●-●-●-●-) curves represent cells preincubated in 2 mM Ca^{++} (upper) and 6 mM calcium (lower). The estimated error was 5%.

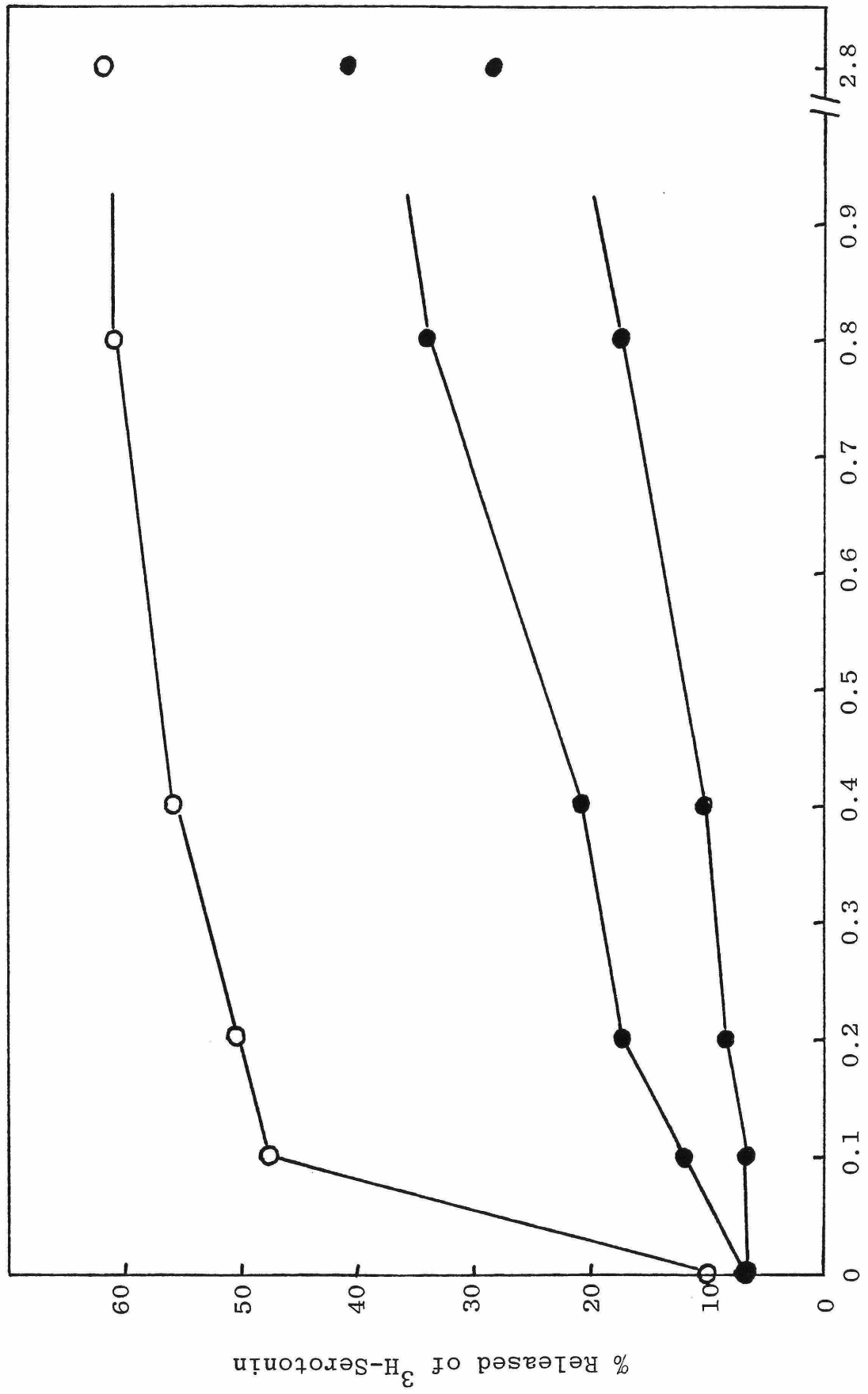

% Released of ^{3}H-Serotonin
0
10
20
30
40
50
60
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
2.8
Concentration of Compound 48/80 (µg/ml)

Figure 14

Effect of calcium concentration on ^{3}H-serotonin release from rat peritoneal cells challenged with verapamil. The rat peritoneal cells were isolated in regular MCB which contains no calcium. The cells were then preincubated in various concentrations of $CaCl_2$ for 10 minutes at 37°C. The cells were then challenged for one hour at 37°C wtih 510 µg/ml of verapamil (-●-●-●-) and 179 µg/ml of verapamil (-▲-▲-▲-▲-). The estimated error was 5%.

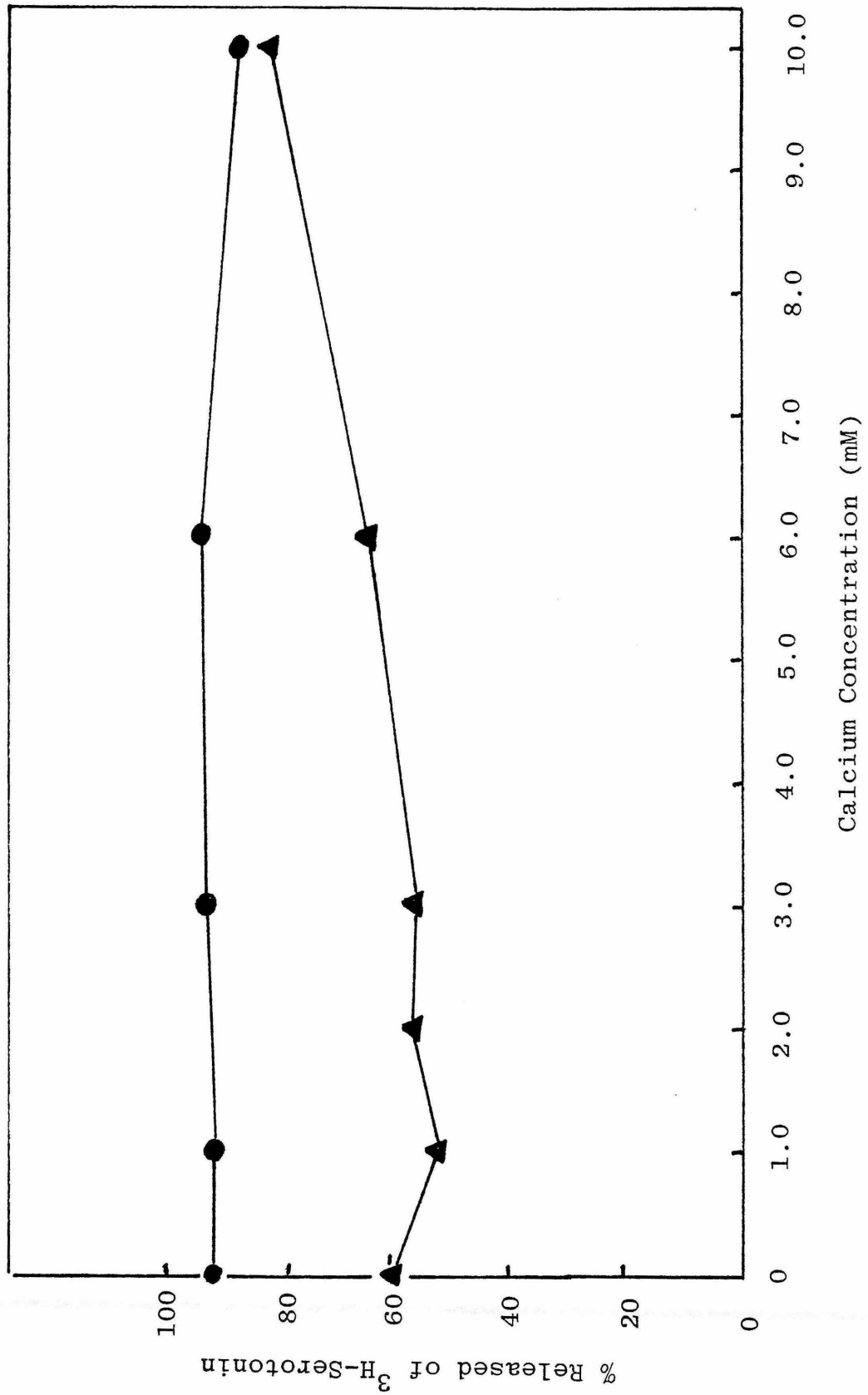

% Released of 3H-Serotonin
100
80
60
40
20
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Calcium Concentration (mM)

Figure 15

The effect of lanthanum concentration on calcium uptake in P815 mastocytoma cells. The P815 cells were aliquoted into MCB containing 2 mM Ca^{++} and ^{45}Ca at a specific activity of 0.58 μmole/10^6 cpm. After a short preincubation the P815 cells (1.3 x 10^6 cells per 1 ml sample) were exposed to various concentrations of $LaCl_3$ for 20 minutes at 37°C. The cells were then worked up on glass filters as described in Materials and Methods. The error in this experiment was large and was estimated from the standard deviation of the values for the blanks to be $\pm$ 3 nmoles Ca/10^6 cells.

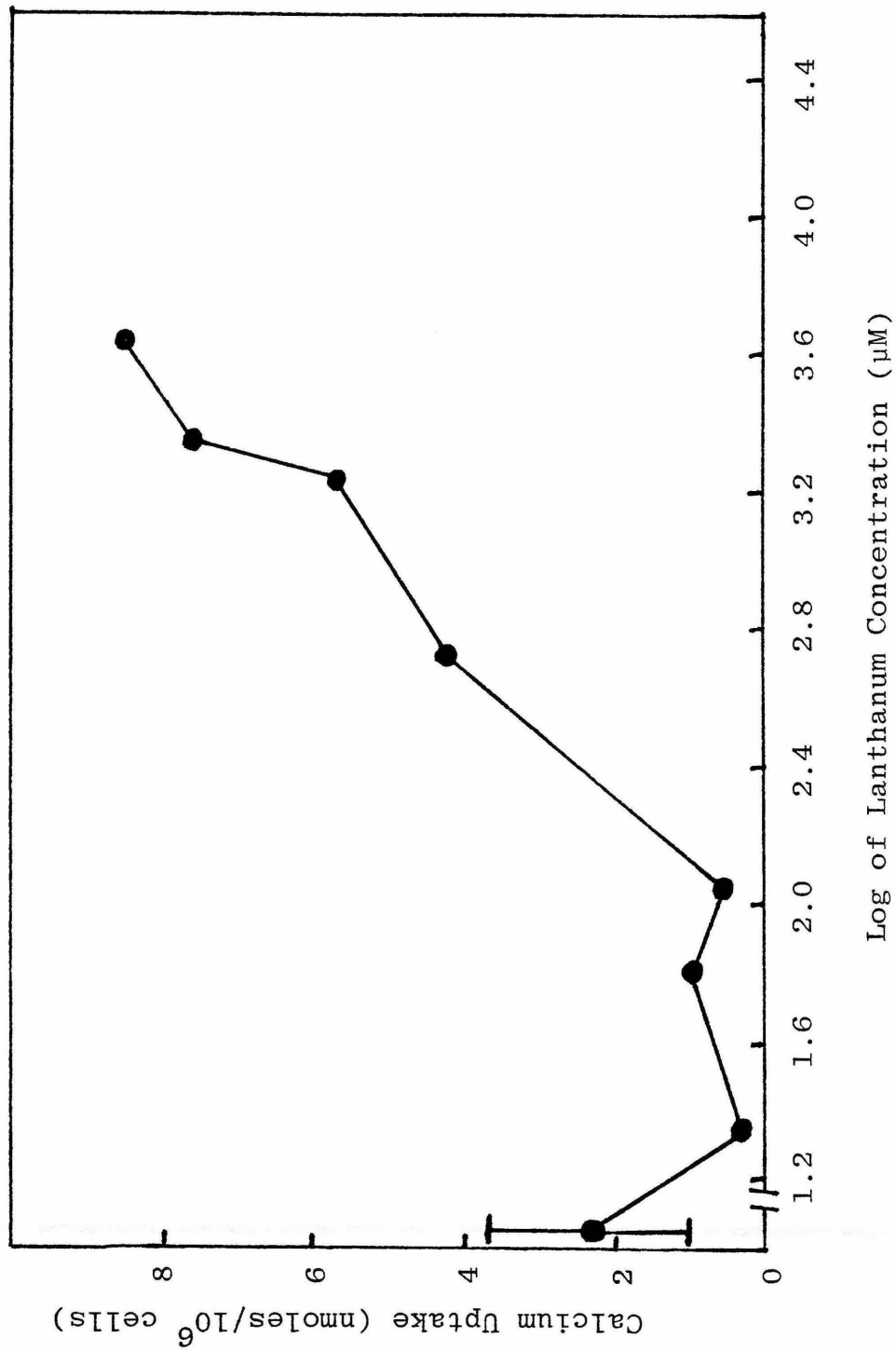

Calcium Uptake (nmoles/10^6 cells)
0
2
4
6
8
1.2
1.6
2.0
2.4
2.8
3.2
3.6
4.0
4.4
Log of Lanthanum Concentration (μM)

Figure 16

Rate of release of ^{3}H-serotonin from rat peritoneal cells challenged with high concentrations of verapamil and crosslinked verapamil. The cells were preincubated and challenged by the usual methods except that aliquots were sampled at various time intervals from large volumes (10 ml). Scintillation counting of the supernatants of the aliquots determined the release of ^{3}H-serotonin. The blank at 0 minutes was 4% of the total ^{3}H-serotonin release and at 91 minutes was 7% of the total ^{3}H-serotonin release. The concentration of verapamil was 880 µg/ml (-●-●-●-●-) and crosslinked verapamil 40 µg/ml (---o---o---).

