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# The Isolation of Plasma Membranes from the Rat Submandibular Salivary Glands: Characterization and Some Aspects of Lipid Metabolism, Enzymic and $Ca^{++}$-Binding Properties

by

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This thesis is dedicated to my dear parents and brother, Gurdial, who have not only supported me financially throughout my student career, but also inspired in me the drive to undertake projects such as this.

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## ABSTRACT

Three distinct plasma membrane fractions were isolated from hypotonic whole homogenates of rat submandibular glands (SMSG) by discontinuous sucrose density gradient centrifugation of the 4,000 x g supernatant.

The characteristic plasma membrane enzymes, 5'-nucleotidase, alkaline phosphatase and ($Na^+ - K^+$)-ATPase were markedly increased in specific activity over the original whole homogenate. 5'-nucleotidase, 30 x; alkaline phosphatase, 8 x; ($Na^+ - K^+$)-ATPase, 8 x.

Plasma membranes were found to be enriched in their content of phospholipid, cholesterol and sialoprotein over that of the original homogenate and their molar ratio of cholesterol to phospholipid was around unity. The spectrum of individual lipids, their fatty acids and protein amino acids were not markedly different from that of the whole tissue homogenate.

The absence of acyl-CoA synthetase activity in plasma membranes was indicated by their inability to utilize free (1-$^{14}$C)-palmitic acid whereas (1-$^{14}$C)-palmitoyl-CoA was readily incorporated into lipids. The presence of acyl transferase was evidenced by the increased incorporation of palmitoyl-CoA into phosphatidylcholine and phosphatidylethanolamine respectively on the addition of lysophosphatidylcholine and lysophosphatidylethanolamine. Various features of the palmitoyl-lysolecithin transferase system of plasma membranes are reported. Acetylcholine, epinephrine, cyclic adenosine-3',5'-monophosphate (cAMP) and theophylline inhibited the incorporation of palmitoyl-CoA into phosphatidylcholine.

The plasma membrane preparations showed carnitine-palmitoyl-CoA transferase activity as they formed palmitoyl-carnitine from palmitoyl-CoA and L-carnitine.

5'-nucleotidase activity was maximum at 2 mM AMP, 10 mM $Mg^{++}$ and pH 7.5 - 10.0. $K^+$ had no effect on this activity. UMP, AMP, CMP, GMP and

IMP were hydrolyzed in that decreasing order. Sodium fluoride (NaF) completely inhibited the enzyme whereas ouabain and cAMP had no effect suggesting the probable non-involvement of 5'-nucleotidase in either cation or exocrine secretion across the plasma membranes.

$(Na^{+} - K^{+})$-ATPase was purified from plasma membranes by deoxycholate and sodium iodide treatments. The enzyme was activated by $Na^{+}$, low levels of $K^{+}$, $Mg^{++}$ and ATP. High levels of $K^{+}$, $Mg^{++}$ and ATP were inhibitory. $Ca^{++}$ very markedly reduced the activity. $(Na^{+} - K^{+})$-ATPase activity was optimum at pH 7.0 - 7.5 and was very specific for ATP as it did not hydrolyze CTP, GTP, ITP, UTP, ADP, AMP, glucose 6-phosphate and p-nitrophenylphosphate. Low levels of acetylcholine, epinephrine, cAMP, and NaF did not alter the activity, however, at higher levels, all were observed to inhibit the enzyme to a variable degree. $(Na^{+} - K^{+})$-ATPase and $(Mg^{++})$-ATPase activities were affected differently by many conditions suggesting that they were separate enzymes.

Plasma membranes can bind $Ca^{++}$ and this binding was slightly increased by ATP without undergoing hydrolysis concomitantly. $Na^{+}$, $K^{+}$ and $Mg^{++}$ inhibited $Ca^{++}$ binding. The binding was maximum at pH 8.0 and was increased by epinephrine, cAMP, ouabain and oxalate but not by NaF or acetylcholine. It was concluded that plasma membranes possess a small innate capacity to bind $Ca^{++}$ and the binding was independent of the ATPase system of plasma membranes.

The significance of all these results in relation to the process of exocrine secretion is discussed.

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## ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine diphosphate |
| AMP | adenosine monophosphate |
| ANSA | aminonaphthol sulfonic acid |
| ATP | adenosine triphosphate |
| CMP | cytosine monophosphate |
| CoA | coenzyme A |
| CTP | cytosine triphosphate |
| Cyclic AMP | cyclic 3',5'-adenosine monophosphate |
| DNA | deoxyribonucleic acid |
| dpm | disintegration per minute (radioactive) |
| EDTA | ethylenediaminetetraacetate |
| FFA | free fatty acids |
| GMP | guanosine monophosphate |
| GTP | guanosine triphosphate |
| $HClO_4$ | perchloric acid |
| IMP | inosine monophosphate |
| INT | 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyltetrazolium chloride |
| Lecithin | phosphatidylcholine |
| lyso-PC | lysophosphatidylcholine (lyso lecithin) |
| lyso-PE | lysophosphatidylethanolamine |
| MES | 2-(N-morpholino)ethanesulfonic acid. $H_2O$ |
| NANA | N-acetylneuraminic acid |
| nm | specific wavelength |
| O.D. | optical density |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |

| | |
|---|---|
| Pi | inorganic phosphate |
| PM | plasma membranes |
| POPOP | 1,4-bis[2-(5-phenyloxazolyl)] benzene |
| PPO | 2,5-diphenyloxazole |
| PS | phosphatidylserine |
| $R_f$ | distance of lipid spot from the origin/distance of solvent front from the origin |
| RNA | ribonucleic acid |
| SMSG | submandibular salivary glands |
| Sph | sphingomyelin |
| TCA | trichloroacetic acid |
| TES | N-tris(hydroxymethyl)methyl-2-aminoethan-esulfonic acid |
| TG | triglycerides |
| TLC | thin-layer chromatography |
| Tricine | N-tris(hydroxymethyl)methylglycine |
| Tris | tris(hydroxymethyl) amino methane |
| UMP | uridine monophosphate |
| UTP | uridine triphosphate |
| DNP | dinitrophenol |

# SECTION I

## A. INTRODUCTION

The outer cell membrane or plasma membrane (PM) is a thin structural layer which encloses the cytoplasm and intracellular organelles and, due to its semipermeable properties, forms a regulatory barrier to overall molecular transport between the cell and its surroundings (1). The balance in this molecular exchange determines, in part, intracellular composition and, consequently, the nature of cellular processes. This molecular transport in both directions seems to be regulated in many instances by the innate nature of the membranes or in some instances by the synthesis of new membrane components under stimulation of inducers (2).

Proteins and lipids constitute the major portion of outer cell membranes while carbohydrates and RNA are present in small amounts (3). Lipids form not only a barrier to the transport of molecules across cell membranes but are also suitable in combination with other macromolecules to form a stable yet flexible boundary giving the cell its innate shape. The expression of innate lipid characteristics is dependent upon lipid-lipid and lipid-protein interactions. Transformations among different arrangements may occur in the membranes stimulated by transport processes (4).

It has been shown that the chemical structure of fatty acid in membrane lipids has a significant effect on physico-chemical properties of membrane lipids and also influences the molecular packing of the membrane components. Enzymes which could regulate the exchange of fatty acid in membrane lipid would contribute considerably to the dynamic nature of the lipid core of the membranes and hence determine much of the physico-chemical properties of the membrane.

Plasma membranes contain enzymes, e.g. $(Na^{+} - K^{+})$-ATPase, which render specificity to the molecular transport across cell membranes. The activity of some enzymes depends upon both lipids and proteins. The induction of a conformational change in protein by either an extra- or intracellular stimulus, might alter the intermolecular arrangement between lipids and proteins and could have a significant effect on membrane permeability (5). The isolation of plasma membranes and a

study of their characteristics in terms of lipid, protein and enzyme content would seem to be a rewarding project not only in helping to understand the structure of membranes, but also to give some information concerning their biological functions. The present study was undertaken to explore some of these aspects of plasma membranes.

Rat submandibular salivary glands (SMSG) were chosen as the tissue source for several reasons. The secretion of electrolytes (e.g. $Na^+$ and $K^+$) from salivary glands is an active transport process ( 6, 11) and is probably mediated through an ATPase system (7, 8). A ($Na^+$ - $K^+$)-ATPase has been shown to be present in the membranous fraction of rat salivary glands (7, 9) and it exhibits properties similar to the transport ATPases found in many other tissues (7, 8). The intra- and extracellular movement of cations in the acini might initiate or regulate the extrusion of macromolecules (10 - 14).

Previous studies from this laboratory (15 - 19) have shown that SMSG exhibit a very active lipid metabolism. It was shown that SMSG slices oxidized various fatty acids to $CO_2$ and that this oxidation was enhanced by L-carnitine and by epinephrine (17). Acetylcholine enhanced both the secretion of proteins from the SMSG slices and the incorporation of $^{32}P$-phosphate into total lipids (15), just as has been reported by Hokin and his colleagues for many secretory tissues (20).

There are three pairs of salivary glands in most mammalian species: the parotids, the submandibulars and the sublinguals. They are innervated by both sympathetic and parasympathetic nerve fibers (21). These glands secrete saliva containing water, electrolytes and proteins, the relative proportion of which depends upon the nature of the stimulus (22). Saliva not only helps to protect the oral epithelium and the upper part of gastro-intestinal tract from acid and proteolytic attack, but also facilitates the chewing and swallowing of food by its moistening and lubricating action (23). The secretion of saliva can be experimentally enhanced by either electrical stimulation of the sympathetic or parasympathetic nerve fibers (21, 24) or by the injection of their respective neuro- transmitters, adrenaline (25, 26) and acetylcholine (21, 26).

The secretion of macromolecules involves the fusion of secretory vesicles with the luminal side of the outer cell membrane, the rupture of the membrane at the point of fusion with subsequent release of secretory material into the acinar ducts (20, 27). It has been demonstrated (20) that the metabolism of certain phospholipids in salivary gland slices is altered during the acetylcholine-stimulated secretion of macromolecules.

## B. OBJECTIVES OF PRESENT STUDY

The purpose of this study was to isolate plasma membranes of rat submandibular salivary glands free from other subcellular contaminants and to determine their enzymic and chemical composition. Furthermore, it was the objective of this investigation to study the lipid metabolism of isolated plasma membranes *in vitro* and to examine the effects of some secretagogues on this lipid metabolism. The properties of plasma membrane enzymes in relation to the secretion process were of further interest. In view of the role of $Ca^{++}$ in the secretion process, a knowledge of $Ca^{++}$ binding properties of isolated plasma membranes was of some interest.

## C. LITERATURE REVIEW

### 1. Structure of Cell Membranes

It was in 1665, when Hook recognized that the cell was the basic unit of vegetable tissue. Von Mohl in 1852 (123) described the cell as a completely closed, globular or elongated vesicle composed of a solid membrane enclosing fluid. In 1855, Nägeli and Cramer (28) described the plasma membrane of a plant cell as a thin, membranous layer of protoplasm and noted some of its properties during exo- or endoosmosis which they produced by using sugar solutions. Overton in 1895 (29) studied the entry of different solutes into living cells and arranged them in classes according to their respective rates of entrance into cells. On the basis of these studies, he suggested that the cell surface was impregnated with lipids such as lecithins and cholesterol. Gorter and Grendel (30) using lipids extracted from red blood cells, measured the surface area of spread monolayers of these lipids. From this data and their calculations of the surface area of erythrocytes, they claimed that lipids of erythrocytes provided just enough surface area to cover twice the cell surface indicating that the cell membrane consisted of a bimolecular leaflet.