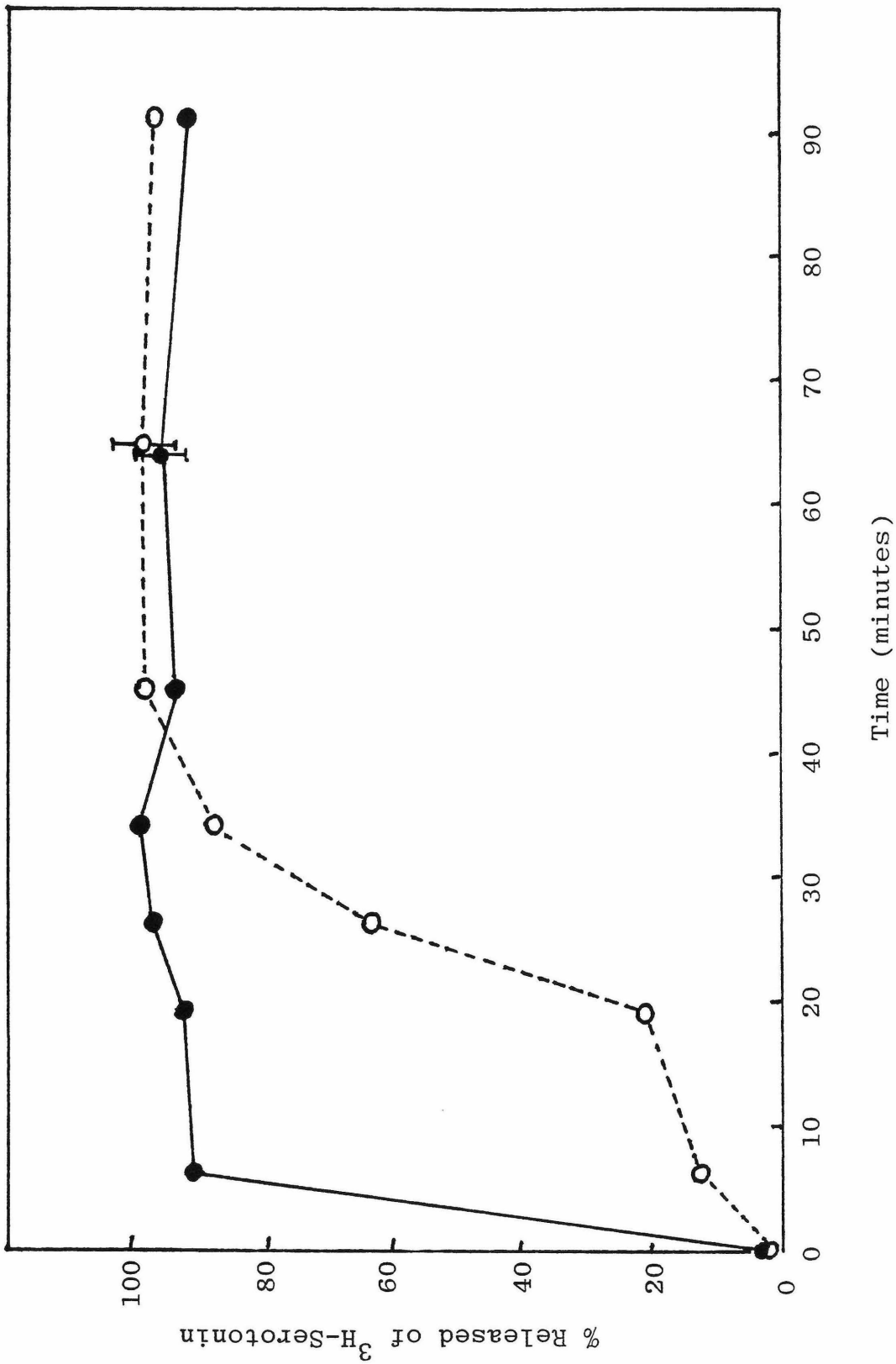
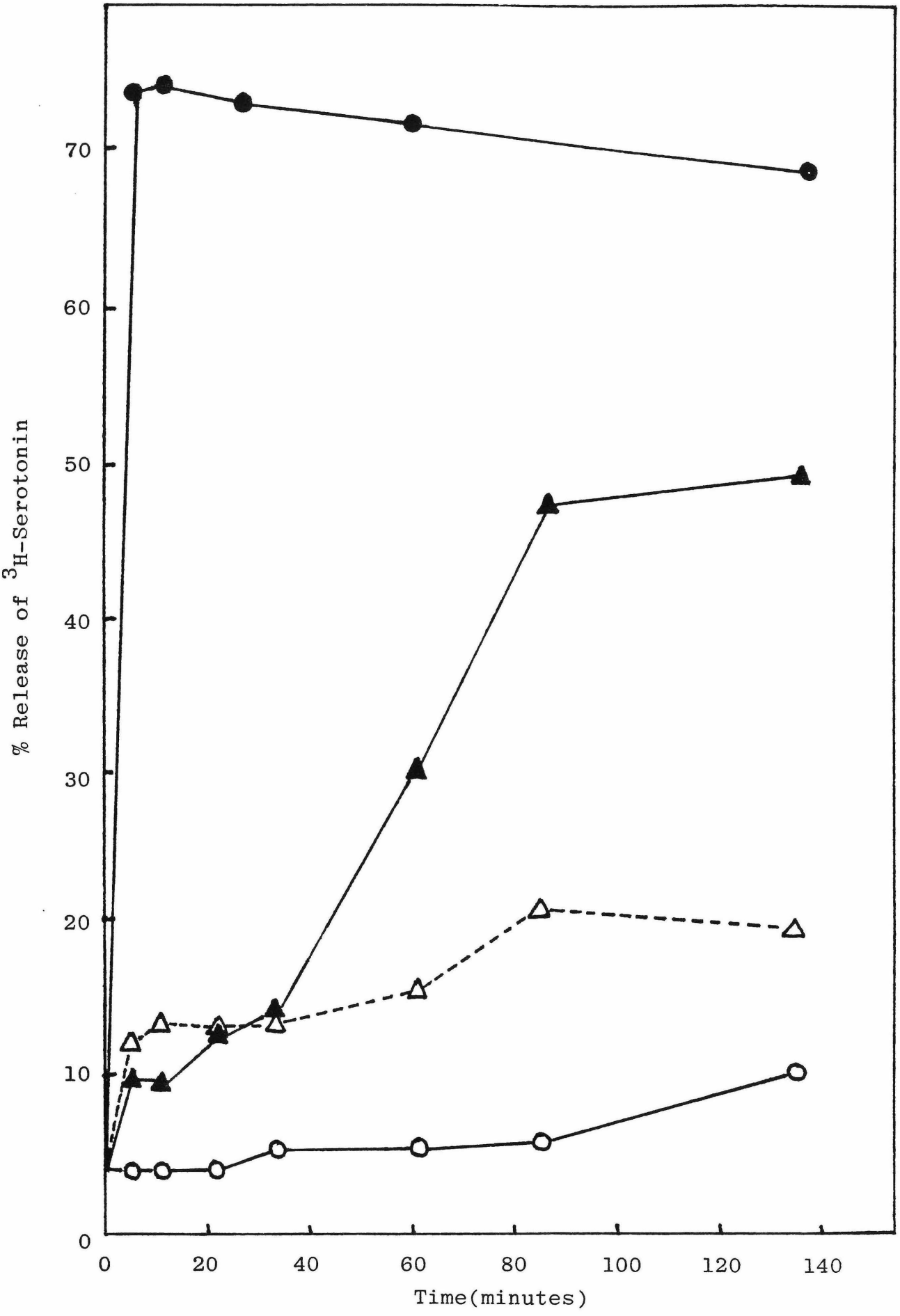

% Released of 3H-Serotonin
0
20
40
60
80
100
0
10
20
30
40
50
60
70
80
90
Time (minutes)

Figure 17

Rate of release of ^{3}H-serotonin from rat peritoneal cells challenged with crosslinked verapamil, verapamil, and compound 48/80. The cells were preincubated and challenged by the usual methods except that aliquots were sampled at various time intervals from large volumes (6 ml) of rat peritoneal cells in MCB. Scintillation counting of the supernatants of the aliquots determined the release of ^{3}H-serotonin from the cells. The various drug concentrations were:

compound 48/80	0.16	µg/ml	-●-●-●-
verapamil	330	µg/ml	-▲-▲-▲-
crosslinked verapamil	5	µg/ml	-Δ-Δ-Δ-
blank		----	-o-o-o-


% Release of ^{3}H-Serotonin
0
10
20
30
40
50
60
70
0
20
40
60
80
100
120
140
Time(minutes)

DISCUSSION

Crosslinking and aggregation of IgE-receptors on the mast cell surface has been proposed as an obligatory event in the degranulation of mast cells (Metzger, 1979). A mechanism of action of verapamil and crosslinked verapamil might feature the drugs crosslinking cell surface structures in a manner analogous to the activation of mast cells by crosslinking IgE-receptors (Hirata and Axelrod, 1980) system. In fact, a chemically crosslinked derivative of verapamil was 30 times more potent by weight at causing the release of ^{3}H-serotonin from rat mast cells relative to monomeric verapamil (Figure 11).

We attempted to further examine the role of crosslinking and aggregation of cell surface structures by comparing the relative ability of chemically crosslinked ovalbumin versus monomeric ovalbumin to cause degranulation of mast cells in the presence of an IgE hybridoma protein that specifically binds to ovalbumin. The data of Table 1 show that ovalbumin crosslinked with Woodward's reagent K caused a greater release of ^{3}H-serotonin from rat mast cells preincubated in the IgE-14-205 protein than monomeric ovalbumin. This result does seem to agree with the general findings of Siraganian et al (1975) and others that chemically crosslinked antigen causes a greater IgE-mediated release of histamines than monomeric antigen.

Moreover, verapamil has a crude symmetry around the tertiary amino group making it a possible candidate for a bifunctional agent capable of crosslinking. The increased ability of crosslinked verapamil to cause the release of ^{3}H-serotonin from mast cells may be due to the availability of more methoxyphenyl rings for binding to cellular target structures.

The increased binding of a crosslinked molecule, relative to its monomeric counterpart, to another structure may be analogous to the so-called thermodynamic "chelate effect" for metal ions (Cotton and Wilkinson, 1972). The chelate effect refers to the enhanced stability of a metal complex system containing ring-forming ligands as compared to a system with similar monomeric ligands. The entropy for the formation of such chelates is significantly more favorable than that for the formation of an analogous complex from a metal ion and several ligands. In forming the chelate, the translational entropy of only one molecule is lost; in forming the multiliganded complex, the translational entropy of several molecules must be sacrificed.

Besides simple thermodynamic considerations, the increased ability of crosslinked verapamil to cause release of ^{3}H-serotonin from mast cells relative to verapamil may be due to steric considerations in the crosslinking potential

of the drugs. For example, crosslinked verapamil may be able to span and consequently link sites that cannot be linked by monomeric verapamil.

IgE binds to a specific glycoprotein on the mast cell surface (Metzger, 1979). Moreover, there is some circumstantial evidence that verapamil and D-600 can also bind to proteins. For example, Kaumann and Uchitel (1976) found significant differences in the (+) and (-) isomers of verapamil in their ability to inhibit potassium-induced contractures in frog skeletal muscle fibers. Furthermore, the (+) and (-) isomers of verapamil were shown to have different abilities to cause the decoupling of excitation from contraction in canine heart (Kaumann and Serur, 1975). The stereoselective pharmacology of (±)-verapamil suggests that the drug is binding to stereoselective sites on proteins.

Discussion of Neuraminidase Results

Experiments were designed to determine if verapamil interacts with the cell surface to cause degranulation of mast cells in a manner analogous to the binding of IgE to its receptor or if verapamil triggers degranulation by some intracellular mechanism of action.

Rat peritoneal cells were treated with neuraminidase, an enzyme that specifically removes sialic acids from cell surface molecules, and then challenged with verapamil, crosslinked verapamil, and compound 48/80

(Figure 12). Pretreatment of the cells with neuraminidase abolished the ability of compound 48/80 to cause release of ^{3}H-serotonin from mast cells (Figure 12). Similarly, Baxter and Adamik (1977) had found that pretreatment with neuraminidase causes a significant decrease in the release of histamine from mast cells exposed to compound 48/80, protamine and polylysine. They also found that the neuraminidase pretreatment did not affect the ability of antigen to evoke IgE-mediated release of histamine from mast cells. Figure 12 shows that pretreatment of the cells with neuraminidase did not significantly affect the ability of verapamil or crosslinked verapamil to cause release of ^{3}H-serotonin from rat peritoneal cells.

These results indicate that the exact sites of action of compound 48/80 and verapamil are different. Compound 48/80 causes degranulation of mast cells by binding to the sialic acids of cell surface molecules that are sensitive to hydrolysis by neuraminidase. In contrast, verapamil almost certainly does not require these neuraminidase-sensitive sialic acids to cause the release of ^{3}H-serotonin from mast cells. Conceivably, the verapamil and compound 48/80 could even by interacting with the same molecule. As a hypothetical example, the verapamil interacts with the protein portion of a glycoprotein and compound 48/80 binds to the saccharide groups of this same glycoprotein but there is not direct evidence for this hypothesis.

A simple way to explain why verapamil and crosslinked verapamil are not inhibited in their ability to cause the release of ^{3}H-serotonin from mast cells when the cells were pretreated with neuraminidase is to propose that these drugs act inside the cell to trigger the release of ^{3}H-serotonin. Other studies have inferred that verapamil not only blocks the so-called "slow channels" for Ca^{++} in the plasma membrane (Fleckenstein, 1977) but causes other effects by acting within the cell. For example, Church and Zsoter (1980) found verapamil inhibited the contraction of rabbit mesenteric vein caused by high concentrations of extracellular potassium. The drug did not alter the lanthanum-resistant uptake* of ^{45}Ca by the vein during the verapamil-induced relaxation, implying that the effect was not due to the drug acting as a specific antagonist of the calcium channels of the plasma membrane but had an intracellular mechanism of action. Moreover, verapamil has been shown to inhibit the norepinephrine-induced development of tension in the isolated rat atrium (Church and Zsoter, 1980). Norepinephrine caused a

*The "lanthanum-method" supposedly allows the direct measurement of ^{45}Ca influx by cell and tissues without interference due to binding of the ^{45}Ca to the cell surface and efflux of the label from the tissue (Van Breeman et al, 1973). However have found that La^{3+}, itself, causes a dose-dependent uptake of ^{45}Ca into P815 mastocytoma cells (Figure 15) and similarly, Foreman and Mongar (1972) found that high concentrations of La^{3+} caused mast cells to release histamine, a process that can be caused by an influx of extracellular Ca^{++} (Hirata and Axelrod, 1980).

rapid increase in tension that could be inhibited by verapamil. Church and Zsoter (1980) proposed that verapamil had an intracellular mechanism of action, supposedly by interfering with the distribution of calcium in the intracellular pools. However, this is not conclusive evidence since verapamil could act at the cell surface and exert its effect on the norepinephrine-induced tension via a mechanism involving a secondary messenger.

Similarly, we have shown that verapamil can cause the release of ^{3}H-serotonin from mast cells in the absence of extracellular calcium; moreover the release was relatively insensitive to the concentration of extracellular calcium (Figure 13). These results are in contrast to those for the release of biogenic amines from mast cells challenged with compound 48/80; in this case release was significantly inhibited by the addition of extracellular calcium (Figure 14). These experiments suggest that the release of ^{3}H-serotonin from mast cells caused by verapamil is not due to an influx of extracellular calcium analogous to the mechanism of degranulation of mast cells by IgE-antigen complexes (Hirata and Axelrod, 1980).