In 1935, Danielli and Harvey (31) noted that the surface tension of biological membranes was low when compared to the high surface tension observed in lipid monolayers. Their measurements of the permeability, surface tension and electrical conductance of thin films of lipids led them to propose a paucimolecular model of membrane structure. Accordingly, the lipids form a continuous bi-molecular leaflet sandwiched between two layers of globular proteins. The polar groups of phospholipids were linked to proteins through electrostatic forces. These cell membranes were considered to be symmetrical (32).

Robertson in 1959 (33), proposed a unit membrane model for membranes on the basis of his electron microscopic studies of the myelin sheath of Schwann cells. He demonstrated that the membrane element of myelin consists of a triple-layered structure having two electron dense strata about 20 A° thick separated

by a light interzone about 35 A° wide making a unit of about 75 A° in thickness. The polar heads of phospholipids and protein contribute to the dense strata and the non-polar portion of the lipids form the inner light area. He suggested that the protein layers are composed of an extended monolayer in the β-configuration. The cell membranes were considered asymmetrical as the outer leaflet was slightly thicker than the inner leaflet because of its content of polysaccharides.

Sjöstrand (34), in contrast to the unit membrane hypothesis, proposed a model of membrane structure where globular lipid micelles have their polar heads oriented outward from the membrane element and are embedded in protein.

Green and Perdue (35) showed that mitochondrial membranes isolated in their laboratory, were composed of discrete and repeating subunits of lipoproteins. The repeating units were composed of a base piece and a detachable sector.

None of these membrane models accounts for all the complex features of cell membranes. The membrane models proposed by Sjostrand and by Green and his associates seem to apply more to the cytoplasmic membranes than to the outer cell membranes. Since there are considerable differences in the enzymic and chemical composition between these two types of membranes, their structure might not be identical.

In view of the various specialized transport processes and other catalytic abilities located in the membranes, and the numerous possibilities of molecular associations between lipids, between different proteins, and between proteins and lipids, it is obvious that considerable research has to be done before a detailed picture of the molecular organization of various membranes can be constructed.

The progress in understanding the molecular structure of plasma membranes has been rather slow because of the problems encountered by investigators in preparing plasma membranes free from other subcellular components.

## 2. Isolation of Plasma Membranes

In 1951 (36) when Palade was studying the subcellular distribution of acid

phosphatase in rat liver, he noted that the 1200 x g pellet, obtained from an homogenate in 0.88 M sucrose, contained nuclei and numerous cell membranes. He described them as 1 μ thick sheets of amorphous refringent material which he suggested as probably representing the membranes of two adjacent cells stuck together with interphase cement.

In 1952, Hogeboom et al. (37) isolated the nuclei from a rat liver homogenate in 0.25 M sucrose containing 1.89 mM $CaCl_2$. Their preparation of nuclei contained a number of irregularly shaped very thin structures which appeared to be collapsed cell membranes.

In 1958, Rajam and Jackson (38) prepared a crude plasma membrane fraction from a rat ascites tumour cell lysate by repeated low speed centrifugation over a layer of sucrose. This translucent material was composed of thin sheets of transparent membranous material. A variable amount of granular material was adsorbed onto these membranous sheets and was considered to be of intracellular origin. Antibody and fluorescence techniques confirmed that most of the membranous material originated from the cell surface.

In 1960, Neville (39) made use of Rajam and Jackson's findings to devise a method for the isolation of plasma membrane from rat liver homogenates. This method involved a gentle homogenization of tissue in 1 mM sodium bicarbonate buffer (pH 7.5) followed by a low speed centrifugation which resulted in a crude plasma membrane fraction overlaying the nuclear pellet. After careful removal from nuclear pellet, the crude plasma membranes were repeatedly washed with buffer to remove contaminants and then further purified by flotation in a discontinous sucrose gradient (d = 1.16 and 1.22).

Emmelot and his co-workers modified this method by introducing an extra layer of sucrose (d = 1.20) (40) and later by another layer, d = 1.18 (41) to separate plasma membrane from contaminating mitochondria. In 1966, (42), they fortified the basic homogenizing medium of Neville with 2 mM $CaCl_2$ which rendered the nuclei resistant to mechanical rupture, thus preventing the release of

nuclear material which hindered the separation of plasma membrane. Bosmann et al. (43) working with HeLa cells overcame the difficulty of separating the plasma membrane from the nuclear pellet by using the 4000 x g supernatant rather than the nuclear pellet.

Since the method of Neville was published, many isolation procedures have been reported, some modifying Neville's original method, others using completely different techniques. These include methods for isolating plasma membrane from liver (41, 44 - 46), brain (47 - 49), red blood cells (50 - 52), toad bladder epithelium (53, 54), intestinal brush borders (55), adipose tissue cells (56, 57), smooth muscle (58), skeletal muscle (59, 60), pancreas (61), kidney (62), HeLa cells (43, 63) and bacteria (64, 65). The isolation of other subcellular particles in addition to plasma membrane has been facilitated by the use of an isotonic homogenizing medium (44, 57). The yield of plasma membrane has been improved by the use of zonal centrifugation (46, 47, 49).

### 3. Composition of Plasma Membranes

In 1913, Burger and Beumer (66) analysed the lipids of sheep and human erythrocytes and found that the cells of one litre of blood contained 0.5 g sphingomyelin, 0.5 g of cephalins and lecithins and 0.5 g of cholesterol. A few years later, Gorter and Grendel (124, 125) estimated the surface area occupied by the individual lipids and described the relative contribution of cholesterol, cephalin and lecithin, and sphingomyelin as 36, 50 and 13 per cent respectively.

Burt and Rossiter (67) prepared ghosts from rabbit erythrocytes and reported their lipid composition. In a number of other investigations, variable lipid compostion of red blood cell ghosts prepared from different species has since been reported (68).

With the exception of these earlier studies on intact erythrocytes and their ghosts, there was essentially nothing known about the lipid composition of plasma membranes of other tissues until recently when the procedure for isolation of plasma

membranes was developed by Neville. It is now established that proteins and lipids are the major components of plasma membranes, however, a significant amount of carbohydrates, seemingly as glycoprotein and RNA are also present (3). Lipids constitute about 35 - 40% of the dry weight of most plasma membranes (3, 71, 73) and include approximately 39% phospholipids, 35% neutral lipids with various other lipids forming the remainder. The phospholipids include phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and a small amount of phosphatidylserine, lysophosphatidylcholine and phosphatidylinositide. A very variable phospholipid composition has been reported for plasma membrane isolated from liver (69 - 73), brain (74, 75, 81), intestinal mucosa (55), pancreas (76) and L cells (77). The neutral lipid fraction contains free cholesterol, triglycerides and free fatty acids (69, 78).

Protein constitutes about 60 - 65% of the dry weight of cell membranes (3, 69, 71, 73, 78). The proportion of protein to lipid varies not only between species, but also between plasma membranes prepared by different methods from the same species.

Carbohydrates constitute a small percentage (1 - 5%) of plasma membranes (3, 73). The most commonly found types are sialic acids, hexose (galactose/glucose) and hexosamine (3, 73, 82). Since almost all the sialic acid can be recovered in the protein fraction of the membranes, it seems that sialic acid is bound to glycoproteins of the outer membrane surface (3).

### 4. Biological Significance of Lipids

The earlier attempts of Parpart and Dziemian (83) to correlate the differences in lipid composition of erythrocytes of various species to their permeability to glycerol were unsuccessful. However, later studies (79) showed that as the ratio of sphingomyelin to lecithin of erythrocytes from various species decreased, their permeability to urea, thiourea, glycerol, ethylene glycol also decreased suggesting a relationship between these lipids and membrane permeability.

Liver mitochondria of rats fed an essential fatty acid deficient diet swell more rapidly than do normal mitochondria (84). Later studies by Wait and van Golde (85) showed that these mitochondria exhibited an altered phospholipid pattern which persumably allowed the ionic imbalance resulting in an influx of water and consequent swelling.

Liposomes prepared from bacterial lipids showed changes in the permeability to glycerol (86) in accordance with the fatty acid composition of the growth media. When liposomes were prepared from natural and synthetic lecithins with increasing amounts of cholesterol, the permeability of liposomes to glucose and glycerol was decreased (87). The effect of cholesterol esters was dependent upon the chain length and degree of saturation of fatty acids (86, 87).

Parpart and Ballentine (88) classified the lipids of red blood cells of various species as loosely-bound, weakly-bound and strongly-bound on the basis of their extraction with dry ether, wet ether, and with alcohol-ether respectively. In a similar study (89), the percentage of loosely-bound lipids varied between species in accordance with their permeability to ethylene glycol, glycerol and thiourea. Analysis of these fractions showed that the loosely-bound fraction contained mainly sterols with minor amounts of sphingomyelins, phosphatidylcholines and phosphatidylethanolamines while the strongly-bound fraction contained mostly polar lipids. These studies showed that a given phospholipid may display different degrees of membrane-binding(stability)depending upon its protein partner. This is supported by Van Deenen (4, 5) who suggested that the intermolecular transformations might occur in a membrane depending upon not only the changes in the hydrocarbon chains but also on the nature of proteins present. The possibility that conformational changes in membrane proteins affect the arrangement and therefore the permeability of the hydrophobic barrier of membranes has been considered (4, 5).

According to the membrane hypothesis (90), a substrate binds to a specific carrier in the membrane forming a lipid-soluble complex which diffuses across the lipid cell membrane, releasing the substrate at the opposite side, thus setting up a concentration gradient for the complex within the membrane in the direction

of transport.

Solomon et al. (91) studied the binding of $Na^+$ and $K^+$ to both synthetic and lipids extracted from red blood cells. It was found that when $Na^+$ or $K^+$ in aqueous buffer were incubated with lipids, these ions complexed with the lipids and subsequently were recovered in the lipid phase. When this lipid extract was exposed to an aqueous medium, the complex dissociated releasing the ions into the aqueous phase. $K^+$ was taken up by these lipids in preference to $Na^+$ showing some specificity.

LeFevre et al. (92) also carried out similar studies with monosaccharides and lipids of red blood cells. They found that there were small differences among the monosaccharides to lipid binding which corresponded to the differences in affinities of monosaccharides for the transport system in intact cells. Moreover, the inhibitors which inhibited monosaccharide transport in intact cells also inhibited their binding to lipids. The binding process did not exhibit stereospecificity. The results of these studies suggested that probably lipids act as carriers during the transport of some molecules across cell membranes.

The treatment of isolated fat cells with phospholipases A (93) and C (94) enhanced the intracellular transport of glucose and the addition of lysolecithin mimicked the phospholipase effect (93). It was suggested that phospholipase acted upon the membrane phospholipids and consequently induced a change in their configuration, thus enhancing the transport of glucose.

Early studies of Hokin and his coworkers with pigeon pancreas slices (95) and rabbit submaxillary (96) and parotid gland slices (97) showed that acetylcholine caused an enhanced secretion of amylase and also markedly stimulated the incorporation of $^{32}P$-orthophosphate into phospholipids. Pancreozymin produced similar effects in pigeon pancreas slices (98). Later studies with pancreas (99, 126) and with the adrenal medulla (127) showed that the greatest increase in specific activity was in phosphoinositide and phosphatidic acid. The work of others with cat submaxillary glands (26), pigeon oesaphageal mucosa (128), leucocytes (129) and pancreas

(100) have also implicated phospholipids in the exocrine secretion of macromolecules. Differential centrifugation (101) and radioautographic studies (102) showed that this effect occurred in the rough surfaced endoplasmic reticulum and in the smooth-surfaced golgi membranes. The finding that the omission of calcium from the incubation medium completely abolished the effect of acetylcholine on the secretion of amylase from pancreatic slices while the incorporation of $^{32}$P-orthophosphate into phosphatidyl inositol and phosphatidic acid was greatly increased (103, 104) suggested that the phospholipid effect of acetylcholine (103) and catecholamines (104) must be concerned with aspects of protein secretion other than those connected with the actual extrusion of macromolecules. Hokin (20) proposed that the effect of acetylcholine on phospholipogenesis involves either the repair or formation of plasma membranes following secretion.

The active extrusion of $Na^+$ and the concomitant accumulation of $K^+$ by erythrocytes (105) and nerve cells (106) was accompanied by the hydrolysis of ATP. Later studies showed the presence of $(Na^+ - K^+)$-ATPase in erythrocyte membranes (107) and crab nerve cells (108). The $K_m$ for the $Na^+$ and $K^+$ transport system corresponds to that of $(Na^+ - K^+)$-ATPase (130) suggesting an intimate relationship between ATP hydrolysis and $Na^+$ and $K^+$ transport. The possible role of $(Na^+ - K^+)$-ATPase in the transport of cations and other substances has been the subject of three reviews (109, 110, 130).

In vivo studies of Barcroft and co-workers showed that the stimulation of parasympathetic (111) and sympathetic (25) nerves to salivary glands promoted the secretion of saliva and also enhanced oxygen consumption. These findings were later confirmed by in vitro studies with slices of salivary glands where acetylcholine, pilocarpine (112, 113) and adrenaline (25) all increased the oxygen consumption. Atropine blocked the effects of acetylcholine or pilocarpine on both secretion and oxygen consumption. These findings indicated that aerobic metabolism was necessary for secretion. In vitro studies of Bdolah et al. (13, 114), Babad and Schramm (115) and perfusion studies of Petersen (116) showed that DNP inhibited both secretion and oxygen consumption when epinephrine (13,

114) or acetylcholine (116) was present indicating the importance of oxidative phosphorylation in the process of secretion.

The studies of Sandhu et al. (117) showed that epinephrine while enhancing secretion from parotid gland slices also increased the oxidation of glucose. In situ studies of Northrop (24) showed that simultaneous stimulation of sympathetic and parasympathetic nerves to submaxillary glands of a dog caused a decrease in glycogen, an increase in lactic acid and a decrease in the creatine phosphate in the gland. These studies indicated that glucose oxidation might have provided energy during secretion. However, Lindsay et al. (118) have shown that at low levels, epinephrine increased the salivary secretion without affecting glucose oxidation, and at higher levels, both the glucose oxidation as well as the secretion were increased. These findings suggested that under resting conditions, the secretion was probably supported by energy derived from source(s) other than directly from glucose. However, under marked stimulation of secretion, glucose might well serve as the prime energy source. Bdolah et al. (13) recently reported that epinephrine could induce secretion when glycolysis was completely inhibited by iodoacetate or N-ethylmaleimide while dinitrophenol and KCN reduced epinephrine-induced secretion of amylase from parotid gland slices. Recent studies in this laboratory with isolated rat SMSG mitochondria have shown that fatty acids can be oxidized very efficiently under resting conditions indicating that fatty acids could readily serve as a source of energy for secretion (19).

Early studies of Lundberg (10) showed that nerve stimulation produced hyperpolarization of the acinar cell of salivary glands which was followed by the release of secretory material. These findings suggested that cations might have a role in the process of secretion. This view has received considerable support from various other investigators (12, 13). Douglas and Poisner (14) have shown that epinephrine-induced salivary secretion varied with the extracellular concentration of calcium and the effect of epinephrine was completely abolished when calcium was omitted. Calcium uptake by the salivary glands is an active process (119). The exact mechanism by which calcium influences the secretion is not yet known

but it does not seem to change the electrical properties of acini (120) nor act on the energy metabolism of the salivary glands (121) nor act through cyclic AMP (122). It might, however, be acting directly at the membrane level by changing the permeability to ions and water.

# SECTION II

## A. ISOLATION OF PLASMA MEMBRANES

### 1. Introduction

A variety of procedures have been used to obtain plasma membranes from liver (39 - 42, 44, 45), erythrocytes (50 - 52) and adipose tissue (56, 57). These procedures involve homogenization of tissue in hypotonic (39, 43, 46, 56), isotonic (44, 45, 57) and hypertonic (59) buffered mediums. Some investigators supplement the homogenizing medium with $CaCl_2$ or $MgCl_2$ (42, 44, 45, 69, 131) or EDTA (43, 57). All the procedures described in the literature, thus far, have emphasized the significance of gentle homogenization of tissue in order to achieve preparations with reproducible characteristics. In some procedures, the plasma membranes are obtained from the pellet after low speed centrifugation of an homogenate by repeated gentle washings with the homogenizing medium (39 - 42, 69); while in others, the heavier material containing nuclei and cell debris are first sedimented by low speed centrifugation and the supernatant fluid is processed for the isolation of plasma membrane (43). A discontinuous sucrose gradient is commonly used to separate plasma membrane from other subcellular contaminants (39 - 42, 44, 45) although in some reports (45, 52, 63) a continuous sucrose gradient has been used. Although sucrose has been most commonly used to prepare gradients, in some procedures, a Ficollgradient has been utilized (57, 131). It has been claimed that on a Ficollgradient, comparable plasma membrane preparations can be obtained in a much shorter time than when a sucrose gradient is used (57). The yield of plasma membranes obtained by different procedures has been rather variable and this could be either an innate feature of an individual procedure, or due to variable degrees of contamination by other subcellular organelles. Plasma membranes prepared by different procedures vary in their relative degree of purity and hence exhibit properties characteristic of the isolation procedure. Generally, the purity of plasma membrane preparations can be increased at the expense of yield and vice versa.

Coleman et al. (44) perfused livers in situ with the warm homogenizing

medium before processing the tissue claiming that the presence of red blood cells could lead to contamination of liver plasma membrane with plasma membrane of red blood cells. Benedetti and Emmelot (3) also noted that about 18 to 33% of the proteins in isolated liver plasma membrane were soluble in physiological saline. These saline-soluble proteins were red in color while the saline-insoluble proteins were colorless upon freeze-drying. On the basis of color and several other properties, these authors concluded that saline-soluble proteins of isolated plasma membrane were cytoplasmic in origin and were probably adsorbed onto the plasma membrane during the isolation procedures.

The composition of the homogenizing medium appeared to be a very important factor in obtaining plasma membrane free from other contaminants and the choice of a proper medium was dependent upon the type of tissue used. It has been demonstrated that the homogenization of tumor cells in a hypotonic buffered medium led to the rupture of nuclei, mitochondria and endoplasmic reticulum (40 - 42, 61) and the plasma membrane isolated from such homogenates was contaminated with material from these subcellular organelles. This problem was resolved by supplementing the medium with either $CaCl_2$ or $MgCl_2$, or both (42, 44, 45, 69), which tended to stabilize the membranes of subcellular organelles thereby preventing their rupture.

Extensive or vigorous homogenization of most tissues also leads to the rupture of subcellular particles and thus increases the contamination of plasma membranes. In those procedures where plasma membranes are obtained from the low speed pellet by repeated washings with the medium (3), vigorous homogenization could lead to low yields as the small fragments of plasma membrane would not be sedimented by high speed centrifugation. It is therefore essential that an appropriate homogenizing medium and the proper homogenization conditions be found, and used consistently, in order to obtain plasma membranes of comparable purity and in good yield. An isotonic homogenizing medium is often preferred over a hypotonic medium as it allows the simultaneous isolation of other subcellular organelles as well as plasma membranes (44, 45, 57).

Plasma membranes are distributed almost equally between the supernatant and the pellet after low speed centrifugation (43). The choice between these two sources of plasma membrane generally has to be made by an individual investigator and would mainly depend upon the characteristics of the tissue under investigation.

Some investigators have used zonal centrifugation rather than isopycnic centrifugation which allows the processing of much more tissue in a single run thus increasing the yield of plasma membrane. Zonal centrifugation is becoming more common and preferred over isopycnic centrifugation especially when a large amount of tissue is available.

2. Procedure

Figure 1 shows a flow diagram of the procedure used to isolate plasma membrane in the present study. This method is essentially that described by Bosmann et al. (43). The rats of the Long-Evans strain, about eight weeks old, were mildly anesthetized with ether and decapitated. The submandibular salivary glands (SMSG) were quickly excised, cleaned of extraneous tissue and placed in a pre-chilled beaker. The glands were weighed and sliced into very small pieces with a sharp razor blade at 4°C. All the subsequent procedures were carried out in the cold room at 2 - 4°C unless otherwise noted. The minced tissue was homogenized in small portions at a time in a buffer containing 20 mM Tris and 10 mM EDTA (pH 7.0) in a Potter-Elvejhem glass homogenizer with 10 - 15 hand-driven strokes, pooled and mixed. The homogenate was filtered through three layers of cheese cloth and the volume adjusted to 14% (w/v). The homogenate was centrifuged at 4,000 x g for 10 min in a refrigerated centrifuge at 2°C. The centrifugation was terminated rather slowly as the loosely packed pellet mixed very readily upon slight disturbance. The supernatant was carefully withdrawn and retained while the pellet was resuspended in 2.5 volumes of the homogenizing medium and spun again at 4,000 x g for 10 min. The supernatants of both spins were pooled and made 45% in sucrose by adding pulverized sucrose slowly while the mixture was constantly stirred. Portions of 10.5 ml of this mixture were placed

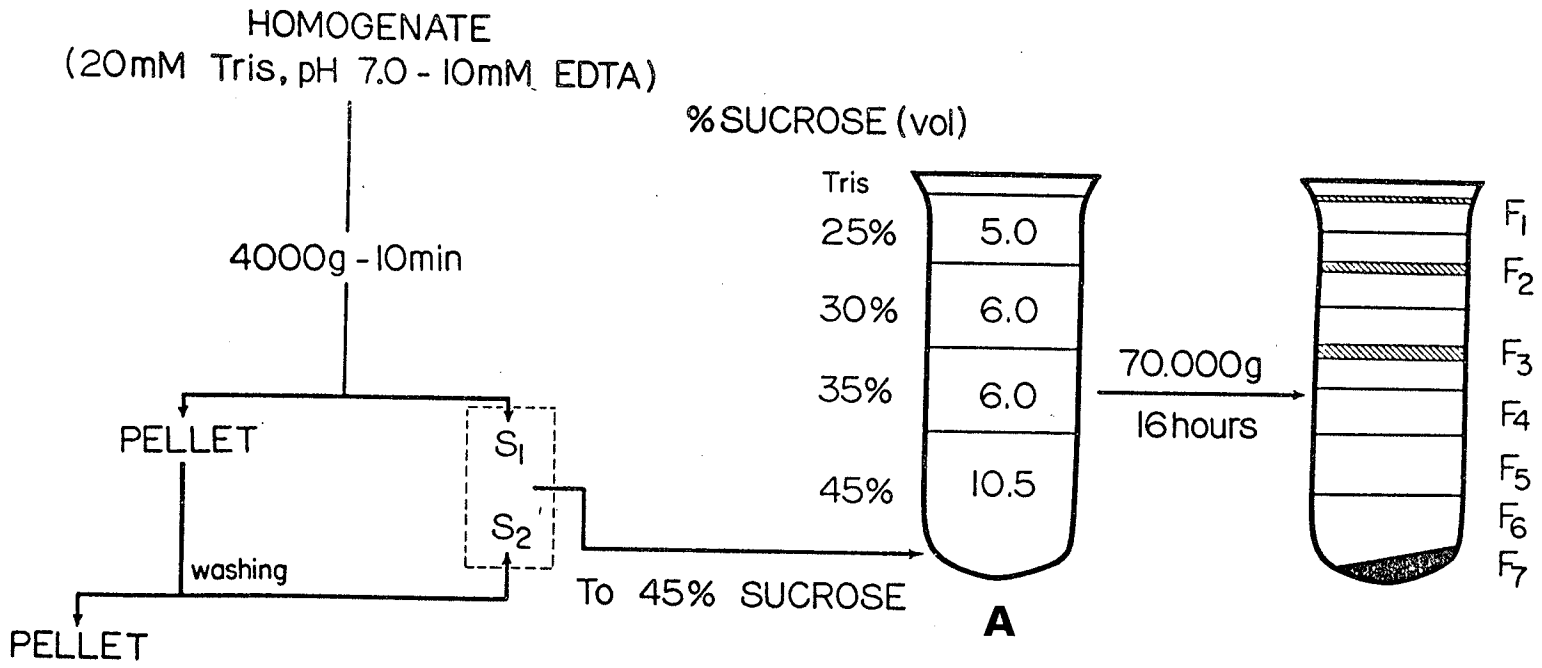



Figure 1. A flow diagram of the procedure used to isolated plasma membranes from rat submandibular salivary glands. The number in tube A refers to ml of sucrose solution. $F_1$, $F_2$ and $F_3$ are the plasma membrane fractions