Discussion of Possible Mechanisms

Verapamil causes a wide variety of effects on mammalian myocardium, some of which cannot be explained by blockage of the so-called "slow calcium channels" (Bayer et al, 1975). For example, Bayer et al (1975)

found that the (±)-isomers of verapamil and D-600 have a quite specific inhibitory effect on the fast Na^+ inward current in feline myocardium. The (+)-isomers also increased the threshold intensity of electrical stimuli needed to elicit conducted action potentials in the myocardium. Bayer et al (1975) also found that the (-)-isomers of verapamil and D-600 were more potent than the (+)-isomers in blocking the so-called specific calcium channels in the myocardium. This local anesthetic effect of verapamil and D-600 has also been shown in frog nerves where the drugs are 1.6X times more active than procaine in blocking action potentials (Singh and Williams, 1972).

A mechanism of action similar to the cationic anaesthetic effect of verapamil could provide a molecular rationale for the ability of verapamil to release ^3H-serotonin from mast cells. Seeman (1972) and Kwant and Seeman (1969) have shown that local anaesthetics such as phenothiazines displace membrane calcium from erthyrocyte ghosts and Sheetz and Singer (1974) suggested that cationic anaesthetics bind to the cytoplasmic side of the membrane bilayer which is relatively rich in the negatively charged phospholipids such as phosphatidylserine (Bretcher, 1972). The cationic drugs supposedly expand the cytoplasmic side of the bilayer and induce cup formations in erythrocytes. Furthermore, Frazier et al (1970) showed with microinjection techniques that the anaesthetic

action of several tertiary amine local anaesthetics was more effective on the cytoplasmic side of the nerve membrane. In accord with the above observations, verapamil may displace membrane-bound calcium from the cytoplasmic side of the plasma membrane and thereby trigger the release of granule-stored amines from mast cells.

Unfortunately, the mechanism of action of cationic anaesthetics have not been resolved, but the anaesthetics do cause a variety of effects that could conceivably increase the cytosolic [Ca^{++}] enough to activate mast cells. For example, positively charged anaesthetics displace loosely adsorbed Ca^{++} from reticulum membranes (Thorpe and Seeman, 1971) and reduce the entry of Ca^{++} across mitochrondrial membranes (Tjioe *et al*, 1971). Furthermore, there is experimental evidence that high concentrations of verapamil affect the translocation of calcium across *in vitro* preparations of subcellular membrane fractions. For example, high concentrations of verapamil have been shown to inhibit calcium uptake by membrane and endoplasmic reticulum fractions from rabbit aorta (Thorens and Haeusler, 1979). Moreover, Crankshaw *et al* (1977) found that D-600 (520 µg/ml) and verapamil (490 µg/ml) significantly reduced Ca^{++} accumulations in subcellular fractions of the plasma membrane, endoplasmic reticulum, and the mitochrondrial membrane of rat myometrium. Lastly, Balzer (1972) found a similar

effect for verapamil in an *in vitro* membrane fraction of the sarcoplasmic reticulum of skeletal muscle. Perhaps, verapamil causes an increase in the concentration of cytosolic Ca^{++} by inhibiting the calcium-ATPase pumps of membranes defining the cytosolic pool. This event may trigger the release of histamine by mast cells challenged by verapamil. An unanswered question is whether the *in vitro* inhibition of calcium-ATPase pumps by verapamil in subcellular membrane fractions is relevant to the intact mast cell.

Verapamil could possibly interact with the ubiquitous intracellular Ca^{++}-receptor, calmodulin, and cause the degranulation of mast cells. Calmodulin has been implicated in a wide variety of calcium-dependent effects such as activation of a phosphodiesterase (Cheung, 1970) an adenyl cyclase (Bronstron *et al*, 1975) a phospholipase A_2 in platelets (Wong and Cheung, 1979) and ATPase-dependent calcium pumps of erythrocyte membranes (Levine and Weiss, 1980). Some or all of these effects could be postulated to affect degranulation of mast cells depending on whether verapamil potentiates or counteracts the action of calmodulin. However, such speculation is pointless until the existence of sufficient calmodulin has been demonstrated in the mast cell.

Kinetics of the Release of ^{3}H-Serotonin from Mast Cells

The kinetics of the release of ^{3}H-serotonin by verapamil and crosslinked verapamil show interesting features (Figures 16 and 17). At concentrations of verapamil and crosslinked verapamil that do not cause maximal release of ^{3}H-serotonin, the pattern of release appears to be biphasic. For release of ^{3}H-serotonin caused by verapamil or crosslinked verapamil, there appears to be a fast release (< 5 minutes) followed by a lag time then a slower release (Figure 17). A possible explanation of this biphasic kinetics is that the drugs first cause a release of ^{3}H-serotonin by interacting with the cell surface, in a manner analogous to compound 48/80, then penetrate the membrane and interact with intracellular targets to bring about the slow release. In accord with this possibility, the crosslinked verapamil showed a larger release of ^{3}H-serotonin in the fast phase then did monomeric verapamil; nevertheless crosslinked verapamil showed a lower overall release of ^{3}H-serotonin from the mast cells. This increased ability of crosslinked verapamil to cause the fast release of ^{3}H-serotonin from mast cells could be due to its ability to aggregate cell surface molecules as a crosslinking agent in a manner analogous to the polymeric compound 48/80. In contrast, monomeric verapamil could be more effective in the slow

phase of release of ^{3}H-serotonin simply because higher concentrations of the drug are penetrating the membrane and interacting with intracellular targets.

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CHAPTER IV

A STUDY OF CONTRACTURE OF SKELETAL MUSCLE CAUSED BY COMPOUND 48/80

INTRODUCTION

Muscle Contracture As A Model for Excitation-Contraction Coupling

A muscle contracture has been defined as a decrease in the length of a muscle or an increase in muscle tension produced without muscle action potential (Gasser, 1930). In contrast, muscle twitches require a muscle action potential and are designated as contractions.

A difficulty in studying excitation-contraction coupling in skeletal muscle is separating the complex ionic events involved in the generation of the action potential from possible ionic or biochemical events associated with the actual coupling of the action potential with the release of calcium from the sarcoplasmic reticulum. Muscle contractures, because they do not depend on muscle action potentials, offer a simpler system for study. However, use of muscle contractures as a basis for inferences on excitation-contraction coupling should involve great caution because of the possible non-physiological nature of the experiment. However, in the past contracture systems have provided useful models for excitation-contraction coupling. For example, there is the so-called potassium contracture which features the de-

polarization of the muscle membrane by replacement of Na^+ by K^+ in the extracellular medium. Frank (1964) demonstrated that the removal of extracellular calcium abolishes the potassium contracture but does not affect the depolarization of the muscle membrane. This result suggested that the extracellular calcium pool was the source of "trigger-Ca^{++}" in the potassium-contracture. A more thorough analysis of the kinetics of the removal of calcium in potassium-contracture suggests that the rate of exchange of "trigger-Ca^{++}" was intermediate between the rate of exchange of extracellular calcium, which was rapid, and the rate of exchange of intracellular calcium of the sarcoplasmic reticulum, which was shown to be slow (Frank, 1979). Similarly, muscle contractions can be abolished in buffer without calcium, but only after Ca^{++} has been removed for approximately ten minutes. This is much longer than the few seconds required for Ca^{++} to diffuse out of the t-tubular system and shorter than the estimated four hours required to significantly deplete the pool of calcium in the sarcoplasmic reticulum (Frank, 1979). The parallel finding for the potassium-contracture of skeletal muscle and the contractions of skeletal muscle caused by an action potential seem to imply that the source of "trigger-Ca^{++}" is the same for both systems and is likely to be membrane-bound calcium.

Caffeine causes a contracture in skeletal muscle at concentrations greater than 3.0 mM (Axelsson and Thesloff, 1958). This contracture does not require extracellular calcium and does not cause depolarization of the sarcolemma. Caffeine is believed to act directly on the sarcoplasmic reticulum and to cause calcium release from that organelle (Ebashi, 1976). The molecular mechanism of this release is not known. This contracture system has limited utility for understanding EC coupling between the t-tubules and the sarcoplasmic reticulum but may be of use in understanding the function of the sarcoplasmic reticulum (Hasselbach, 1980).

This study involves the development of an experimental model of excitation-contraction coupling. We have found that compound 48/80, a promoter of stimulus-secretion coupling in mast cells causes a contracture in muscle. The possibility of a mechanistic link between stimulus-secretion coupling in mast cells and excitation-contraction coupling in skeletal muscle was investigated using the compound 48/80 to induce muscle contracture.

Protein Turnover in Muscle

Continuous protein turnover is a feature of muscle and many other tissues and cells (Goldberg and St. John, 1977). Protein degradation. as well as protein synthesis is precisely regulated in the normal cell. Protein breakdown may function to remove abnormal proteins, to degrade enzymes or structures not needed in the immediate function of the cell, and to provide amino acids for other tissues under nutritional stress. The rates of protein synthesis and degradation in tissues can be changed by extracellular signals such as hormones and by the activity of the tissue. Protein turnover ultimately can determine the growth, stability or atrophy of tissue.

There is evidence that protein turnover in normal and pathological muscles is sensitive to the distribution of calcium in the intracellular pools. Kameyama and Etlinger (1979) had found that protein turnover in rat soleus muscles could be affected by treatment of the muscles with the Ca-ionophore, A23187. Pennington (1978) proposed that muscular dystrophy, a disease characterized by abnormalities in protein turnover, might also exhibit abnormalities in cellular calcium metabolism.

Chapter II of this thesis presents evidence that, in mast cells, compound 48/80 can mobilize intracellular stores of calcium leading to degranulation of the cell. In the normal excitation-contraction cycle of skeletal muscle the muscle action potential triggers a series of events which lead to the mobilization of calcium from the intracellular organelle, the sarcoplasmic reticulum (Hasselbach, 1980). Since compound 48/80 causes a contracture in frog skeletal muscle, we were interested in a possible relation between protein turnover and the mobilization of intracellular calcium in frog skeletal muscle challenged with compound 48/80.

MATERIALS AND METHODS

Isolation of Frog Skeletal Muscles

The muscles were isolated from frogs of the genus Rana pipiens (Carolina Biological). The frogs were double pithed and pinned down. The skin was cut away from the leg and the exposed muscles were kept wet by oxygenated frog Ringer's solution. All muscles were removed by a technique such that the tendons of origin and insertion could be easily cut with no damage to the muscle membrane. Fine pointed forceps and corneal scissors proved indispensible to this task. The muscles removed, with the ranges in masses, were: the sartorius (80-400 mg), semitendinosus (70-300 mg), semimenbranosus (280-1200 mg), gracilis major (240-950 mg), gastrocnemius (420-1940 mg), and a tightly bound complex of the peronius, tibialis articus and tibialis posticus (220-800 mg). This "shin muscle" complex was not separated for fear of damaging the muscle membrane. When more than one frog was used in an experiment care was taken to match their sizes.

Denervation of the Sartorius Muscle

Intact frogs were set in 10% ethanol until they ceased to show any movement reflexes. A small (∿ 2 cm) incision was made over the sartorius muscle. The other leg was left intact to later serve as a control. With fine point forceps and corneal scissors the sartorius muscle was separated from the gracilis major muscle to expose the sartorius nerve. Care was taken to avoid damaging the muscles. The nerve was cut causing a contraction/twitch of the sartorius. The skin was closed with wound clips and after a week the sartorius muscles were isolated and submitted to pharmacological challenge.

Treatment of Frog Muscles with Neuraminidase

Frog skeletal muscles were treated with neuraminidase derived from Vibrio cholerae (Calbiochem-Behring). . Typical experiments exposed the muscle to 0.25 units/ml in 0.7 ml of PAB for 75-90 minutes at 25°C. The muscles were then transferred to another PAB aliquot and subjected to the various pharmacological challenges. One unit of neuraminidase activity is defined as the amount of enzyme that will release 1 μmole of N-acetylneuraminic acid from human acid α_1-glycoprotein per minute at 37°C in 0.05 M sodium acetate buffer, pH 5.5 containing 5 mM calcium chloride.

Measurement of the Efflux of $^{86}Rb^{+}$ from Frog Muscle

Muscles were preincubated in 10 μCi/ml of $^{86}RbCl$ in PAB at 25°C for 1.5 hours. The excess $^{86}Rb^{+}$ was washed from the muscles by dipping them in a large volume of PAB then incubating the muscles in a 5 ml volume of the buffer. The wash was then repeated. The rationale for this procedure was to remove loosely associated $^{86}Rb^{+}$ from the muscle. After this preparation, the muscles were put into plastic tubes containing PAB and challenged with the various pharmacological agents. Aliquots of the extramuscular buffer were sampled and analyzed by the standard methods of scintillation counting.

Protein Degradation Measurements

All experiments involving protein degradation were performed in protein anabolism buffer (PAB). PAB is a balanced buffer specifically designed to maintain protein synthesis. The amphibium PAB was modified from the basic frog Ringer buffer with adaptation from a mammalian muscle system (Kameyama and Etlinger, 1979). PAB consisted of 116 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 10 mM Tris(hydroxymethyl)amino-methane, 10 mM glucose, 1.8 mM $CaCl_2$, 1.0 mM isoleucine, 1.0 mM leucine, 1.0 mM valine and 10 units/ℓ insulin. The PAB was titrated to pH 7.4 with concentrated hydrochloric acid.

The isolated frog muscle was incubated in PAB at 25°C. After a specific drug challenge the muscle was placed in PAB containing 10% trichloroacetic acid (TCA). The original PAB was also made 10% in TCA. The TCA served three purposes: to precipitate any exogenous protein released by the muscle; to precipitate the drug 48/80 to prevent interference with the subsequent tyrosine assay and to precipitate the protein of the intact muscle so that the muscle could easily be broken up to release internal pools of tyrosine. The muscles were generally left overnight at 5°C before being broken up by manual manipulation with a glass stirring rod.

Each muscle of a given experiment was manipulated for approximately the same time. The precipitated muscle debris was pelleted by centrifugation and the supernatant was analyzed. It was found that the muscles could be broken up very easily by the addition of an aliquot of saturated sodium carbonate solution to the TCA precipitated muscle. This approach was tried in several experiments and was believed not to the assay.

The supernatant of the original reaction volume (containing released tyrosine) and the supernatant of the precipitated muscle (internal tyrosine pool) were analyzed by the spectrofluorimetric method of Waalkes and Udenfriend (1957). The assay consisted of derivatizing tyrosine with 1-nitroso-2-napthol and analyzing the extracted product spectrofluorimetrically (activation 460 mμ; measurement 570 mμ). The instrument used was a Perkin-Elmer MPF-4 fluorescence spectrometer kindly loaned by Dr. M. A. Raftery. In most experiments the total protein degradation was cited as the sum of the released tyrosine and the internal tyrosine pool.

Origin and Maintenance of L8 Myoblast Cell Line

The L8 cell line was a gift of Dr. H. Blau of Stanford University (Blau and Epstein, 1979). The line was originally derived from newborn rat skeletal (thigh) muscles (Yaffe, 1968). These cells retain the ability to fuse to form multi-nuclear syncytia (myotubules). Upon formation of the myotubules the L8 cells cease division and DNA synthesis and synthesize a number of proteins of myotubules including myosin and the muscle isozymes of creatine kinase.

The L8 myoblasts were grown at 37°C in 5% CO_2/100% H_2O in Waymouths media supplemented with 10% fetal calf serum. The preconfinement monolayers were passed by first washing the cells with Ca and Mg - free Dulbecco's phosphate buffered saline and then digesting the monolayer for three minutes at 37°C with 0.125% trypsin. The cells were split 1:10 to 1:20.

Protein Degradation in L8 Cells

The cells were grown from low density to approximately 5 x 10^5 cells per plate. The growth medium was washed off the monolayer with several rinses of PBS. 1.5 µℓ of PAB, adjusted with NaCl for mammalian cells, was added to the plates. The cells were challenged with 48/80 for one hour. After this treatment 0.25 ml of 30% TCA was added to 1 ml of the supernatant to precipitate protein and drug. One ml of 30% TCA was then added to the remaining 0.5 ml of PAB. The monolayer of precipitated cells was mechanically detached from the tissue culture plate by a rubber policeman. Both aliquots were centrifuged to remove the TCA precipitate and the supernatant analyzed for tyrosine. The total of the two aliquots represented the total free tyrosine in the L8 cells. Differences in free tyrosine levels indicated protein degradation by the assumptions of Kameyama and Etlinger (1979).

Measurement of ^{45}Ca Uptake

In a typical muscle calcium exchange experiment, muscles were exposed to $^{45}CaCl_2$ in the presence of 25°C PAB. The ^{45}Ca was 1 μCi/ml in a 2 ml volume. The specific activity of the ^{45}Ca was 5×10^4 cpm/μmole Ca at a concentration of 1.8 mM $CaCl_2$. After the designated time period for the challenge the muscles are serially washed in 5 ml PAB. After four washes (∿ 15, 15, 30, 30 minutes) the external ^{45}Ca counts have stabilized at a level of ∿ 0.4% of the original specific acitivity. Because the wash counts had stabilized at such a low value, it was assumed that all "loosely" bound surface ^{45}Ca had been removed from the muscle. The muscles were then digested in 3 N nitric acid on a steam bath for two hours. Muscle debris, if any was present after the digestion, was centrifuged and an aliquot of the supernatant was taken. The aliquots were neutralized with an aqueous sodium hydroxide solution. The ^{45}Ca was then counted on a Beckman scintillation counter.

Assay of Protein Synthesis in Frog Skeletal Muscle

The muscles were preincubated in oxygenated protein anabolism buffer (PAB) at 25°C for 20 minutes. The PAB had been made 0.6 μCi/ml with ^{14}C-tyrosine (Amersham) and 0.5 mM with cold tyrosine. The muscle was challenged in this medium for ∿ 1 hour in presence of a drug or in the absence of drug as a control. Then the muscle was treated with 30% trichloroacetic acid (TCA) at 5°C for at least 12 hrs. The muscle-TCA tube was spun at 2,000g for 10 minutes. The supernatant was collected and extracted three times with diethyl ether to remove the TCA which can interfere with the scintillation counting. An aliquot was counted to determine the amount of ^{14}C-tyrosine present. Another aliquot was taken to determine the amount of tyrosine present in the cytoplasmic pools. The tyrosine was measured by the Waalkes and Udenfriend (1957) method described elsewhere in this study. The TCA precipitate (the bulk of the muscle) was washed then solubilized in 6N NaOH for several hours in a steambath. An aliquot of this mixture was neutralized and counted. This count represented the amount of ^{14}C-tyrosine incorporated into the muscle protein. Total tyrosine incorporation was determined by dividing the specific activity of the cytoplasmic pool of tyrosine by the total ^{14}C-tyrosine incorporated into the muscle protein.

Protein synthesis was expressed as pmoles tyrosine incorporated in 1 mg of muscle per hr.

RESULTS

General

Isolated frog muscles undergo a readily observable contracture when exposed to the drugs; compound 48/80 and verapamil. Typical concentrations that would cause a readily observable contracture in the muscle were: 500 µg/ml-2 mg/ml for compound 48/80 and 1.3 mg/ml of verapamil. The contractures were characterized by a significant change of length in the muscle (∿ 40% for the sartorius) and the development of rigor or loss of plasticity. The general description of the contracture caused by compound 48/80 was very similar to the caffeine-induced contracture/rigor described by Schwartz et al (1978).

The contractures were often scored on a relative basis. A slight change in the morphology of a muscle relative to a untreated control muscle was given a value of one plus (+). A large or maximum contracture was given a value of four pluses (++++). If no difference was observed between the untreated control muscle and the treated muscle, the treated muscle was scored as "no contracture". The scoring system though semi-quantitative, is reproducible. For example, no contractures were ever observed on the muscles exhaustively pretreated with neuraminidase. Control muscles for the neuraminidase experiment treated with compound 48/80

(between 1 mg/ml and 2 mg/ml) always elicited a strong contracture response.

Our focus was on the ionic events associated with contracture and with protein turnover during the contracture. The experiments were usually designed to get the maximum contracture response such that differences in ionic flux or protein turnover between treated muscles and control muscles would be significant for a small number of samples.

Muscle contracture caused by compound 48/80 was tested for its dependence on a nerve-muscle interaction Denervated frog sartorii in which the nerve had been allowed to atrophy were observed to undergo the same contracture in the presence of compound 48/80 as a sartorius muscle not surgically altered. The denervated sartorii showed the same characteristic compound 48/80-dependent increase in ^{45}Ca-uptake (2-3 fold) relative to control as the non-surgically altered sartorius muscle.

A preliminary experiment indicated that treatment of the sartorius muscle with EDTA (2 mM for 20 minutes) could diminish, but did not abolish, the contracture caused by 48/80. Frog skeletal muscles bathed in PAB with no Ca^{++} and challenged with compound 48/80 showed no observable difference in contracture or rate of protein degradation relative to muscles incubated in the normal 1.8 mM Ca^{++} PAB.

Neuraminidase and Skeletal Muscle

Neuraminidase treatment of frog skeletal muscles prevented the observable contracture caused by compound 48/80. Similarly, the release of ^{3}H-serotonin from mast cells was inhibited by pretreatment of the cells with neuraminidase (see Chapter III, Figure 12).

Efflux of $^{86}Rb^{+}$ From Skeletal Muscle

The results of Figures 18 and 19 show the effects of verapamil and compound 48/80 on the efflux of $^{86}Rb^{+}$ from frog skeletal muscles.

Figure 18 illustrates the dose-dependent increase in the initial rate of efflux of $^{86}Rb^{+}$ from frog skeletal muscle caused by compound 48/80. This result was similar to that seen in Chapter II for P815 cells challenged with compound 48/80. (Figure 5) Figure 19 shows the dependence in the $^{86}Rb^{+}$-efflux in the frog sartorius muscle as a function of concentration of 48/80 and as a function of the time during which the muscle was exposed to the agent. At all doses of compound 48/80 the rate of $^{86}Rb^{+}$-efflux was fairly constant during the 62 minutes in which samples were taken.

Figure 20 shows the effect of verapamil on $^{86}Rb^{+}$-efflux in frog skeletal muscle challenged with verapamil. The verapamil at low concentrations (< 400 µg/ml) caused an inhibition of the spontaneous release of $^{86}Rb^{+}$ from the muscle while at higher concentrations the drug causes an increase in the $^{86}Rb^{+}$-efflux from the muscle. This result is very similar to that in Chapter II obtained for $^{86}Rb^{+}$-efflux from rat mast cells loaded with $^{86}Rb^{+}$-^{3}H-serotonin and challenged by verapamil.

Figure 18

Initial rates of $^{86}Rb^{+}$-efflux from frog skeletal muscle challenged with compound 48/80. The muscles were preincubated in 10 μCi/ml of $^{86}RbCl$ in PAB at 25°C for 1.5 hours. The muscles were prepared for pharmacological challenge by sequential incubation in two 5 ml volumes of PAB. Prior to each 10 minute incubation the muscles were dipped in a large volume of PAB. After preparation, the muscles were challenged with compound 48/80 for 10 minutes. Aliquots of the extramuscular buffer were sampled and counted by the usual methods of scintillation counting. The rates are expressed in cpm/mg per min. The muscles used were the semimembranosus (-■-■-■-) and the sartorius (-●-●-●-●-●-). The estimated error was ± 5%.

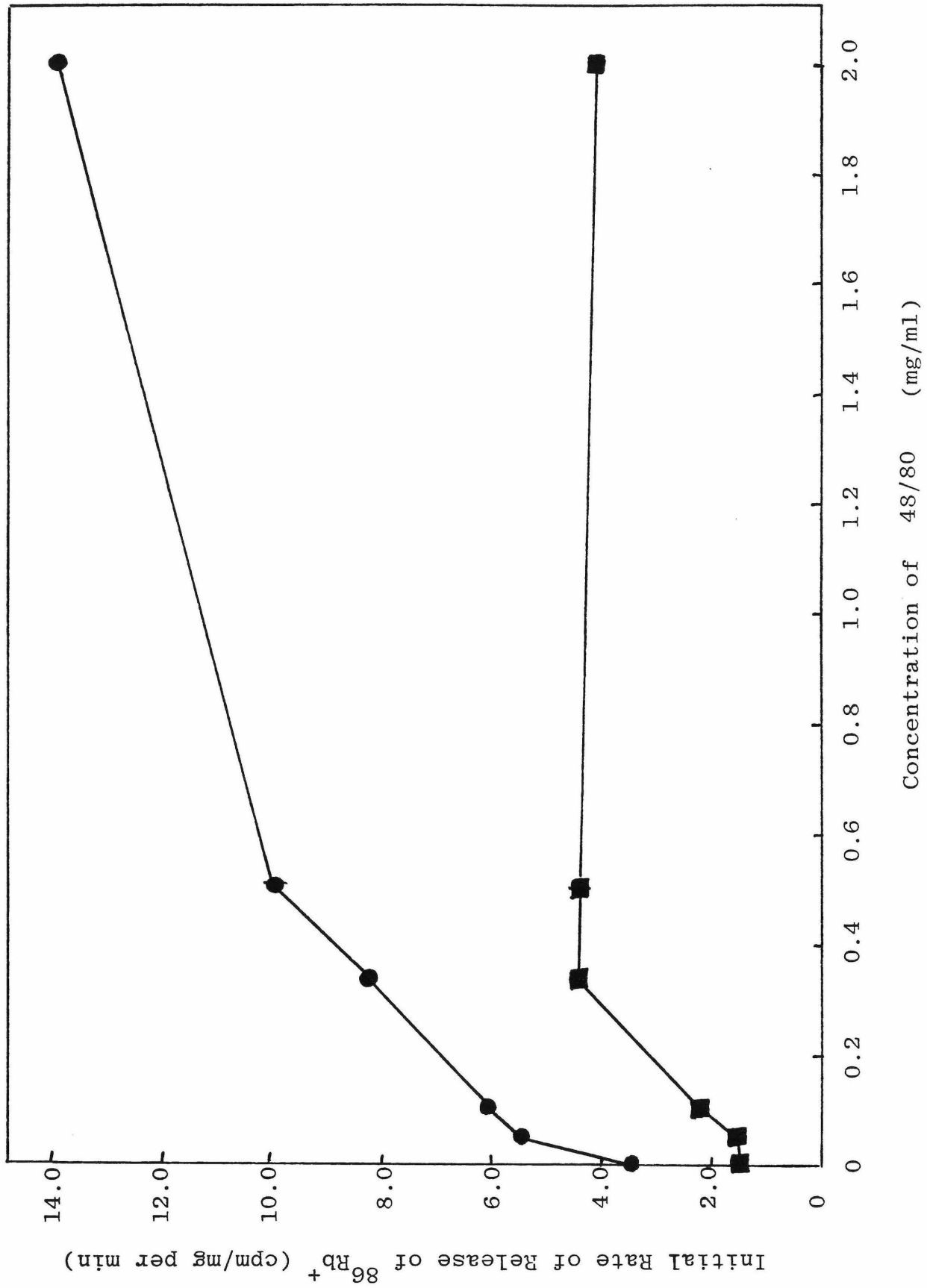

Initial Rate of Release of $^{86}Rb^{+}$ (cpm/mg per min)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Concentration of 48/80 (mg/ml)

Figure 19

Efflux of $^{86}Rb^{+}$ from a frog sartorius muscle challenged with compound 48/80 for different periods of time. The details of this experiment are described in Figure 18. The curves represent a direct count of an aliquot of the buffer containing the sartorius divided by the muscle mass. The aliquots were sampled at 10 minutes (lowermost curve), 37 minutes (middle curve) and 62 minutes (uppermost curve). The estimated error for this experiment was 50 cpm/mg.