in each tube of 30 ml capacity upon which layers of 35% (6 ml), 30% (6 ml), 25% (5 ml) sucrose and finally 50 mM Tris-HCl buffer (1 ml) were placed. The gradient was spun at 70,000 x g for 16 h in a fixed-angle rotor in an International centrifuge at 2°C. Starting from the top of the gradient, fractions of 3.0 ml ($F_1$), 5.5 ml ($F_2$), 6.0 ml ($F_3$), 3.5 ml ($F_4$), 4.5 ml ($F_5$) and 6.0 ml ($F_6$) were removed with a pipette. The pellet ($F_7$) was suspended in a small volume of 50 mM Tris-HCl buffer (pH 7.4). The pooled fractions were diluted four fold with the buffer and centrifuged at 70,000 x g for 1 h. The supernatant was decanted and the pellet was washed once more in the same manner. The resulting pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) and made to a suitable final volume. The fractions were divided into suitable small portions and stored frozen at -70° for chemical analysis. Enzyme assays were performed as soon as possible.

### 3. Discussion

At the onset of the present study, the original method of Neville (39) was used. This method involves a gentle homogenization of the tissue in a hypotonic bicarbonate buffer (0.001 M, pH 7.5) followed by repeated low-speed centrifugation of the homogenate to sediment plasma membrane along with nuclei and cell debris. Plasma membranes are separated from other components of the nuclear pellet by repeated washing with the buffer and finally purified by gradient centrifugation. However, this method did not yield satisfactory results. The difficulty was encountered at a step where a white fluffy layer was to be separated from a relatively pinkish layer by repeated washings. In rat SMSG pellets, these layers were indistinct and thus a satisfactory separation was rather difficult.

The isolation of plasma membranes from SMSG homogenates in isotonic sucrose medium was also carried out in our laboratory but the plasma membranes were not as pure as those obtained by the procedure of Bosmann *et al.* (43) which was used to isolate plasma membranes used in all the results reported in this thesis. The degree of plasma membrane purity was based upon the specific

activity of 5'-nucleotidase. An attempt was also made to reduce the time of plasma membrane preparation by using a Ficoll gradient as suggested by McKeel and Jarrett (57), however, these plasma membrane preparations exhibited a 5'-nucleotidase activity lower than that of plasma membranes prepared with sucrose gradients.

The procedure of Bosmann et al. (43) was then tried. As in the method of Neville, the tissue was homogenized in an hypotonic medium, however, the medium contained 20 mM Tris-HCl buffer (pH 7.0) and 10 mM EDTA instead of the 1 mM $NaHCO_3$ used by Neville. Moreover, unlike Neville's method, where plasma membranes were obtained from the pellet after low speed centrifugation, the isolation of plasma membranes was carried out using the 4,000 x g supernatant. In this way the difficulties usually encountered in isolating plasma membranes from nuclear contaminants were avoided.

Approximately 15 to 20% of the total proteins applied to the sucrose gradient were recovered in the seven distinct fractions ($F_1$ - $F_7$). The remaining proteins were presumably soluble and were distributed in the interfacial spaces. The yield of plasma membranes in $F_1$, $F_2$ and $F_3$ was about 0.76 mg plasma membrane protein per g wet weight of tissue and was comparable to the yield of plasma membrane reported for various other tissues (44, 71, 73, 140).

In this procedure, as in others, the nature of the homogenization step was critical to reproducible results. Ten to fifteen hand-driven strokes in a Potter-Elvehjem homogenizer (clearance 0.004 inch) were found to yield reproducible plasma membrane preparations. Excessive and vigorous homogenization led to both poor yields of plasma membranes and somewhat higher levels of contamination with other subcellular organelles.

In the present study, the sucrose gradients were spun in a fixed-angle rotor instead of a swing-out rotor because more tissue could be processed in a single run and thus more plasma membrane would be obtained. No difficulty was encountered in recovering the various layered fractions with this procedure.

Residual sucrose in the final fractions interfered with both enzymic and chemical analysis and, therefore, the final pellets were always washed routinely with Tris buffer before suspending them in an appropriate volume of buffer.

## B. CHARACTERISTICS OF PLASMA MEMBRANES

### 1. Introduction

The relative purity of plasma membrane preparations is commonly checked by such criteria as the relative specific activities of marker enzymes, chemical composition and the morphological appearance. The activity of no single enzyme can be used to identify a subcellular fraction, as many enzymes appear to have a wide distribution within the cell. One enzyme, 5'-nucleotidase, however, does appear to be rather exclusively present in plasma membrane, at least of liver cells (132, 133, 135). Some activity has been reported in liver microsomes (134) but it is conceivable that this represents the contamination of microsomes by plasma membranes (132). 5'-nucleotidase activity is, therefore, most commonly used as an indicator of plasma membranes (43 - 45, 132) during isolation procedures. Other enzymes, whose specific activity appears to be highest in plasma membranes and can be used as marker enzymes, are adenyl cyclase (52, 136, 137), $(Na^{+} - K^{+})$-ATPase (3, 138), $(Mg^{++})$-ATPase (41, 44, 45), p-nitrophenyl phosphatase (135, 150), 1-leucyl naphthylamidase (3, 135), phosphatidylinositol-kinase (139) and glycosidase (132).

Enzymes which are absent from plasma membranes and have been established as characteristic of other subcellular particles, are used to estimate contamination of plasma membranes by such particulates. Plasma membranes, either lack, or demonstrate a very low level of activity of galactosyltransferase (141) and UDPase (43), a characteristic of golgi bodies; glucose 6-phosphatase (43, 45, 73), esterase (43, 142) and NADH-cytochrome c reductase (145), microsomal enzymes; succinate cytochrome c reductase (45, 143), succinic dehydrogenase (57), monoamine oxidase (144), cytochrome c oxidase (73), mitochondrial enzymes; acid phosphatase (73) and β-glucosidase (146), lysosomal enzymes and $NAD^{+}$-pyrophosphatase (161), enzyme characteristic of nuclei. Thus relative contamination of plasma membrane preparations can be estimated and the probable source identified.

The chemical composition of plasma membranes appears to differ from that of the other subcellular organelles in several respects (68, 76). Plasma membranes contain sphingomyelin and cholesterol in large amounts while the former lipid is lacking in smooth endoplasmic reticular membranes and the latter is present in only small amounts (68, 76, 135). The absence of S-lipid from plasma membranes is also considered as an indicator of purity of the plasma membrane preparation (71). The cytoplasmic membranes contain a high proportion of unsaturated fatty acids (68), whereas saturated fatty acids predominate in both erythrocyte membranes (79) and in liver plasma membranes (78). Per unit weight of protein, rat liver plasma membranes contain more sialic acid and hexosamine than do the other subcellular fractions derived from liver (3). Cytochrome c representing mitochondria and cytochrome $P_{450}$ representing endoplasmic reticular membranes are not found in plasma membranes (3). RNA is virtually absent from plasma membranes while found in considerable amounts in microsomes and nuclei (3). A molar ratio of cholesterol to phospholipids near one is considered characteristic of plasma membranes while a ratio lower or higher than one indicates that plasma membranes are associated with other subcellular structures (135). Nuclear membranes can be differentiated from plasma membranes on the basis of low phospholipid content, the presence of DNA and a RNA/DNA ratio of 0.1 - 0.2 (147, 161).

Liver plasma membranes can also be distinguished from membranes of other subcellular organelles by the use of electron microscopy. Plasma membranes display a triple layer structure of 80 - 90 A° in width and are asymmetric, the inner leaflet being slightly thicker than the outer (3). In contrast, the endoplasmic reticular membranes are symmetrical, 40 - 60 A° in width and are globular in appearance. Mitochondrial elementary particles are 90 - 100 A° in width and form a repeating pattern of discrete sub-units which are absent from plasma membranes, and thus can be easily distinguished from plasma membranes. In addition, plasma membranes possess some specialized areas such as the tight junctions and desmosomes which are not found in other membranes (3).

On negative staining, some plasma membranes have been shown to display a hexagonal array of globular knobs about 50 - 60 A°; such a configuration is restricted to areas about tight junctions and desmosomes. These globular sub-units do not appear as independent particles extending from the surface and budding at the membrane edge as in the endoplasmic reticular membranes where they are independent sub-units forming the membrane itself (3). Nuclear membranes viewed under similar conditions show pits and pores. It is possible, therefore, to distinguish membranes of different subcellular origin on the basis of morphological evidence. It should be stressed, however, that the above mentioned features are noted only under appropriate conditions of fixing and staining.

## 2. Methods Used For Characterization of Isolated Salivary Gland Plasma Membranes

Plasma membranes were localized by assaying the various fractions obtained during the isolation procedure for activities of 5'-nucleotidase, alkaline phosphatase, $(Na^{+} - K^{+})$-ATPase, $(Mg^{++})$-ATPase and adenyl cyclase. The contamination of plasma membranes with endoplasmic reticulum and mitochondria was estimated by determining the activities of glucose 6-phosphatase and succinate-INT reductase respectively. In addition these fractions were characterized morphologically with the aid of electron microscopy and on the basis of their chemical composition.

### a) Enzyme Assays

#### (i) 5'-nucleotidase (E.C.3.1.3.5.)

The activity was measured by the method of Michell and Hawthorne (148). The reaction mixture in a final volume of 0.5 ml contained 100 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 10 mM sodium potassium tartrate 2 mM AMP and about 50 µg of protein. The reaction was started by adding AMP, continued for 15 min at 37°C and stopped by the addition of 0.5 ml ice-cold TCA (12.5% final concentration). The amount of Pi released during incubation was estimated by the method of Fisk and Subbarow (149) adapted for use on a Technicon autoanalyser. Controls contained all ingredients except AMP.

(ii) $(Na^{+} - K^{+})$-ATPase (E.C. 3.6.1.3.) and $(Mg^{++})$-ATPase (E.C. 3.6.1.4.)