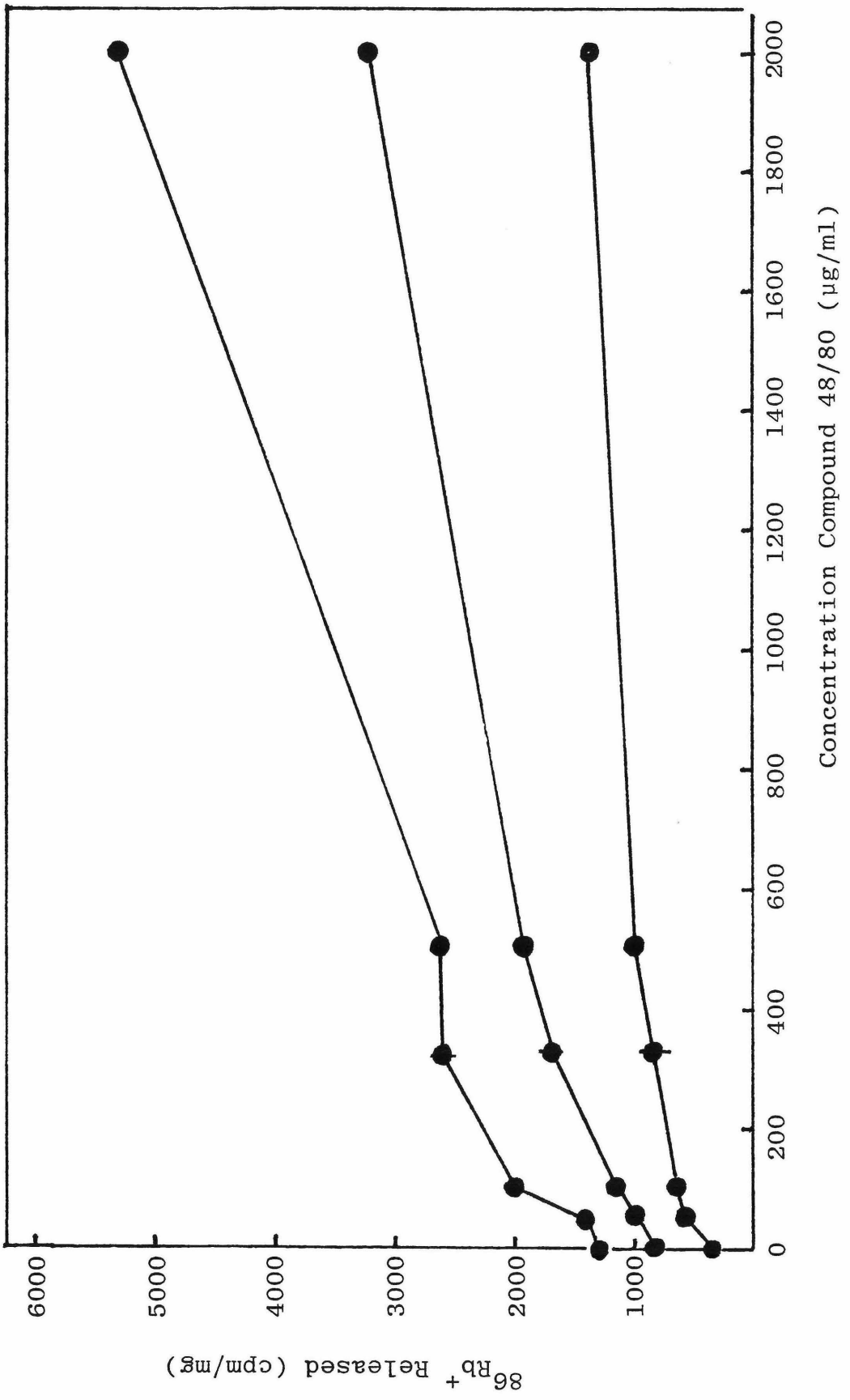
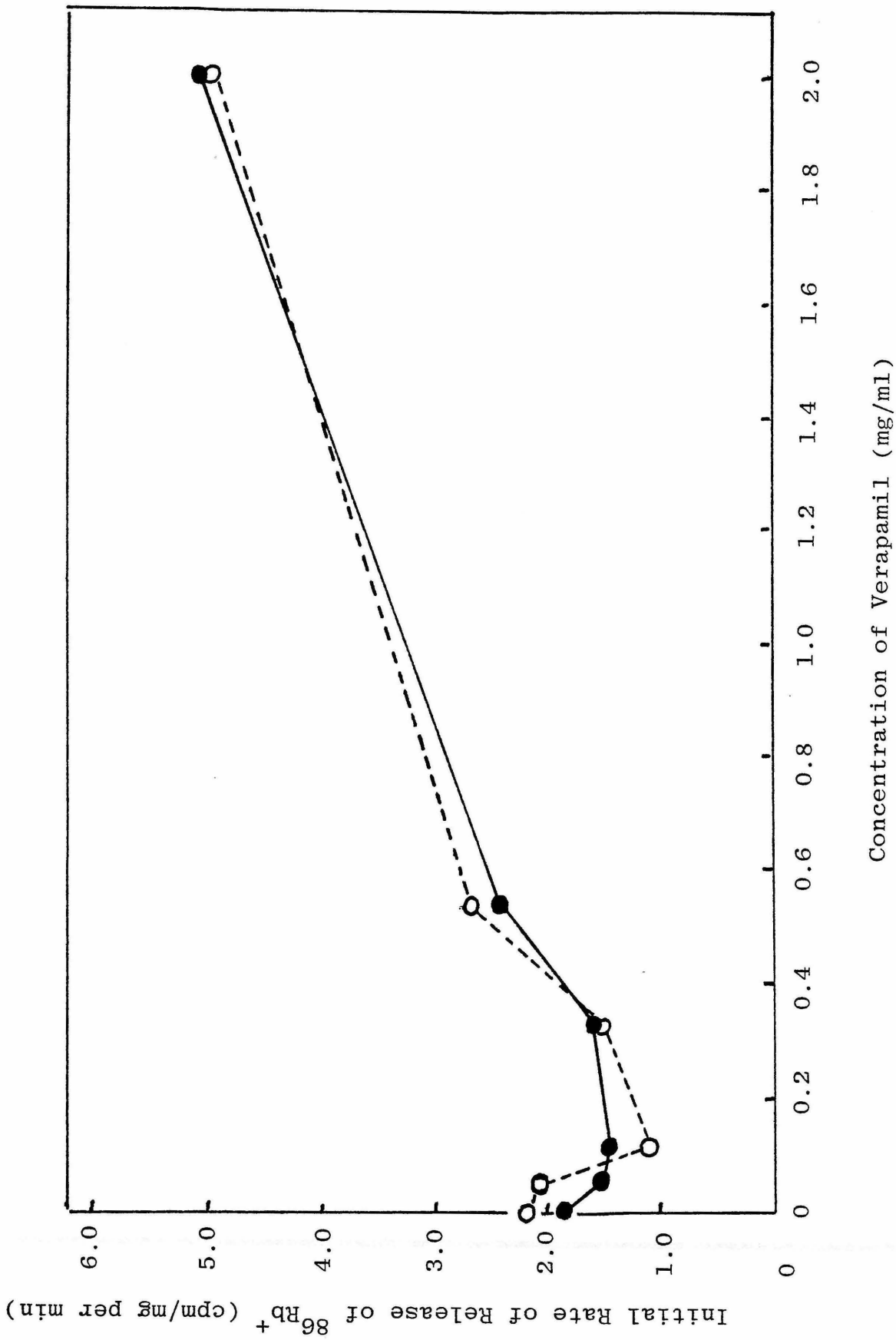

$^{86}Rb^{+}$ Released (cpm/mg)
6000
5000
4000
3000
2000
1000
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Concentration Compound 48/80 (µg/ml)

Figure 20

Initial rates of $^{86}Rb^{+}$-efflux from frog skeletal muscle challenged with verapamil. Details of the experimental procedure are described in Figure 18. Semitendinosus muscle (---o---o---o---) and muscles of the shin complex (-●-●-●-) were challenged by the drug for 10 minutes at 25°C. $^{86}Rb^{+}$-efflux was expressed in units of cpm/mg per min. The estimated error was 5%.


Initial Rate of Release of $^{86}Rb^{+}$ (cpm/mg per min)
6.0
5.0
4.0
3.0
2.0
1.0
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Concentration of Verapamil (mg/ml)

Protein Degradation in Skeletal Muscle

Compound 48/80 (2 mg/ml) caused a large increase in the rate of protein degradation in frog skeletal muscles relative to controls (Figure 21). Protein degradation was estimated to be a function of release of free tyrosine into the medium and into the internal pools of the muscle. This amino acid was chosen because it is not metabolized and does not itself alter the rates of protein synthesis or degradation (Kameyama and Etlinger, 1979, Fulks et al, 1975). This effect was relatively fast because significant differences in the tyrosine levels released into the extracellular pool could be detected in the first 15 minutes after challenge by the drug. The approximately 2-fold increase in net protein degradation was correlated with the characteristic contracture of the muscle caused by compound 48/80.

Figure 21 shows the effect of compound 48/80 on protein degradation in frog skeletal muscle. The results are pooled for four quartets of muscles; the sartorius, the semitendinosus, the gracilis major and the so-called shin complex. The statistical analysis using the t-test for paired observations (Sokal and Rohlf, 1969) is summarized in the following Table:

control vs 48/80 (2 mg/ml)	significant ($P < 0.05$)
control vs neuraminidase	not significant
neuraminidase vs neuraminidase then 48/80	not significant

The results of a preliminary experiment on protein degradation were very similar to Figure 12 and are summarized in the following Table:

Treatment	Relative Degradation Rate
control (normalized)	100%
compound 48/80 (2 mg/ml)	296%
neuraminidase	139%
neuraminidase then compound 48/80	108%

In all experiments involving protein degradation only the muscles challenged with compound 48/80 and not pretreated with neuraminidase showed a contracture.

Figure 22 shows that compound 48/80 caused a dose-dependent increase in protein degradation in L8 myoblast cells.

Figure 21

Protein degradation in frog skeletal muscle: effect of neuraminidase and compound 48/80. This histogram summarizes the pooled results derived from four quartets of muscles in one controlled experiment. The muscles used were the sartorius, semitendinosus, gracilis major and the shin complex.

The control values were normalized within the quartets and a ratio determined for values of the muscles treated with neuraminidase and/or compound 48/80 (2 mg/ml). The error bars on the treated muscles represent the standard deviation of these ratios of treatments to controls. The standard deviation of the control values before normalization was 78 $\pm$ 12 pmoles Tyr/mg per hr.

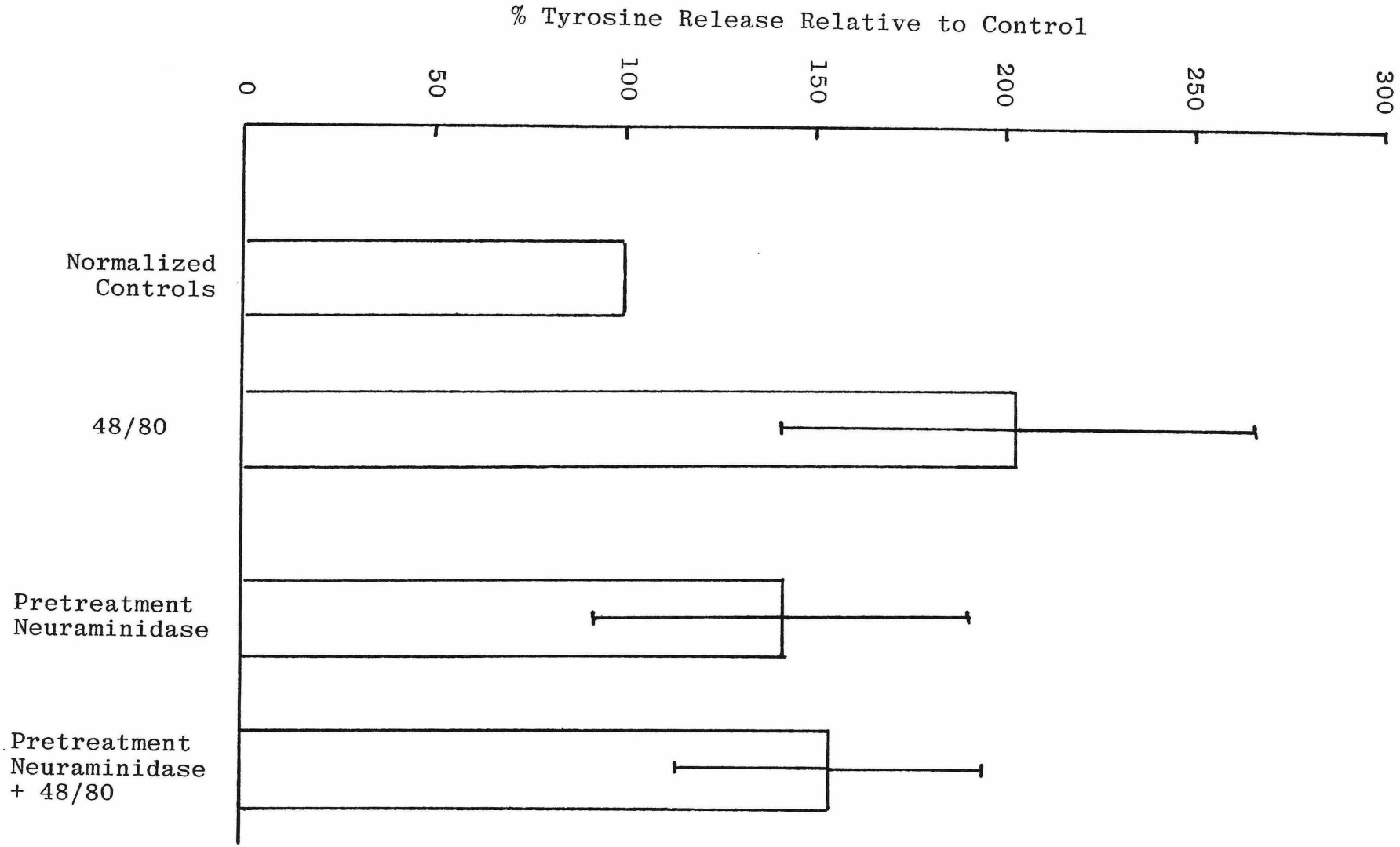
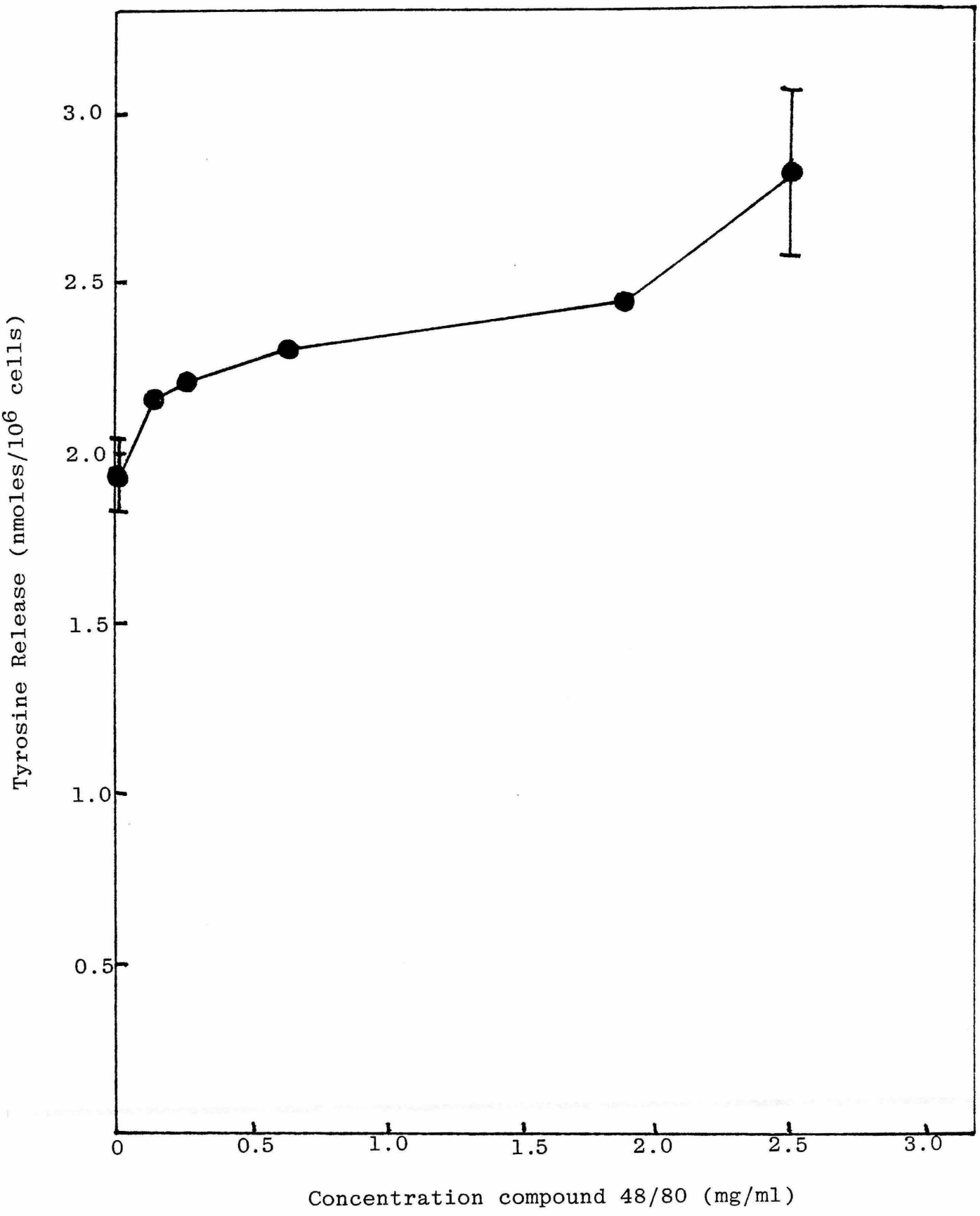

% Tyrosine Release Relative to Control
0
50
100
150
200
250
300
Normalized Controls
48/80
Pretreatment Neuraminidase
Pretreatment Neuraminidase + 48/80

Figure 22

Effect of compound 48/80: protein degradation in L8 myoblast cells. The L8 myoblast cells were challenged with compound 48/80 for one hour at $37^{o}C$. Total protein degradation was measured as the sum of the tyrosine released into the extracellular buffer and the free tyrosine in the intracellular pools. The units were nmoles Tyr/10^{6} cells. The details of the method for measuring protein degradation in tissue culture cells are described in Materials and Methods, Chapter IV).