The method used to estimate the activity of ATPase was essentially that described by Quigley and Gotterer (138). Total ATPase activity was measured in the presence of 50 mM Tris-HCl buffer (pH 7.4), 120 mM NaCl, 20 mM KCl, 7.5 mM $MgCl_2$, 5 mM ATP (disodium salt) and 25µg of tissue protein in a total volume of 0.5 ml. $(Mg^{++})$-ATPase (ouabain-insensitive ATPase activity) was determined as for total ATPase except that 1 mM ouabain was present in the medium. The reactants were equilibrated for 5 min at 37°C before the initiation of reaction by the addition of ATP. After 15 min of incubation, the reaction was terminated by the addition of an equal volume of ice-cold TCA (12.5%). Pi was estimated in the supernatants by a slightly modified procedure of Fisk and Subbarow (149) as described on page 27. The activity of $(Na^{+} - K^{+})$-ATPase was obtained by subtracting the ouabain-insensitive activity from total ATPase activity. The amount of Pi released non-enzymically under similar conditions without tissue was always examined. The control samples were identical to the test samples except that the tissue was pre-killed with TCA.

(iii) Alkaline phosphatase (E.C. 3.1.3.1.)

The reaction mixture, containing 50 mM glycine buffer (pH 10.5), 0.5 mM $MgCl_2$, 5.5 mM p-nitrophenylphosphate (sodium salt) and approximately 50 µg of protein in a final volume of 0.5 ml, was incubated for 30 min at 37°C. The reaction was terminated by the addition of 5 ml of 20 mM NaOH. The controls were treated exactly in the same manner except that the tissue was added after stopping the reaction with NaOH. The test samples were read against their own controls at 405 nm in a 0.5 ml cuvette with a 1 cm light path in a Unicam SP-600 spectrophotometer. One milliunit of enzyme activity represented the liberation of 1 nmole of p-nitrophenol from p-nitrophenylphosphate. This method was similar to that described by Emmelot and Bos (150).

(iv) Adenyl cyclase

The activity was estimated according to the procedure of Krishna et al. (151) with the modification suggested by Bär and Hechter (152). The reaction mixture in a final volume of 0.2 ml contained 2 mM ATP mixed with ( $\alpha$-$^{32}$P-ATP), 20 mM KF, 20 mM aminophylline, 5 mM $MgCl_2$, 50 mM TES buffer (pH 7.4), 1 mM cyclic 3',5'-adenosine monophosphate and 50 - 100 µg of protein. The incubation was carried out in 2 ml glass-stoppered test tubes for 15 min at 37°C. Reaction was terminated by placing the test tubes in boiling water bath for 2 min. The mixture was cooled in an ice bath for 5 min then centrifuged at 2,000 rpm for 10 min. The supernatant was applied to a Dowex-50-$H^+$ column (0.4 x 3.3 cm), then washed with 3 ml of distilled water which was collected in a centrifuge tube . Equal volumes (0.2 ml each) of 250 mM $Ba(OH)_2$ and 250 mM $ZnSO_4$ were added to the eluant and mixed well. The pH was adjusted to 7.0 with either $Ba(OH)_2$ or $ZnSO_4$ and centrifuged for 10 min at 2,000 rpm. The supernatant was withdrawn and the treatment with $Ba(OH)_2$ and $ZnSO_4$ repeated twice more. A 3 ml aliquot of clear pooled supernatants was mixed with 17 ml of Bray's solution and the radioactivity measured in a liquid scintillation counter (Nuclear Chicago Inc., Model Unilux II). Counts were corrected for quench and adjusted to 100% efficiency. The recovery of cyclic 3',5'-adenosine monophosphate was determined spectrophotometrically by measuring absorption at 260 nm in a Unicam SP-800 spectrophotometer.

(v) Glucose 6-phosphatase (E.C. 3.1.3.9.)

The procedure used to estimate the activity of this enzyme was that described by de Duve et al. (143). The activity was measured at 37°C in a total volume of 0.5 ml containing 40 mM glucose 6-phosphate, 70 mM histidine buffer (pH 6.5) containing 10 mM EDTA and about 50 µg of enzyme protein. The reaction was carried out for 20 min and stopped by the addition of an equal volume of ice-cold TCA (12.5%). The amount of Pi liberated was measured manually as described on page 28 (King's Method).

(vi) Succinate-INT reductase (E.C. 1.3.99.1.)

The activity of this enzyme was measured by the method described by Pennington (153). The reaction mixture, containing 50 mM potassium phosphate buffer (pH 7.4), 50 mM sodium succinate, 25 mM sucrose, 0.05% (w/v) INT (p-iodophenyl tetrazolium salt), 50 µg of protein in a final volume of 0.5 ml, was incubated at 37°C for 15 min in glass-stoppered tubes. The reaction was initiated by the addition of INT and stopped by adding an equal volume of ice-cold TCA (10%). The resulting mixture was shaken with 2 ml of ethyl acetate to extract the formazan complex, cleared by centrifugation for 5 min at 2,000 rpm and the optical density of the upper phase containing formazan was measured at 490 nm in a Unicam Model SP-600 spectrophotometer. The activity was expressed as the change in O.D. units per mg protein per h.

b) Electron Microscopy

Tissue fractions were prefixed with 2.5% glutaraldehyde in a solution of 0.54% d-glucose (pH 7.4) and postfixed in 1% osmic acid. Samples were dehydrated with ethanol. Fractions 2, 3, 4 were embedded in Vestopal W while fraction 1 was embedded in methacrylate. The sections were cut and viewed with a Philips 300 electron microscope.

3. Chemical Methods

a) Inorganic Phosphate (Pi) Estimation

(i) Automated measurement of inorganic phosphate (Pi) with the Technicon Autoanalyser System

This method is based upon the formation of phosphomolybdic acid from acidic ammonium molybdate and Pi. Phosphomolybdate is then reduced with ANSA (1-amino-2-naphthol-4-sulfonic acid) to form a blue complex, essentially according to Fisk and Subbarow (149) as described in Technicon Method File N-4b.

The TCA-precipitated proteins were sedimented by centrifugation at 2,000 rpm in a clinical centrifuge for 10 to 15 min and the clear supernatants analysed

for Pi with a Technicon Autoanalyser (Technicon Instruments, Inc. New York, U.S.A.).

(ii) Manual measurement of Pi

King's Method: Occasionally, Pi analysis was carried out manually according to King's modification (154) of the Fisk and Subbarow procedure (149). The clear supernatants from TCA-treated tissue (0.7 ml) were diluted to 1.9 ml with water, mixed with 0.3 ml of ammonium molybdate solution (5% (w/v) in 15% (v/v) $H_2SO_4$) and 0.075 ml of ANSA (0.2% (w/v) made in a solution containing 12% (v/v) sodium bisulfite and 2.5% (w/v) sodium sulfite). The optical density was measured after 5 min at 680 nm in a 0.5 ml quartz cell having a 1 cm light path with a Unicam SP-600 spectrophotometer.

Taussky and Shorr's Method: In experiments with calcium binding and ($Na^+$ - $K^+$)-ATPase, this method was used to estimate Pi as it allowed the efficient measurement of very small amounts of Pi (155). According to this method, ferrous sulphate ($FeSO_4.7H_20$) was used as a reducing agent instead of ANSA as in the Fisk and Subbarow method. Ammonium molybdate solution (10%, w/v) was made in 10 N $H_2SO_4$. The color reagent was prepared fresh on the day of use as follows: Ammonium molybdate solution (10 ml) was diluted to about 70 ml with distilled water to which 5 g of ferrous sulphate was added. The final volume was adjusted to 100 ml and the solution was kept in a dark bottle.

In calcium binding studies, an aliquot of protein free filtrate was made to 12.5% (v/v) with TCA. In ($Na^+$ - $K^+$)-ATPase experiments, the supernatants of TCA (12.5%)-killed tissue were used directly.

To an aliquot of 0.4 ml containing about 5 - 10 µg of Pi, 0.2 ml of the color reagent was added, mixed and the reaction was allowed to proceed for 5 min at room temperature. The absorbance of the blue color was then measured against a blank at 600 nm on a Unicam spectrophotometer. Standards containing $KH_2PO_4$ were analysed simultaneously and used for quantitation of Pi.

b) Protein Estimation

Proteins were estimated by the colorimetric method of Lowry et al. (156). The color reaction involves the formation of a protein-$Cu^{++}$ complex followed by reduction of phosphomolybdic-phosphotungstic reagent by the copper-treated protein under alkaline conditions. The fractions were diluted with 50 mM Tris-HCl buffer (pH 7.4) such that 100 µl of each fraction contained about 20 - 30 µg of protein. They were then solubilized in 1 N NaOH. Samples were diluted to 0.5 N for analysis.

Copper-carbonate solution was prepared fresh as follows: equal volumes (50 ml) of sodium tartrate (2%, w/v) and copper sulphate (1%, w/v); and 2.5 ml of 1 N NaOH were mixed. To 1 ml of the resulting solution, 50 ml of sodium carbonate (2%, w/v) was added and mixed thoroughly.

One ml of copper-carbonate solution was added to each sample, mixed and allowed to stand for at least 10 min at room temperature. 100 µl of 1 N Folin-phenol reagent was then added, mixed and the color allowed to develop for 30 min at room temperature. The absorbance was measured at 750 nm in a 0.5 ml quartz cell having a 1 cm light path with a Unicam-SP 600 spectrophotometer.

Tris buffer has been shown to interfere with the color reaction and the error due to this buffer in the test samples was corrected by adding an equal volume of this buffer to standard samples. A series of protein standards containing bovine serum albumin (Fraction V) were always included in every analysis.

c) N-acetylneuraminic Acid (NANA) Measurement

NANA was estimated by the method described by Warren (157). The method involves at first the liberation of NANA from tissue protein by acid hydrolysis and then oxidation of NANA by periodate in 3 M phosphoric acid to form a chromogen which reacts with 2-thiobarbituric acid to yield a colored compound. This chromophore is then extracted into cyclohexanone and the optical density is measured at 549 and 532 nm. The intensity of color is a linear function of NANA between 10 and 60 nmoles.

(i) Tissue hydrolysis to liberate NANA

A 0.1 ml aliquot of sample was placed in a 12 ml glass-stoppered tube and 0.1 ml of 0.2 N $H_2SO_4$ was added. The tubes were heated at 80°C for 2.5 h in autoregulated heating blocks. Hydrolysis for this length of time was shown to be necessary for the optimum liberation of NANA from sialoproteins of rat SMSG (158). At the end of hydrolysis, the test tubes were cooled and centrifuged for 2 min in a clinical centrifuge. A blank containing 0.2 ml of distilled water only and a set of samples containing known amounts of NANA in 0.2 ml of 0.1 N $H_2SO_4$ were prepared and treated similarly as test samples.

(ii) Estimation of NANA

To each sample, 0.1 ml of 0.2 M sodium metaperiodate in 9 M $H_3PO_4$ was added and mixed thoroughly. After exactly 20 min, 1 ml of 10% (w/v) sodium arsenite solution in 0.5 M sodium sulphate and 0.1 N $H_2SO_4$ was added to each tube and mixed until the yellow color disappeared. Finally, 3 ml of 0.6% (w/v) 2-thiobarbituric acid in 0.5 M sodium sulphate was added to each tube with thorough mixing. The samples were then heated in boiling water for 15 min when they were removed and immersed in cold water for 5 min. The color was extracted into 4.3 ml of cyclohexanone and separated by centrifugation for 10 min in a clinical centrifuge. The absorbance of color was measured at 549 nm and at 532 nm. To minimize the variation due to setting of wavelength after every sample, all samples were first read at 549 nm and then at 532 nm. Sialic acid content was calculated by using the following expression (157).

$$\mu\text{moles NANA} = 0.090 \times O.D._{549nm} - 0.033 \times O.D._{532nm}$$

d) Lipid Analysis

(i) Extraction of lipids

The method of Bligh and Dyer (159) was used to extract and purify lipids. The tissue is treated with a mixture of chloroform and methanol which breaks most of the linkages between the lipids and proteins. The resulting crude lipid extract contains appreciable amounts of water-soluble contaminants such as in-

organic salts, proteins, amino acids, gangliosides, etc. These are removed by a further addition of chloroform and water to give a biphasic system. The upper methanol-water phase containing most of the contaminants is removed from the lower lipid-containing phase.