Tyrosine Release (nmoles/10^6 cells)
3.0
2.5
2.0
1.5
1.0
0.5
0
0.5
1.0
1.5
2.0
2.5
3.0
Concentration compound 48/80 (mg/ml)

Uptake of ^{45}Ca in Skeletal Muscles

Compound 48/80, at concentrations sufficient to cause an observable contracture, also caused a significant increase in ^{45}Ca-uptake into frog skeletal muscle. In four distinct experiments involving seven muscle pairs, compound 48/80 (2 mg/ml) caused a (2.2 $\pm$ 0.5)-fold increase over controls in initial ^{45}Ca-uptake. Neuraminidase pretreatment of the muscles did not abolish this effect. For example, three muscle pairs, pretreated with neuraminidase then challenged with compound 48/80 (2 mg/ml) showed a (2.2 $\pm$ 0.6)-fold increase over muscles only treated with neuraminidase in initial ^{45}Ca-uptake.

Figure 23 shows that neuraminidase-treated muscles have a higher rate of ^{45}Ca-uptake relative to non-treated muscles. The results from another pair of muscles were pooled with the two pairs of muscles in Figure 21 for a test of statistical significance. Using a t-test for paired comparisons (Sokal and Rohlf, 1969) the difference between ^{45}Ca-uptake in muscles treated with neuraminidase and control muscles was found to be statistically significant ($P < 0.05$).

The measured "^{45}Ca-uptake" represents the amount of label that has been taken up during the exposure of the muscle to the ^{45}Ca and then retained by the muscle until completion of a workup consisting of four washes over a

period of approximately 1.5 hours. The purpose of these washings is to exchange membrane-bound ^{45}Ca with cold calcium. Since some intracellular ^{45}Ca could exchange with calcium in the extracellular medium during the workups, the measured ^{45}Ca retained in the muscle need not represent total uptake of ^{45}Ca during the pharmacological challenge but instead represents the difference between the ^{45}Ca gained during exposure of the muscle to ^{45}Ca during the incubation with drug and the subsequent loss of the ^{45}Ca during the workup of the muscle. Indeed, calcium exchange does occur in frog sartorius muscle when exposed to frog Ringer's solution. For example, Cosmos and Harris (1961) found that in muscle incubation in frog Ringer's there was a steady net inflow of Ca^{++}; yet there was some exchange of label indicating an efflux of ^{45}Ca out of the muscle.

Assuming that a gram of muscle equals a milliliter of myoplasm, the increase in ^{45}Ca-uptake caused by compound 48/80 translates to a rate of increase in myoplasmic $[Ca^{++}]$ of $\sim$ 3 μM per minute if the "influx" of calcium calculated from ^{45}Ca-uptake was totally directed into the myoplasm with no uptake by other intracellular calcium pools.

Figure 23 shows that verapamil inhibits the uptake of ^{45}Ca caused by compound 48/80. Interestingly, verapamil, alone, causes a contracture and seems moreover to enhance the contracture caused by compound 48/80.

Figure 23

Calcium uptake in frog skeletal muscle: effect of neuramindase and compound 48/80. Figure shows the results of a representative experiment derived from two quartets of frog skeletal muscle. The muscles were incubated in PAB at 37°C with ^{45}Ca at a specific activity of 0.724 pmole/cpm. The challenge by 48/80 was for 12 minutes. The neuramindase pretreatment was ∿ 85 minutes at 0.25 units/ml. The key observation was that only the muscles challenged by compound 48/80 (2 mg/ml) showed the characteristic contracture. The control muscles, muscles pretreated with neuramindase, and muscles pretreated with neuramindase and subsequently challenged by compound 48/80 were uniformly flaccid.

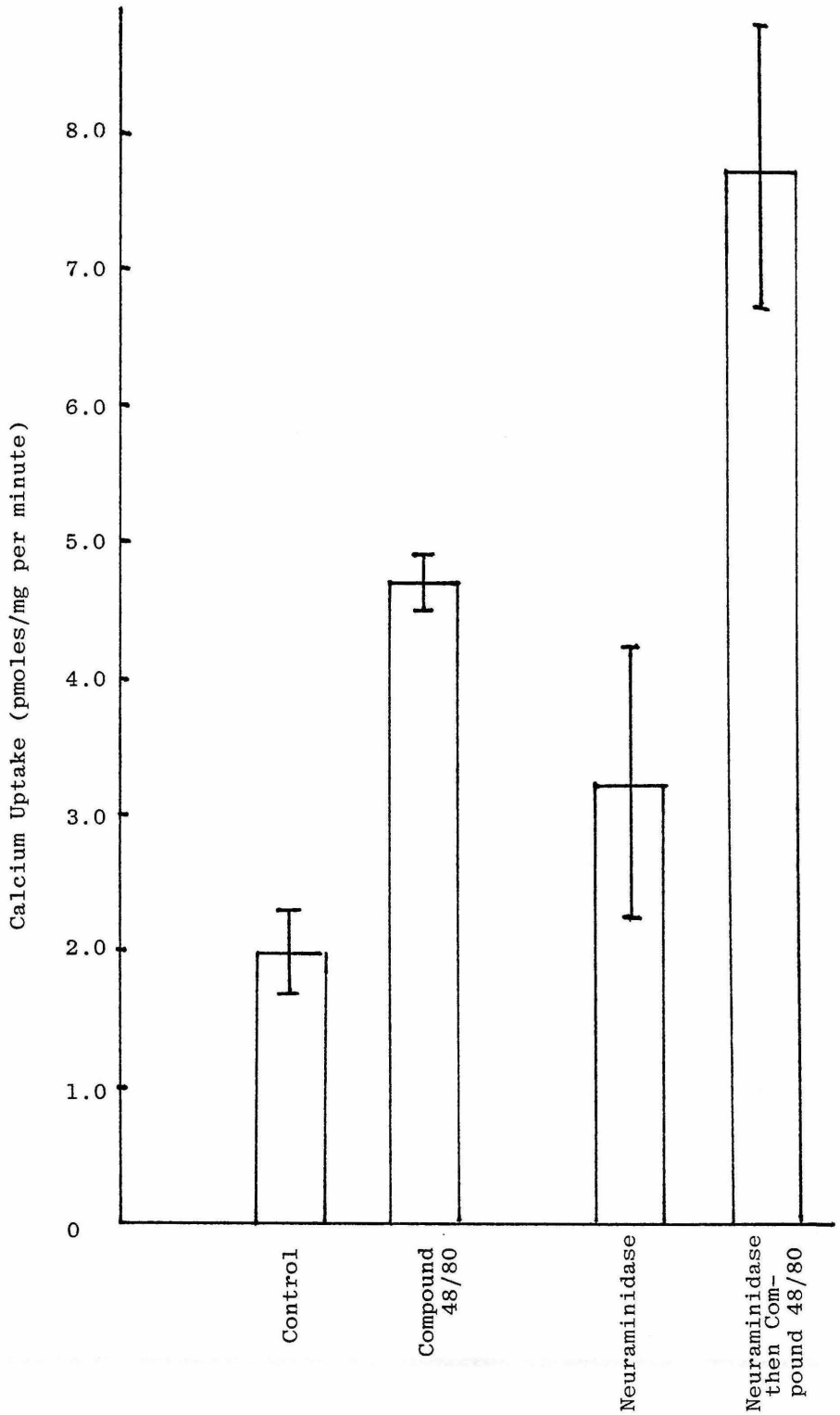
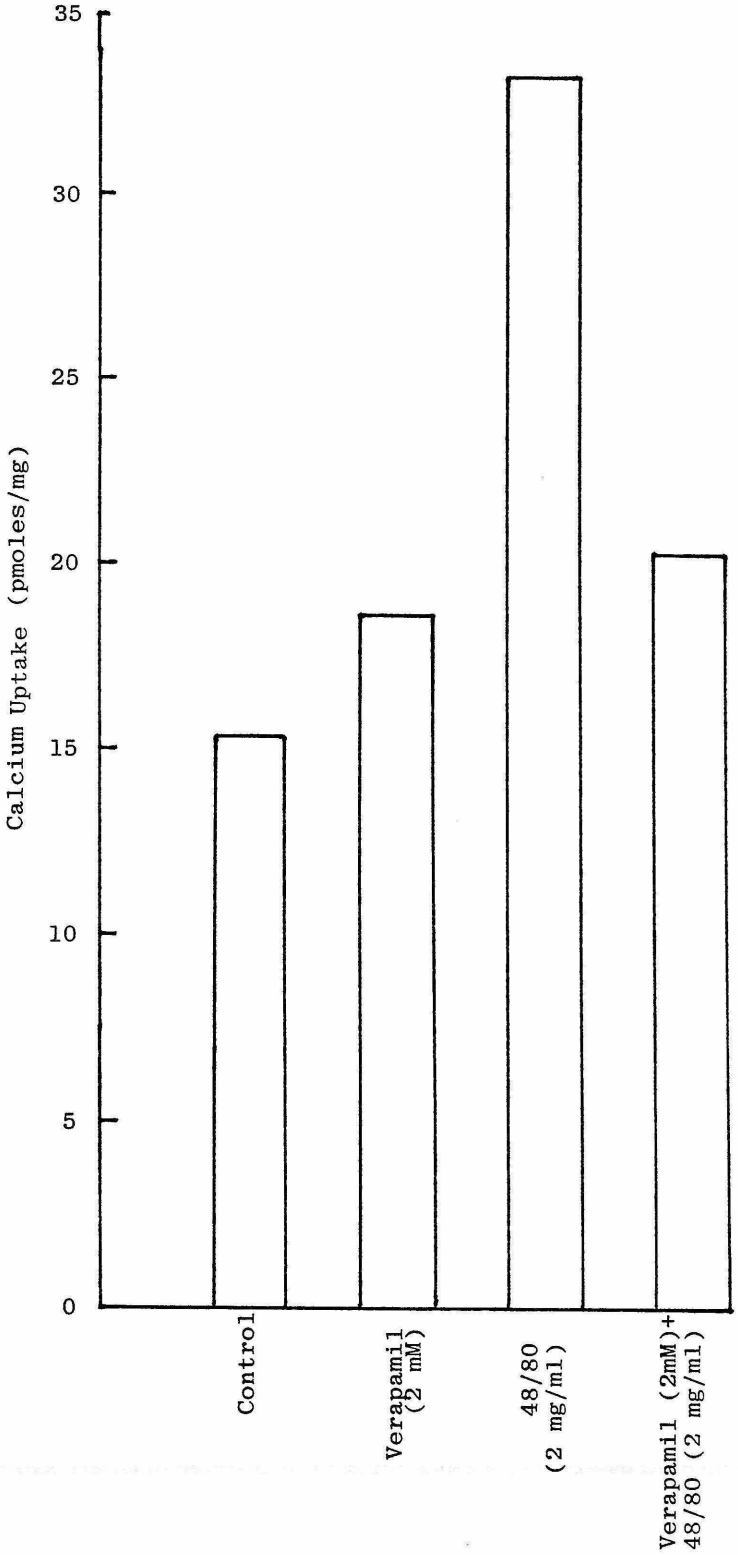

Calcium Uptake (pmoles/mg per minute)
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Control
Compound 48/80
Neuraminidase
Neuraminidase then Compound 48/80

Figure 24

Effect of verapamil and compound 48/80 on calcium uptake in frog skeletal muscle. The histogram represents an experiment involving a quartet of frog semimembranosus muscles. The challenge by the various drugs was for five minutes with the verapamil and compound 48/80 being added simultaneously to the one muscle. The control muscles did not show any observable contracture. Muscles treated only with verapamil or compound 48/80 showed an observable contracture. The muscle treated with both drugs simultaneously was observed to have a "stronger" contracture than muscle treated with only compound 48/80 or only verapamil. The estimated error was $\pm$ 3 pmoles/mg.