Generally a 1 ml aliquot of aqueous tissue suspension was treated with 3.75 ml of a mixture of chloroform-methanol (1:2, v/v). After thorough mixing the tubes were centrifuged to sediment non-lipid material mostly containing denatured proteins. The supernate was decanted into graduated centrifuge tubes to which 1.25 ml each of chloroform and water was added, mixing thoroughly after each addition. The tubes were then centrifuged in a clinical centrifuge for 10 min to obtain two separate phases. The upper phase containing water-soluble contaminants was removed and discarded in most instances. The lower phase containing most of the lipids was reduced to dryness at 37°C under a stream of nitrogen. The residue was redissolved in a mixture containing chloroform and methanol (2:1, v/v) to an appropriate volume. Aliquots of this lipid extract were used for the analysis of total lipid phosphorus total cholesterol and for the separation of lipids by thin-layer chromatography.

(ii) Total lipid phosphorus

A portion of the lipid extract was placed in a thick walled tube (2 x 20 mm) and evaporated to complete dryness at room temperature. Perchloric acid (60%), 0.7 ml, and a glass bead to prevent abrupt boiling was added to each tube and the samples were refluxed for 20 min to liberate inorganic phosphate. After cooling, 3 ml distilled water was added in two portions such that the material along the walls of the tubes was washed down, followed by 2 ml of 1.25% (w/v) ammonium molybdate solution. After thorough mixing, 4 ml of 100 fold diluted solution of ANSA (0.064% (w/v) made in 15% sodium bisulfite) was added. The mixture was mixed thoroughly and let stand for 45 to 60 min at room temperature when the intensity of color was measured with a Unicam SP-600 spectrophotometer at 680 nm. Standards, containing $KH_2PO_4$, were processed for the quantitation of phosphate.

(iii) Total cholesterol estimation

The method used to estimate cholesterol was that described by Momose et al. (160). It involves preliminary dehydration of cholesterol to form 2,4-cholestadiene which then polymerizes to form a dimer or trimer. This is followed by oxidation in an acid which is coupled with the reduction of iron to ferrous state which produced a color maximally absorbed at 450 nm. The reduction of iron to ferrous state is directly proportional to the amount of cholesterol.

The color reagent was prepared by dissolving 4 g of ferric chloride ($FeCl_3.7H_2O$) in 150 ml of 85% phosphoric acid under a fume hood. After dissolution, 50 ml of 70% perchloric acid was added slowly with continual stirring. The resulting solution was stored in a dark bottle at 2 - 4°C.

A portion of lipid extract was evaporated to dryness and the residue dissolved in 2 ml of glacial acetic acid. One ml of color reagent was added and mixed well by inversion. The tubes were stoppered and heated at 100°C for 20 min. They were then removed and cooled in a cold water bath. The absorbance of color was measured at 450 nm against a reagent blank. Samples containing known amounts of cholesterol were treated similarly.

(iv) Separation of lipids by thin-layer chromatography

Preparation of Thin-layer Chromatographic (TLC) Plates: A 20 x 20 cm glass plate was thoroughly cleaned with petroleum ether prior to use. A 50% (w/v) slurry of silica gel G (E. Merck AG, Darmstadt, Germany) in distilled water was prepared by thorough mixing and a uniform layer, 0.5 mm thick, was applied to the TLC plate by means of an applicator (Model 200-11, Research Specialities Co., U.S.A.). The plates were allowed to dry at room temperature overnight, activated for 45 min at 110°C on the day of use and cooled in a desiccator. Seven to nine narrow channels were drawn so as to give 6 - 8, 2.5 cm lanes of silica gel.

Separation of lipids: A suitable aliquot of lipid extract was reduced to dryness at 37°C under a stream of nitrogen. The residue was dissolved in a small volume of a mixture of chloroform and methanol (2:1, v/v) and quantitatively

transferred to a centrally located point 3 cm from the bottom edge of a thin-layer chromatographic plate. After the spots were dry, the TLC plate was placed in a closed chamber containing sufficient chloroform : methanol : water (14 : 6 : 1, v/v) to cover 2 cm on the bottom of the plate. When the solvent front was approximately 2 cm from the top, the TLC plate was removed and allowed to stand at room temperature for 3 - 4 h. The individual lipids were visualized by exposing the TLC plate to iodine vapors. A permanent record was made by tracing freshly-exposed plates. The lipids were identified on the basis of $R_f$ values of known lipids which were run simultaneously. The iodine color was allowed to fade away overnight. The silica gel containing individual lipids was scraped into thick-walled tubes and digested for 20 min with an appropriate volume of 60% $HClO_4$ to liberate Pi. The silica gel was removed by centrifugation prior to measuring the optical density of the color developed by the procedure described in Section II, page 31.

Cholesterol, both free and esterified, was extracted from silica gel with glacial acetic acid. The silica gel was sedimented by centrifugation and an aliquot of the clear supernatant was analysed for cholesterol according to the procedure described in Section II, page 32.

(v) Fatty acid analysis by gas-liquid chromatography

Preparation of Fatty Acid Methyl Esters: Fatty acid esters were prepared from whole lipid extracts by a direct transesterification reaction in methanolic-$H_2SO_4$. In the case of TLC-separated lipids, the silica gel containing a lipid was transferred directly to centrifuge tubes without prior extraction as this was found to produce a much higher yield than attempting to elute the lipid from the gel.

The amount of methanolic-$H_2SO_4$ added to lipid contained in graduated centrifuge tubes was dependent upon the quantity of lipid present. Usually, 1 - 2 ml were adequate. The samples were heated at 70°C for 3 h or overnight at 37°C. One to two ml of water was added to each tube and then the methyl esters were

extracted with three 1 ml portions of petroleum ether. The combined extracts were washed with water, dried over $Na_2SO_4$ and reduced to dryness at 30°C under a stream of nitrogen.

Gas-liquid Chromatography: The residue containing methyl esters was redissolved in 50 - 100 µl of 2,2,4-trimethylpentane. An aliquot of 1 - 5 µl depending upon the methyl esters content, was injected into a column (Stainless steel, 5 feet x 1/8 inch) packed with 3% SE-30, 100 - 200 mesh on Gas Chrom Q, contained in Pye "Series 104" gas chromatograph Model 64 (W.G. Pye & Co., Ltd., Cambridge, England). Nitrogen flowing at a rate of 40 ml/min was used as a carrier gas. Standard samples containing known methyl esters were chromatographed similarly and a calibration curve from known methyl esters was constructed. Methyl esters contained in unknown samples were identified on the basis of their retention time as compared with the retention time of known standards. The area under each chromatographic peak was calculated by half height analysis, e.g. 1/2 x height x base and the mole percent composition calculated.

(vi) Amino acid analysis

Hydrolysis of Proteins: The sample containing about 10 - 20 µg of protein was hydrolysed in 6 N HCl for 21 h at 121°C in an evacuated sealed tube.

Separation of Amino Acids: The hydrolysate was applied onto a column packed with Chromabeads (type C) and separated into individual amino acids on the Technicon TSM-1 amino acid analyzer (Technicon Instruments, Inc.). Amino acid peaks resulting from an unknown sample were identified by comparing them with those of known amino acids. The area under each amino acid peak was determined by half-height analysis e.g., 1/2 x base x height and the amount of amino acid present was calculated by using the following expression:

$$\text{µmoles of unknown amino acid} = \frac{\text{Area of an unknown amino acid}}{\text{Area of a standard amino acid}} \times \text{µmoles of standard amino acid}$$

An internal standard containing norleucine was always analysed with each run of unknown sample and the amount of each amino acid was corrected to 100% recovery.

## C. RESULTS AND DISCUSSION

### 1. Enzymic Studies

The activity of 5'-nucleotidase, a plasma membrane marker enzyme, in $F_1$, $F_2$ and $F_3$ of approximate densities 1.01, 1.10 and 1.14 respectively, was much higher than the activity in other fractions of higher density ($F_4$ - $F_7$) collected simultaneously (Table 1). The activities of other plasma marker enzymes such as ($Na^+$ - $K^+$)-ATPase, ($Mg^{++}$)-ATPase and alkaline phosphatase was also high in $F_1$, $F_2$ and $F_3$ although the activity of alkaline phosphatase and ($Mg^{++}$)-ATPase in $F_1$ was not distinct from $F_4$. Similarly, ($Na^+$ - $K^+$)-ATPase activity in $F_3$ was not different from $F_4$ and $F_5$ (Table 1).

Electron microscopic examination of $F_1$, $F_2$, $F_3$ and $F_4$ revealed that $F_1$, $F_2$ and $F_3$ had the characteristic appearance of a plasma membrane fraction while $F_4$ appeared to be more like smooth endoplasmic membranes (Figures 2 a, b, c, d).

Since a molar ratio of cholesterol to phospholipid around unity is considered as an indicator of plasma membranes (135), the molar ratios in $F_1$, $F_2$, and $F_3$ of 1.15, 0.94, 0.92 (Table III) lend support to these fractions being composed predominantly of plasma membranes.

The activities of various enzymes in rat SMSG plasma membranes ($F_2$) were compared with those in plasma membranes of other tissues (Table II). The specific activity of 5'-nucleotidase in $F_1$, $F_2$ and $F_3$ showed an increase of about 25 - 40 fold over the whole homogenate. The activity of the final preparation was similar to the reported activities for most liver plasma membrane preparations (41, 44, 69, 70, 78) except those reported by Henning et al. (73) and Ray et al. (71). Recently, Meldosi et al. (140) and Zentgraf (161) have reported a very low activity of this enzyme in pancreatic and red blood cell plasma membrane respectively, suggesting that the uncritical use of this enzyme as a unique plasma membrane marker is not warranted.

The specific activity of ($Na^+$ - $K^+$)-ATPase, ($Mg^{++}$)-ATPase and alkaline

TABLE I

The Distribution of Enzymic Activities in Various Fractions Obtained During the Isolation of Plasma Membranes from Rat Submandibular Salivary Glands

| Tissue Fraction | Protein as % of whole homogenate | 5'-Nucleotidase | Alkaline phosphatase | $(Na^+ + K^+)$-ATPase | $(Mg^{++})$-ATPase | Glucose-6 phosphatase | Adenyl cyclase | Succinic-INT reductase |
|---|---|---|---|---|---|---|---|---|
| Whole Homogenate | 100* | 1.24 ± 0.09 | 42.1 ± 2.4 | 3.9 ± 0.2 | 10.8 ± 1.1 | 0.4 ± 0.0 | n.d. | 16.3 ± 3.5 |
| $F_1$ | 0.07 ± 0.01[1] | 22.62 ± 4.31 | 79.8 ± 20.9 | 35.3 ± 4.7 | 93.4 ± 20.9 | 2.1 ± 0.5 | 0.0 | 0.0 |
| $F_2$ | 0.19 ± 0.01 | 47.64 ± 7.38 | 371.6 ± 114.0 | 16.7 ± 5.2 | 218.0 ± 56.0 | 2.2 ± 0.3 | 243 ± | 0.0 |
| $F_3$ | 0.60 ± 0.01 | 29.79 ± 4.37 | 279.3 ± 49.6 | 9.4 ± 3.2 | 168.2 ± 12.3 | 1.4 ± 0.4 | 637 ± 192 | 1.5 ± 0.4 |
| $F_4$ | 0.50 ± 0.01 | 10.32 ± 1.93 | 84.7 ± 19.0 | 8.9 ± 2.7 | 79.6 ± 17.6 | 0.9 ± 0.1 | 5060 ± 290 | 7.3 ± 2.2 |
| $F_5$ | 0.55 ± 0.08 | 3.05 ± 0.74 | 27.6 ± 6.6 | 7.3 ± 4.8 | 55.0 ± 18.1 | 0.7 ± 0.1 | 4847 ± 2097 | 15.1 ± 5.0 |
| $F_6$ | 3.79 ± 0.41 | 1.87 ± 0.27 | 18.6 ± 5.3 | 3.7 ± 0.1 | 19.5 ± 1.8 | 0.8 ± 0.1 | 1907 ± 959 | 16.5 ± 8.3 |
| $F_7$ | 0.98 ± 0.30 | 1.01 ± 0.17 | 12.8 ± 2.8 | 4.0 ± 2.2 | 23.0 ± 7.5 | 0.8 ± 0.1 | 912 ± 138 | 11.5 ± 6.9 |

All enzyme units (mean ± S.E) are expressed as μmoles $P_i$ released /mg protein /h except for adenyl cyclase (pmoles cAMP formed /mg protein /15 min) and succinic-INT reductase (O.D. units /mg protein /h) and alkaline phosphatase (mUnits /mg protein /h).

*88.23 ± 7.45 mg protein /g wet weight of tissue

[1] Figures in table refer to mean ± S.E.

TABLE II

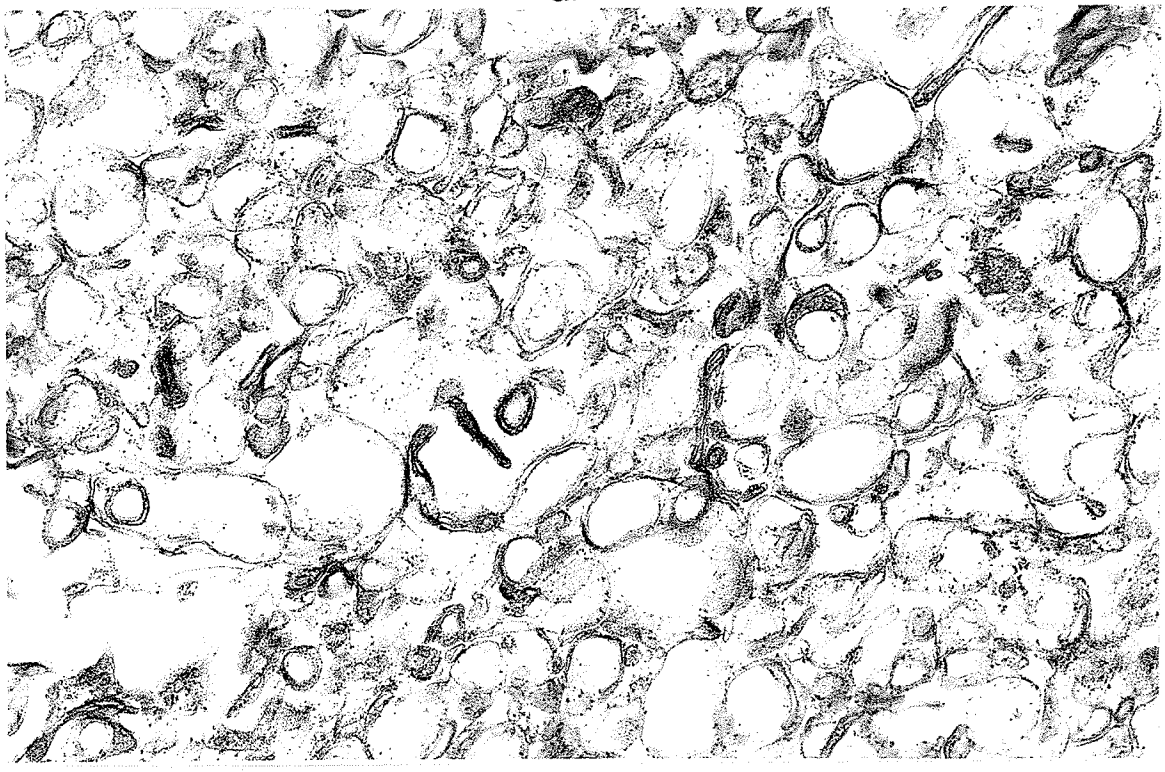
Comparison of Enzyme Activities of Plasma Membranes Isolated from Different Tissues

| Source | Enzymes | | | | | Yield of PM mg PM protein/ g wet wt tissue | Reference |
|---|---|---|---|---|---|---|---|
| | 5'-Nucleotidase | $(Na^{+}+K^{+})$-ATPase | $(Mg^{++})$-ATPase | Glucose 6-Phosphatase | Succinate-INT Reductase | | |
| | µmoles of Pi released/mg protein/h | | | | µmoles/mg/h | | |
| Liver | 32.2 | 11.6 | 40.1 - 44.2 | 1.44 | Nil | - | 41 |
| Liver | 31.3 - 91.0 | - | 8.5 - 39.3 | 0.78 - 6.90 | 0.0 - 0.022 | 0.48 | 44 |
| Liver | 44.3 | 7.9 | 43.5 | 0.90 | 1.2 | - | 45 |
| Liver | 69.0 | - | - | 2.16 | - | 0.46 | 73 |
| Liver | 85.2 | - | 58.0 (Total) | 0.13 | - | 0.52 | 71 |
| Hela Cells | 23.7 - 35.2 | - | 25.0 (Total) | - | Nil | - | 43 |
| Pancreas | 69.0 | - | - | 2.16 | - | 0.46 | 140 |
| SMSG* | 22.6 - 47.6 | 9.4 - 35.3 | 93.4 - 218.0 | 1.40 - 2.20 | Nil | 0.17 - 0.53 | Present Findings |

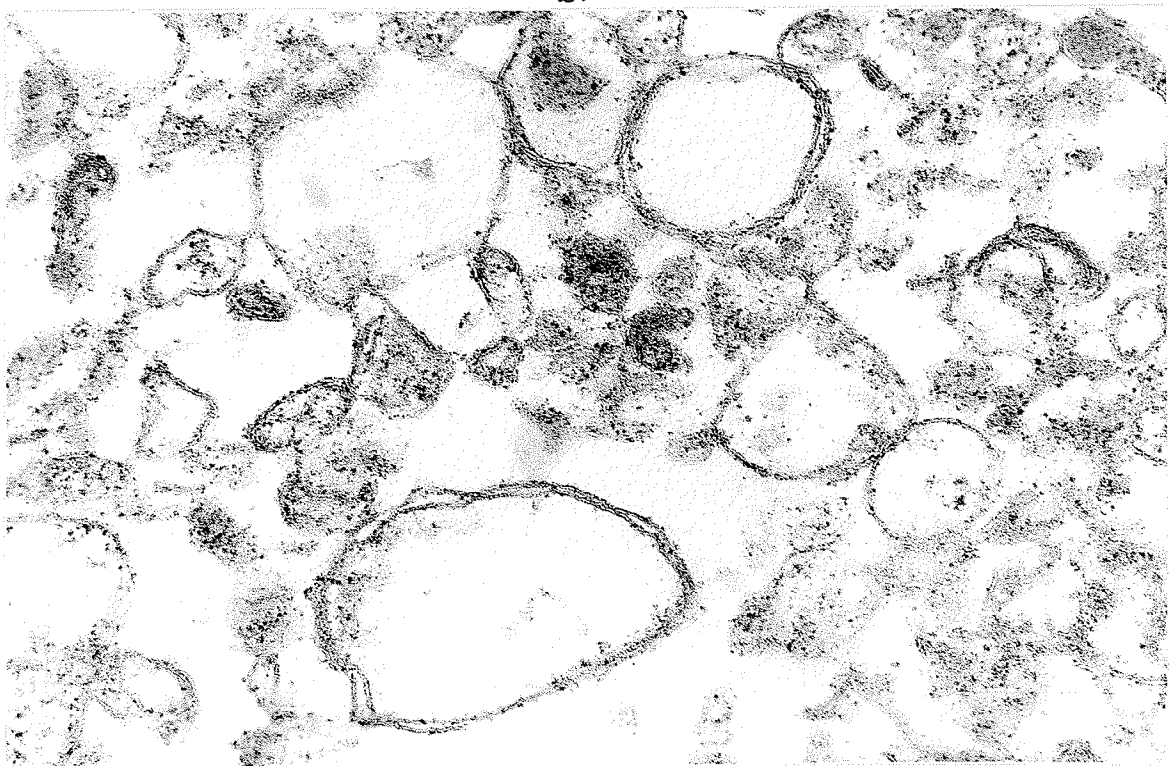
*Submandibular salivary glands

Figure 2. Electron micrographs of isolated fractions from the salivary glands. a) $F_1$ x 53,352; b) $F_2$ x 65,208; c) $F_3$ x 32,604; and d) $F_4$ x 53,352. The tissue was fixed and stained as described under Methods. $F_1$, $F_2$ and $F_3$ contained plasma membrane vesicles and showed little contamination with other subcellular particulates. $F_4$ was predominately composed of smooth endoplasmic reticular tubules.

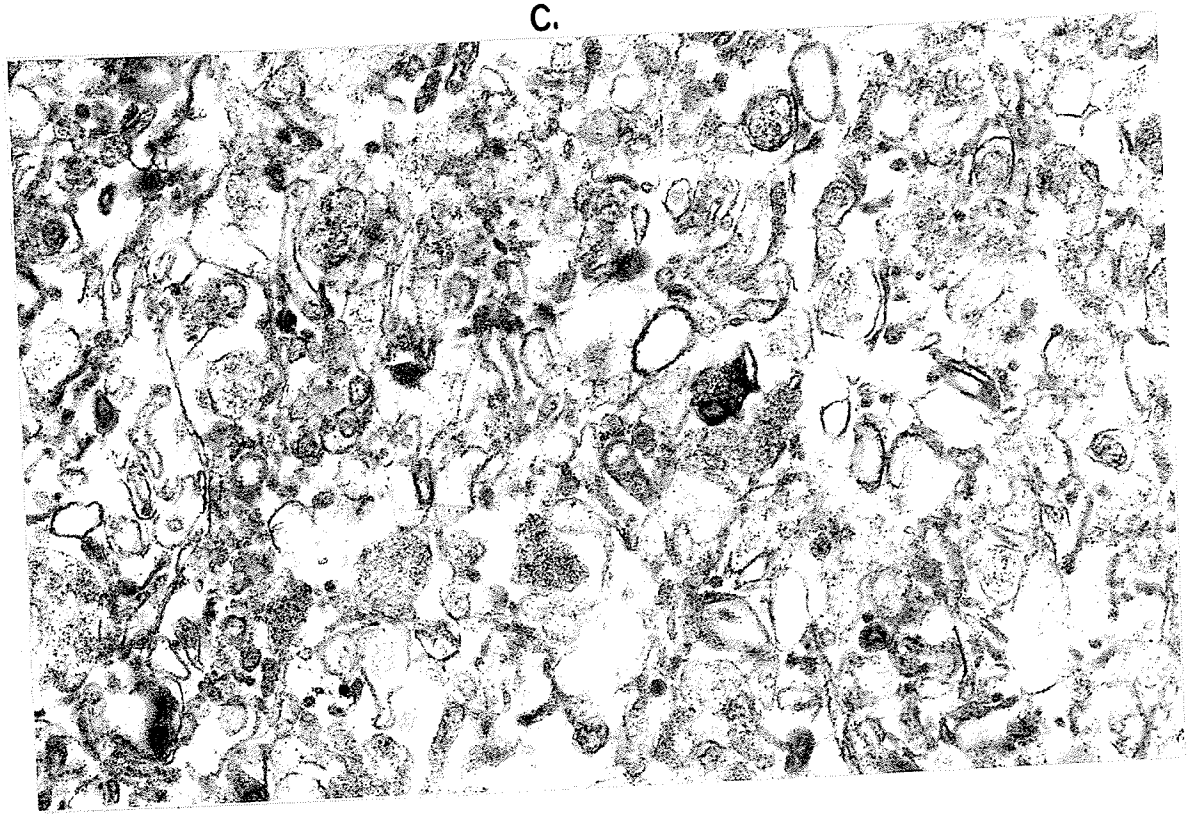
a.