Calcium Uptake (pmoles/mg)
35
30
25
20
15
10
5
0
Control
Verapamil (2 mM)
48/80 (2 mg/ml)
Verapamil (2mM)+ 48/80 (2 mg/ml)

Protein Synthesis in Skeletal Muscle

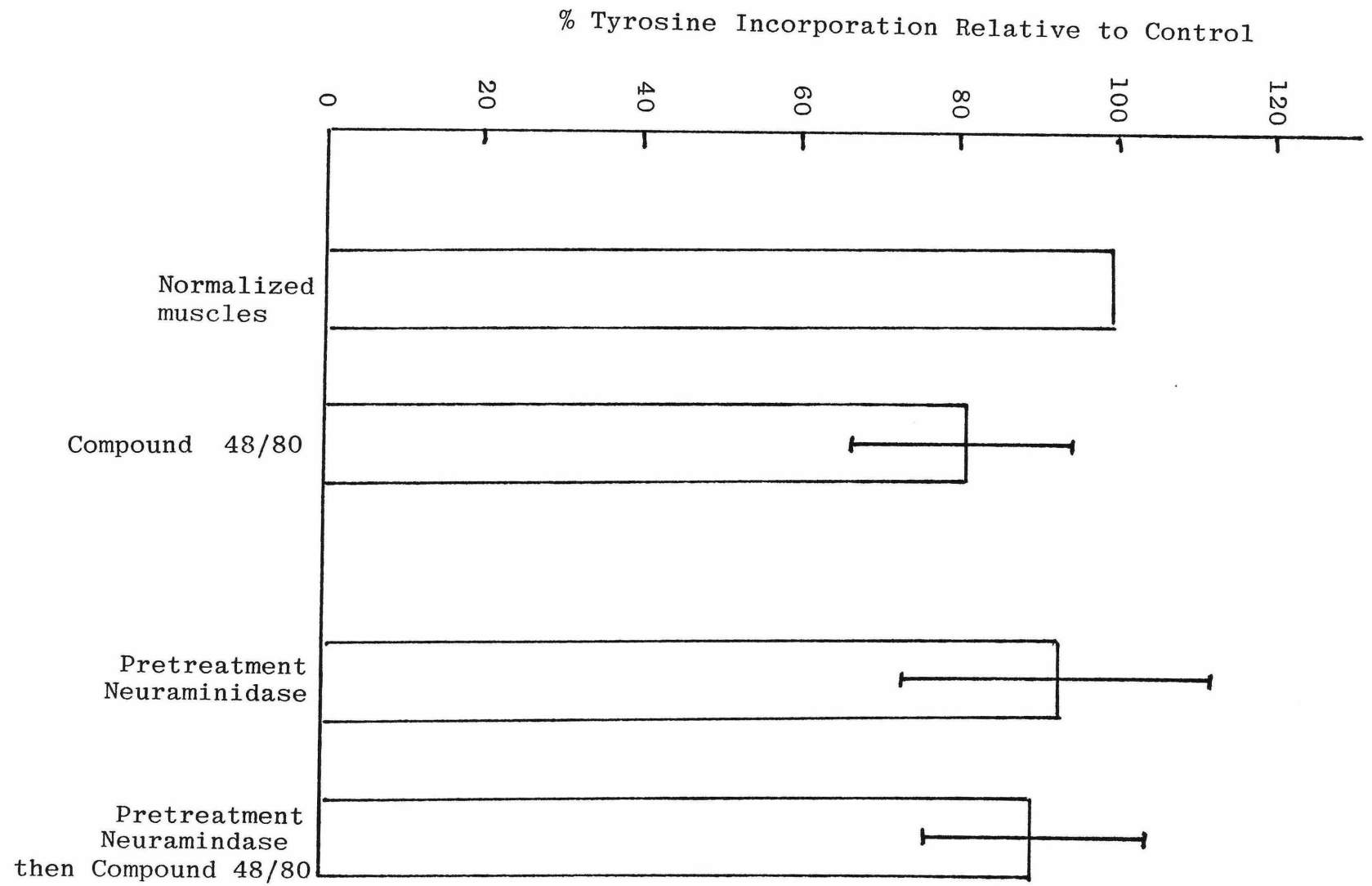
Figure 25 shows the effect of compound 48/80 on the rate of protein synthesis in frog skeletal muscle. In these experiments only muscles challenged with compound 48/80 and not pretreated with neuraminidase showed an observable contracture. A statistical analysis of the results of two experiments examining protein synthesis in frog skeletal muscle indicated no significant difference in the rate of protein synthesis between any two of the experimental treatments. The statistical test used was a t-test comparison for paired values (Sokal and Rohlf, 1973).

Figure 25

Protein synthesis in frog skeletal muscles: effect of neuraminidase and compound 48/80. This histogram summarizes the results from five quartets of muscles in two separate experiments. The control muscles were normalized to the 100% level of tyrosine incorporation. Examples of the incorporation of tyrosine into control muscles are:

semitendinosus	7.0 pmole Tyr/mg per hr
sartorius	7.8 pmole Tyr/mg per hr
gracilis major	18.5 pmole Tyr/mg per hr
semimembranosus	15.9 pmole Tyr/mg per hr

The incorporation of Tyr into muscles pretreated with neuroaminidase and/or challenged with 2 mg/ml compound 48/80 was divided by the control values. A standard deviation of these ratios for each group was calculated and is represented by the error bars on the histogram.


% Tyrosine Incorporation Relative to Control
0
20
40
60
80
100
120
Normalized muscles
Compound 48/80
Pretreatment Neuraminidase
Pretreatment Neuramindase then Compound 48/80

DISCUSSION

Model for the Contracture of Frog Skeletal Muscle Caused by Compound 48/80

In order to simplify the following, the discussion begins with the presentation of a hypothetical model for the mechanism by which pharmacological agents such as compound 48/80 interact with muscles and thereby cause effects such as contracture. Subsequently, the results obtained in these studies, and of others, will be discussed in terms of this postulate.

The model features two, unconnected processes which can be initiated by agents such as compound 48/80: (i) release of Ca^{++} from the sarcoplasmic reticulum into the myoplasm which causes the muscle to contract. (ii) an independent increase in the rate of Ca^{++} influx across the cell membrane. (This influx need not result in a net increase in $[Ca^{++}]$ in the myoplasm as the incoming calcium may be taken up by the sarcoplasmic reticulum and/or pumped back out of the cell as rapidly as it enters.)

The mechanistic pathway of the contractures caused by compound 48/80 is initiated by the binding of the drug to a surface molecule situated on the membrane of the t-tubule; this surface molecule includes sialic acid

residues which must be intact for interaction with compound 48/80. The interaction of compound 48/80 with a surface molecule in the t-tubules transmits a "signal" to a region of the membrane of the sarcoplasmic reticulum which is essentially in contact with the membrane of the t-tubule; this causes a sharp increase in the calcium permeability across the membrane of the sarcoplasmic reticulum such that large amounts of calcium flow out of this organelle into the surrounding myoplasm. (The nature of the "signal" is not known but could be an important component of the coupling of excitation to contraction in skeletal muscle. Chapter I). This increase in myoplasmic [Ca^{++}] in turn activates the contractile apparatus causing the muscle to contract. Moreover, the increase in myoplasmic [Ca^{++}] activates calcium-dependent protein degradation in the muscle and also causes an increase in K^{+} permeability of the outer membranes of the muscle.

In a separate process, compound 48/80 causes an influx of extracellular Ca^{++} across those regions of the muscle membrane not in direct contact with the terminal cisternae of the SR. This entering Ca^{++} can be rapidly taken up by the intracellular calcium pools, mainly the sarcoplasmic reticulum. As noted earlier, the sarcoplasmic reticulum has the ability to remove very rapidly calcium from the myoplasm by virtue of the high rate and capacity of its ATP-dependent Ca^{++} pumps; indeed, such removal of

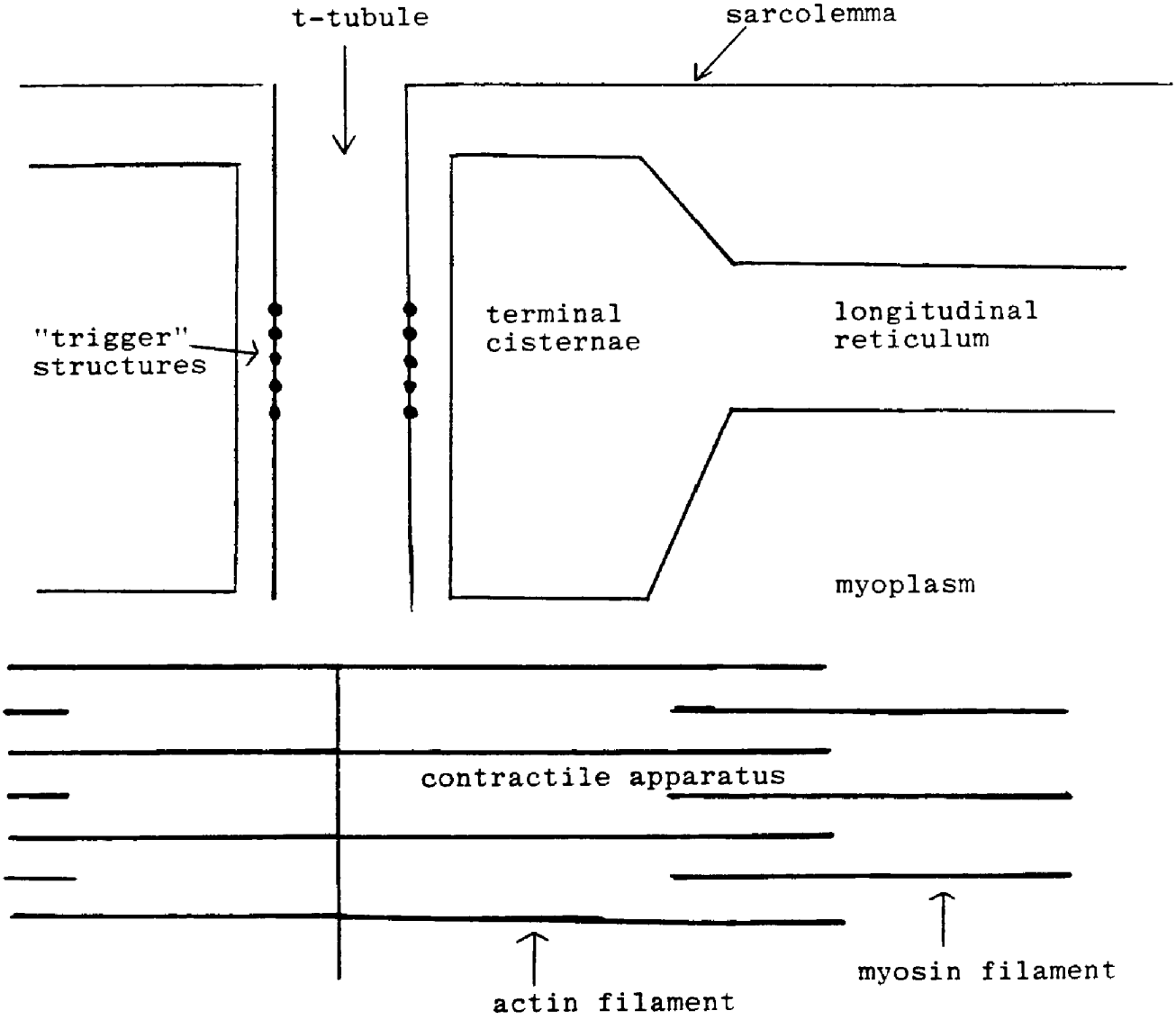
calcium from the myoplasm by the sarcoplasmic reticulum leads in normal circumstances to the rapid relaxation of a contracted muscle.

If compound 48/80 does not interact with the t-tubular "trigger" structures but can still cause the influx of extracellular Ca^{++} across the muscle membrane, Ca^{++} pumping capacity of the SR may well be sufficient to prevent the myoplasmic $[Ca^{++}]$ from reaching the threshold required for contractures. Thus one may observe an uptake of Ca^{++} without concomitant contraction. However, when compound 48/80 also activates the "trigger" structures causing release of Ca^{++} from the SR and also allows the concentration of myoplasmic $[Ca^{++}]$ to reach the threshold required for contraction. (The amount of calcium released in this way from the sarcoplasmic reticulum greatly exceeds the capacity of any ATP-dependent Ca^{++} pumps to remove Ca^{++} from the myoplasm.)

Figure 26 is a schematic diagram of skeletal muscle illustrating the structures of skeletal muscle relevant to the proposed model.

Figure 26 - Schematic of Skeletal Muscle

Structure	Function
sarcolemma	propagation of initial action potential
t-tubule	excitation-contraction coupling
terminal cisternae	release of Ca^{++}
longitudinal reticulum	reuptake of Ca^{++}
"trigger" structure	stimulus-contracture coupling ? excitation-contraction coupling ?



Evidence Relevant to Location of "Trigger" Structures in t-Tubules

The contracture of frog skeletal muscle caused by compound 48/80 is inhibited by pretreatment of the muscle with neuraminidase (Figures 21 and 25). Neuraminidase does not penetrate the plasma membrane (Gottschalk, 1972), thus the target molecule for compound 48/80 that "triggers" the contracture should reside on the surface of the muscle and its activity should depend on sialic acid residues susceptible to removal by neuraminidase.

In this regard, Dorrscheit-Kafer (1977) found that pretreatment of frog sartorii with neuraminidase caused the threshold membrane potential for contraction to be raised and found that increases in extracellular Ca^{++}, an ion that preferentially binds to sialic acids over other physiological ions (Jaques et al, 1977), also raised the contraction threshold. This finding indicated that a cell surface structure which required sialic acids for maximal activity was involved in excitation-contraction coupling in skeletal muscles. This molecule and the neuraminidase-sensitive "trigger" molecule of our model may well be identical.

The model proposes that the interaction of compound 48/80 occurs with the "trigger" structure located on the surface of the t-tubules. In this regard, the study of glycerol-treated muscles demonstrated that the t-tubules were essential for excitation-contraction coupling. Micrographs indicate that glycerol treatment causes a reorganization of the t-tubules in such a way that they no longer appear as the intermediate element of the triad of the SR, t-tubule and the sarcolemma and seem physically pinched off from the sarcolemma (Howell and Jenlon 1967). Muscles treated in this way with glycerol quickly loose their twitch response and their ability to contract in 35 mM K^+ Ringers but still respond to caffeine, a direct activator of the SR. These observations indicate that the coupling between the muscle action potential and the SR has been disrupted by the glycerol treatment,

Morphologically, the t-tubules lie is close proximity to the terminal cisternae of the SR and approximately 80% of the surface area of the t-tubules about the terminal cisternae (Peachey, 1965).

Compound 48/80 likely causes the activation of Ca^{++} release from the sarcoplasmic reticulum by a process which depends on receptors which contain sialic acid residues and which are localized in the t-tubules.

Similarly, normal excitation-contraction depends on a coupling process localized in the t-tubules and dependent on sialic acid susceptible to neuraminidase (Dorrsheidt-Kafer, 1977). These similarities lead to the suggestion in the model that the cell surface molecules involved in these two processes are the same.

Incidentally, both compound 48/80 (MW 800) and V. Cholerae neuraminidase (MW 90,000) should be able to enter the lumen of the t-tubules as even larger molecules such as ferritin (MW 460,000) and peroxidase (MW 200,000) can easily enter these regions (Constantin, 1975).

Evidence for an Increase in Myoplasmic [Ca^{++}]

A central feature of the model for contracture caused by compound 48/80 is that in normal muscle the drug triggers a process that leads to an increase in myoplasmic [Ca^{++}]. Likewise, the normal excitation-contraction cycle of skeletal muscle features a transient increase in myoplasmic [Ca^{++}] from 0.1 μM to approximately 2 μM (Caputo, 1978) with the source of this increase being the sarcoplasmic reticulum (Hasselbach, 1980). Therefore, we will examine the evidence that compound 48/80 does indeed cause an increase in myoplasmic [Ca^{++}].

1) $^{86}Rb^{+}$-Efflux

Compound 48/80 causes a dose-dependent increae in the efflux of $^{86}Rb^{+}$ from frog skeletal muscle (Figure 18). In non-excilatory cells, K^{+}-efflux (Rb^{+} is an analogue of K^{+}) is correlated with an increase in the cytosolic concentration of Ca^{++}. This increase in the cytosolic [Ca^{++}] is believed to increase the permeability of K^{+} across the plasma membrane which causes the K^{+} to flow out of the cell down it concentration gradient (Putney, 1979). If one extends this hypothesis to skeletal muscle, the increase in the $^{86}Rb^{+}$-efflux caused by compound 48/80 suggests an increase in the myoplasmic [Ca^{++}].

2) Protein Degradation

Further evidence that contracture of frog skeletal muscle caused by compound 48/80 features an increase in myoplasmic [Ca^{++}] was derived from studies of protein degradation in the muscles. We will briefly discuss the results and than will review the findings of others that show the rate of intracellular proton degradation reflects cytosolic [Ca^{++}].

Figure 21 shows a significant increase in the rate of protein degradation in muscles challenged with compound 48/80 relative to control muscles. This increase in the rate of protein degradation correlates with the observable contracture of frog skeletal muscle caused by compound 48/80. In contrast, there was no significant increase in the rate of protein degradation in muscles pretreated with neuraminidase and subsequently challenged with compound 48/80 relative to control muscles pretreated with neuraminidase but then not challenged with compound 48/80 (Figure 21). Pretreatment of the muscle with neuraminidase also abolishes the contracture caused by compound 48/80 further implying that increase in the rate of protein degradation may correlate with contracture of the muscle. Further evidence along these lines is provided by our finding that a significant dose-dependent increase in the rate of protein degradation

was seen in L8 myoblast cells treated with compound 48/80 (Figure 22).

We suggest that during contracture the relative increase in the rate of protein degradation was due to an increase in the concentration of myoplasmic [Ca^{++}]. This correlation has been previously proposed by others. Kameyama and Etlinger (1979) showed that the rate of protein degradation in rat soleus muscles in the presence of extracellular Ca^{++} was increased when the muscles were exposed to the calcium ionophore, A23187 relative to controls in the same medium but not treated with the ionophore. Cochrane and Douglas (1973) demonstrated that mast cells in the presence of extracellular Ca^{++}, and treated with A23187 underwent degranulation, a process likewise thought to reflect on an increase in cytosolic [Ca^{++}] (Chapter II, Theoharides and Douglas, 1978). Therefore, if A23187 treatment of mast cells and rat soleus muscle effects intracellular [Ca^{++}] in the same manner, then rat soleus muscles should experience an increase in myoplasmic [Ca^{++}] on treatment with A23187 and such an increase in [Ca^{++}] would lead to an increase in protein degradation.

There is circumstantial biochemical evidence showing that protein degradation in skeletal muscle depends on the intracellular concentrations of calcium. For example, Reddy et al (1975) and Kar and Pearson (1978) have isolated neutral proteases that are activated by Ca^{++} and hydrolyze a variety of muscle proteins. Moreover, Dayton et al (1976) have purified a Ca^{++}-activated protease, possibly involved in myofubrillar protein turnover, from porcine skeletal muscle.

3) Summary

In summary, the increase in protein degradation which correlates with contracture caused by compound 48/80 (Figure 21) and the increase in the efflux of $^{86}Rb^{+}$ (Figure 18) which depends on the dose of compound 48/80 suggest that an increase in the concentration of myoplasmic $[Ca^{++}]$ may be the central feature of the mechanism of contracture in frog skeletal muscle caused by challenges with compound 48/80.

Possible Source of Ca^{++} for Contracture

In our proposed model for contracture caused by compound 48/80, there are two independent processes that could effect the concentration of myoplasmic $[Ca^{++}]$: an increase in the rate of Ca^{++} influx across the cell membrane and/or the net release of Ca^{++} from the SR. Experimental evidence suggests that extracellular calcium is not sufficient to cause contracture of frog skeletal muscle challenged with compound 48/80. Accordingly, the sarcoplasmic reticulum seems the most likely source of calcium required for contraction.

1) Extracellular Calcium

Evidence that extracellular calcium is not the source includes the following observation. Compound 48/80 could cause a contracture of isolated frog skeletal muscle incubated in physiological buffer containing no calcium. This contracture was similar to a normal contracture casued by the drug in the presence of the regular 1.8 mM Ca^{++} physiological buffer. This observed independence of the contracture on extracellular calcium is similar to the lack of a requirement for extracellular calcium in the degranulation of mast cells caused by compound 48/80 (Uvnas and Thon, 1961), we have also discussed (Chapter III)

a) Measurement of ^{45}Ca-uptake - The uptake of extracellular calcium by muscles challenged with compound 48/80 was increased relative to control muscles; however this increase was not correlated with a contracture of the muscle (Figure 23). Specifically challenge with compound 48/80 of muscles pretreated with neuraminidase causes the same relative increase in ^{45}Ca uptake as does challenge with compound 48/80 of muscles not treated with the enzyme; these muscles however did not undergo a contacture.

The model asserts that compound 48/80 causes an increase in the influx across the sarcolemma of extracellular calcium; this process leads to an increased uptake of ^{45}Ca without, necessarily an increase in myoplasmic $[Ca^{++}]$, because the ATP-dependent pumps of the SR take up the ^{45}Ca upon entry into the myoplasm and prevent its exchange with the extracellular medium. When the SR is not activated by compound 48/80 because of neuraminidase pretreatment of the muscle, the influx of extracellular calcium caused by compound 48/80 is not sufficient to activate the contractile apparatus because the SR takes up the entering Ca^{++} sufficiently rapidly to not increase the myoplasmic $[Ca^{++}]$ enough to cause the muscle to contract.

b) Effect of Verapamil - Verapamil caused a decrease in the uptake of ^{45}Ca in frog skeletal muscles simultaneously challenged with compound 48/80 relative to muscles only treated with 48/80 but does not inhibit the contracture (Figure 24). In fact, the two drugs where added simultaneously to a muscle seen to cause a "stronger" contracture than either did alone. Verapamil blocks the influx of calcium across the plasma membrane in a variety of preparations of smooth and cardiac muscle (Fleckenstein, 1977). If this mechanism of action could be extended to skeletal muscle, then the results of Figure 24 are readily explained; the drug inhibited the increase in uptake of ^{45}Ca caused by compound 48/80 by specifically blocking the influx of extracellular calcium in the muscle. Since this blockage by verapamil of the influx of extracellular calcium does not inhibit contracture caused by compound 48/80, we conclude that an increase in uptake of extracellular calcium does not correlate with contracture of the muscle. According to our model, this suggests that verapamil blocks calcium influx across the sarcolemma but does not interfere with the operation of the receptors in the t-tubules which on binding of compound 48/80 mediate the contracture.

Interestingly, verapamil, alone, causes a contracture of frog skeletal muscle. This is a surprising result for verapamil has been defined as a specific blocker of calcium channels of the plasma membrane of a number of cells and tissues (Fleckenstein, 1977) but the contraction of muscle depends on the mobilization of Ca^{++} from the SR into the myoplasmic pool (Hasselbach, 1980) not calcium influx across the sarcolemma. Indeed, verapamil may be acting as a blocker of calcium channels when the drug at low concentrations causes a decrease in the spontaneous efflux of $^{86}Rb^{+}$ from frog muscles (Figure 20). This decrease in $^{86}Rb^{+}$-efflux could be reflective of a decrease in myoplasmic $[Ca^{++}]$ (see Chapter II, Putney (1979)) caused by verapamil blocking the spontaneous flux of Ca^{++} into the myoplasm from membrane bound pools and/or the extracellular medium. However, at higher concentrations of verapamil this effect due to the blockage of calcium channels is overcome and there is an increase in myoplasmic $[Ca^{++}]$ which leads to observed increases in $^{86}Rb^{+}$-efflux and to the observed contracture. In this case, the mechanism of contracture of skeletal muscle caused by verapamil is unknown but may be related to the mechanism of degranulation of mast cells caused by the drug (Chapter III) in verapamil is proposed to act only after entry into the cell.

c) Summary - In summary, we have examined several lines of experimental evidence and made an analogy to mast cells to propose that in skeletal muscle the increase in calcium influx from the extracellular environment caused by compound 48/80 is neither a sufficient condition nor a necessary condition for contracture. Accordingly, the extracellular pool does not seem to be a likely source of Ca^{++} that causes the increase in myoplasmic $[Ca^{++}]$ and subsequently causes contracture.

The mechanism of the increased uptake of ^{45}Ca caused by compound 48/80 probably depends on a membrane component which interacts with compound 48/80 to increase the rate of influx of calcium into the muscle. The function of this component of the sarolemma is not affected by treatment of the muscle with neuraminidase and the influx of ^{45}Ca is taken up by an intracellular calcium pool, presumably, the sarcoplasmic reticulum.

2) Sarcoplasmic Reticulum

Possible indirect evidence that the sarcoplasmic reticulum is the source of Ca^{++} required for the contracture of muscle caused by compound 48/80 is that contracture caused by high concentrations of caffeine is generally similar in appearance ie shortened muscle and loss of plasticity (Schwarz et at, 1978). Moreover, caffeine contracture is caused by the direct interaction of the drug with the SR causing the release of Ca^{++} into the myoplasm (Weber and Herz, 1968).

Conclusion and Further Tests of the Model

The contracture of skeletal muscle challenged with compound 48/80 correlates with an increase in the rate of protein degradation and the rate of $^{86}Rb^{+}$-efflux in the muscle; these observations suggest an increase in myoplasmic [Ca^{++}]. Compound 48/80 also caused an increase in the uptake of ^{45}Ca, however this increase does not correlate with contracture. Pretreatment with neuraminidase of the muscles abolished the contracture indicating that compound 48/80 interacts with a "trigger" structure that contains sialic acid residues essential for activity, such pretreatment does not however effect the increase in rate of influx of Ca^{++} in response to compound 48/80.

The model proposes that the "trigger" structure is located on the t-tubular membrane proximate to the terminal cisternae of the sarcoplasmic reticulum. Thus a test of the model would involve treatment of the muscle with glycerol causing a separation of the t-tubules from the sarcolemma and subsequent challenge with compound 48/80. If the contracture does not occur under these conditions the site of the "trigger" structure would likely be in the t-tubules. As a positive control, caffeine should still be able to cause a contracture under these conditions otherwise the glycerol treatment would

also be affecting the function of the SR. (Note, glycerol-treated muscles are still activated by caffeine, Howell and Jenlon, 1967).

The increase in $^{86}Rb^{+}$-efflux caused by compound 48/80 implies that there was also an increase in myoplasmic $[Ca^{++}]$. The question is whether the increase in myoplasmic $[Ca^{++}]$ was directly the result of the interaction of compound 48/80 with the "trigger" structure proposed in the model. If pretreatment of the muscle with neuraminidase lowers the increase of $^{86}Rb^{+}$-efflux caused by 48/80 then one would conclude that the "trigger" structure sensitive to neuraminidase is indeed important in controlling myoplasmic $[Ca^{++}]$. If, however, $^{86}Rb^{+}$-efflux caused by compound 48/80 is not affected by neuraminidase then the model is not valid unless other factors besides the release of calcium from the SR into the myoplasm can influence $^{86}Rb^{+}$-efflux. For example, the $K^{+}(^{86}Rb^{+})$ channels could "sense" the increased influx of Ca^{++} across the muscle membrane seen in muscles pretreated with neuraminidase and challenged with compound 48/80. Furthermore, neuraminidase itself causes an increase in ^{45}Ca uptake in skeletal muscle (Figure 23) and similarly in cultured heart cells (Langer et al, 1976) Again, this increased influx of Ca^{++} across the muscle membrane (as distinct from increased myoplasmic $[Ca^{++}]$)

caused by neuraminidase could be "sensed" by the K^+ ($^{86}Rb^+$) channels thus causing an increase in $^{86}Rb^+$-efflux such that the experiment would not reveal any change in myoplasmic [Ca^{++}] caused by compound 48/80.

The existence of a highly specialized organelle, such as the sarcoplasmic reticulum, for the generation and removal of transient increases in cytosolic [Ca^{++}] has not been demonstrated in mast cells, nevertheless compound 48/80 causes the degranulation of mast cells presumably by mobilizing an intracellular pool of calcium (see Chapter II). An experiment examining the effect of neuraminidase treatment on uptake of ^{45}Ca into mast cells challenged with compound 48/80 could show a result similar to skeletal muscle (Figure 23); neuraminidase treatment of mast cells inhibits degranulation but does not change the increase in ^{45}Ca-uptake caused by challenge with compound 48/80. Using our model for skeletal muscle, such a result would indicate that the activation of an intracellular calcium pool in the mast cell was dependent on the close proximity of the pool to neuraminidase-sensitive "trigger" structures of the plasma membrane and the increase in ^{45}Ca-uptake was due to ATP-dependent calcium pumps on an intracellular pool rapidly taking up the increase in the influx of extracellular Ca^{++} and preventing the cytosolic [Ca^{++}] from reaching the

threshold for degranulation. This finding would suggest that the functional internal structure of the mast cell is complex and similar to skeletal muscle. However, influx of extracellular Ca^{++} caused by crosslinking of receptors to IgE is sufficient and necessary to cause degranulation of mast cells (Hirata and Axelrod, 1980) indicating that this, possibly large, influx of Ca^{++} is <u>not</u> prevented from increasing cytosolic $[Ca^{++}]$ to the threshold for degranulation by being taken up by an intracellular calcium pool analagous to the SR in skeletal muscle.

Parallels Between Pharmacological Challenge of Rat Mast Cells and Skeletal Muscle. A Summary

Table IV lists the findings of this thesis on rat mast cells and frog skeletal muscle that are similar for both tissues.

These similar findings indicate that certain steps of the mechanistic pathway of stimulus-secretion coupling in mast cells might be analogous to steps in the coupling of pharmacological stimulus to contracture or even to excitation-contraction coupling in skeletal muscle. Such steps include the binding of compound 48/80 to receptors of the cell surface which have sialic acid residues. This in turn causes the mobilization of calcium from intracellular pools into the cytosol consequently activating the function characteristic of the tissue; degranulation of mast cells and activation of the contractile apparatus in skeletal muscle.

Interestingly, mast cells also contain a "contractile apparatus" composed of actin microfilaments, that have been proposed upon activation to eject the granules from exocytotic pits at the cell surface (Cochrane and Douglas, 1974).

Table IV, Chapter IV - Summary of Similar Findings on Rat Mast Cells and Frog Skeletal Muscle of this Thesis

	Similar Findings	Implication
1.	compound 48/80 causes an increase in $^{86}Rb^{+}$-efflux	increase in cytosolic [Ca^{++}]
2.	no absolute requirement for extracellular Ca^{++}	influx of Ca^{++} into the tissue not required for activation
3.	low doses of verapamil inhibit spontaneous release of $^{86}Rb^{+}$	verapamil inhibits mobilization of Ca^{++} at those concentrations
4.	high doses of verapamil caused increase release of $^{86}Rb^{+}$ and activation	verapamil overcomes the inhibition of mobilization of Ca^{++} and itself causes mobilization of Ca^{++}
5.	processes of activation are neuraminidase-sensitive	the target of compound 48/80 contains essential sialic acid.

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