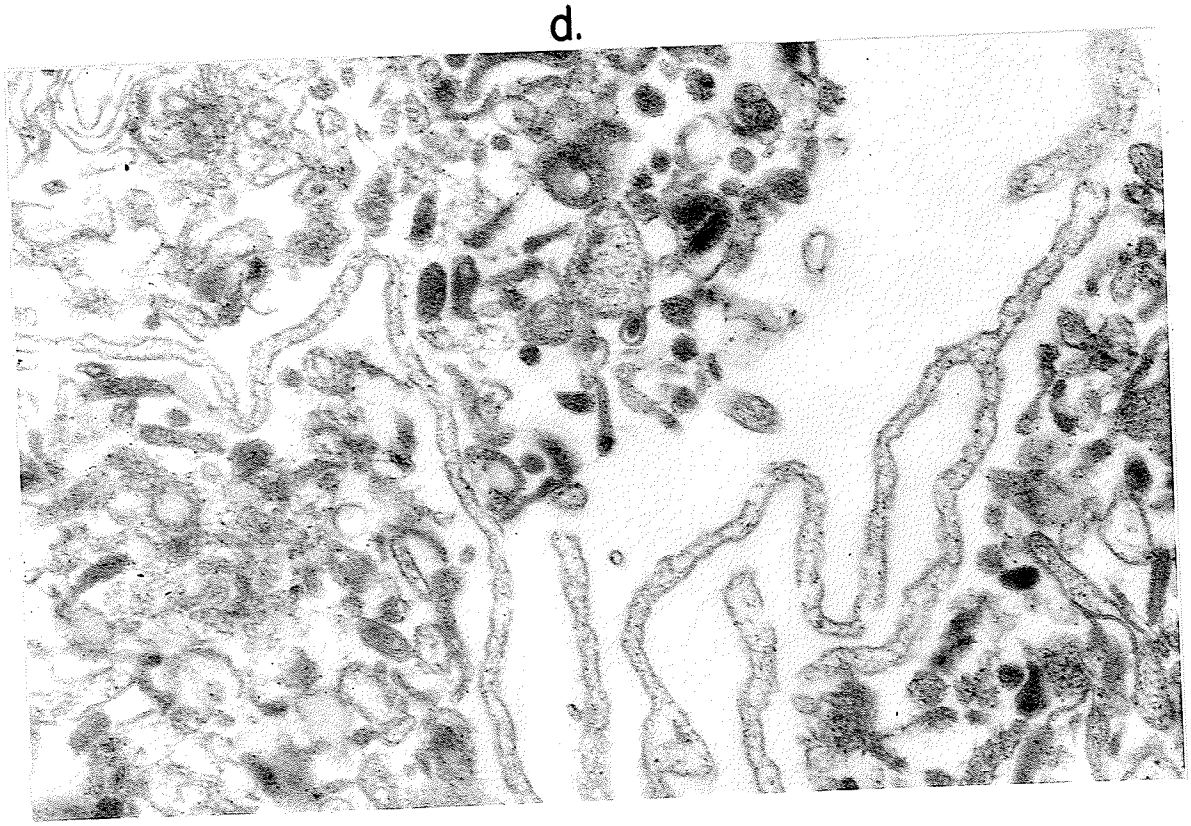
b.



c.



d.



phosphatase in plasma membrane fractions showed an increase of 2.5 - 9, 2 - 10 and 8 - 12 fold respectively over the whole homogenate (Table I). $(Na^{+} - K^{+})$-ATPase specific activity was similar to that reported for plasma membranes derived from other tissue (Table II). However, the activity of $(Mg^{++})$-ATPase in rat SMSG plasma membranes was higher than those values reported for liver plasma membranes (41, 44, 45) except as reported by Fleischer and Fleischer (132) for bovine liver plasma membranes. The exceptionally high activity of $(Mg^{++})$-ATPase in SMSG plasma membranes could be a characteristic of this tissue or could be due to the fact that assays were always done with fresh plasma membrane preparations. It has been demonstrated that on storage, the activity of $(Mg^{++})$-ATPase in many tissue preparations decreases considerably (162, 217).

There were some differences among levels of some enzyme specific activities in $F_1$, $F_2$ and $F_3$. While 5'-nucleotidase, $(Mg^{++})$-ATPase and alkaline phosphatase expressed their highest activities in $F_2$, $(Na^{+} - K^{+})$-ATPase activity was highest in $F_1$ (Table I). There were also differences in the activity of acyl-transferases (Table X) and hydrolases (Figure 5) among these fractions (to be discussed later in Section III). These differences, along with the differences in their chemical composition (Table III), could suggest that plasma membranes in these fractions were probably derived from different regions of cell surface or could have resulted from the isolation procedure where fragmentation of plasma membranes into variable size particles might have occurred.

Contrary to the earlier reports with liver plasma membranes where adenyl cyclase was shown to be almost exclusively bound to plasma membranes (136, 137), the adenyl cyclase activity was lower in plasma membrane fractions of rat SMSG than in other isolated particulates ($F_4 - F_7$) (Table I). These findings indicate that adenyl cyclase although present in plasma membranes of rat SMSG, expressed its highest specific activity in other membranous fractions. Schramm and Naim (163) have recently reported the subcellular distribution of adenyl cyclase in the rat parotid glands. They found 52% of total activity in the 200 x g pellet which they presumed to contain cell membranes, and the remaining activity was mainly

found in the microsomal fraction although a little activity was also present in other particulates which might contain golgi membranes and rough endoplasmic reticulum. Adenyl cyclase activity of their preparation was higher than that found in the present preparation ($F_4$, $F_5$). McKeel and Jarrett (57) also reported the activity of adenyl cyclase both in the microsomes and plasma membranes of adipose tissue; plasma membranes exhibiting the highest activity. The activity of their preparation was lower than that noted in the present study.

Since adenyl cyclase activity was measured in the presence of 20 mM KF in the present study, the results were therefore compared with the activity found by others under similar conditions. Since KF has been shown to activate adenyl cyclase in many different tissues (178), some of the differences in adenyl cyclase activity among the isolated particulates noted above might be explained on the basis of variable degree of activation of this enzyme by KF.

The contamination of plasma membranes with mitochondria was estimated by analyzing these fractions for the activity of succinate-INT reductase. A majority of succinate-INT reductase activity was found in the 4000 x g pellet, indicating that the supernatant was almost free of mitochondria (unreported observations). Plasma membrane fractions ($F_1$, $F_2$ and $F_3$) displayed negligible activity (Table I). The absence of mitochondria was also confirmed by electron microscopy (Figures 2a, b, c). $F_4$, $F_5$, $F_6$ and $F_7$ showed increasing order of the succinate-INT reductase activity (Table I). These results show that fractions ($F_4$ - $F_7$) contained some mitochondria.

Gluscose 6-phosphatase activity has been considered representative of smooth endoplasmic reticular membranes (43, 45, 73). A low activity of this enzyme was noted in the plasma membrane fractions (Table I). Similar low specific activity of this enzyme has been reported in plasma membranes prepared from different tissue sources and by different techniques (Table II). It is becoming apparent that the presence of some glucose 6-phosphatase activity is an innate property of many plasma membranes and may indicate either

that some glucose 6-phosphatase is actually part of the plasma membrane or that an irreducible amount of endoplasmic reticulum (perhaps through plasma membrane-endoplasmic reticulum units) is present. Some evidence in support of such a view stems from the findings that the properties of plasma membranes and microsomal glucose 6-phosphatase were different in liver (41, 164). It has also been argued that if glucose 6-phosphatase activity in plasma membranes truly represented the microsomal contamination, then the other microsomal marker enzyme such as inorganic pyrophosphatase should show a similar degree of contamination. However, this has not been found (164).

2. Chemical Composition of Plasma Membranes

Plasma membranes were enriched in total phospholipids, cholesterol and sialic acids by about 10, 12 and 2 fold respectively over the whole homogenate (Table III). Similar observations with respect to the levels of cholesterol (71, 73), phospholipids (43) and NANA (43) in plasma membranes have been made by others.

Phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphotidylinositide and phosphatidylserine accounted for 49, 24, 18 and 5% respectively of total phospholipids in the whole homogenate (Table IV). In plasma membranes, these phosphatides constituted about 30, 29, 25 and 17% respectively of the total phospholipids (Table IV). These results show a decrease in the level of phosphatidylcholine and an increase in the levels of phosphatidylethanolamine, sphingomyelin, phosphotidylinositide and phosphatidylserine in plasma membranes in comparison to the whole homogenate. Similar changes in the phospholipid composition of pancreatic plasma membrane over the whole homogenate have been reported by Meldosi et al. (76). The enrichment of plasma membranes with sphingomyelin has also been well documented (3, 71 - 73, 76).

A comparison of phospholipid composition of plasma membranes isolated from different tissues by different techniques is given in Table V. It is evident that in most plasma membrane preparations, phosphatidylcholine, phosphatidyl-

TABLE III

Content of Cholesterol, Phospholipid and Sialoprotein in Salivary Gland Preparations

| Tissue Fraction | μg cholesterol / mg protein | μg phospholipid / mg protein | Molar ratio of cholesterol to phospholipid | nmoles NANA / mg protein |
|---|---|---|---|---|
| Whole Homogenate | 57 ± 7 | 215 ± 36 | 0.576 | 40 ± 10 |
| $F_1$ | 1292 ± 191 | 2322 ± 464 | 1.151 | 101 ± 28 |
| $F_2$ | 677 ± 100 | 1447 ± 142 | 0.938 | 49 ± 23 |
| $F_3$ | 484 ± 67 | 1053 ± 52 | 0.916 | 43 ± 10 |
| $F_4$ | 247 ± 28 | 638 ± 33 | 0.771 | 33 ± 6 |
| $F_5$ | 207 ± 34 | 668 ± 126 | 0.710 | 25 ± 7 |
| $F_6$ | 155 ± 34 | 551 ± 88 | 0.576 | 18 ± 6 |
| $F_7$ | 83 ± 16 | 402 ± 68 | 0.454 | 13 ± 3 |

Figures in table refer to mean ± S.E. To calculate moles 386 was used as molecular weight of cholesterol and 725 for average molecular weight of phospholipid. Phospholipid weight was obtained by multiplying the lipid phosphorus by 25. Sialoprotein is expressed as N-acetylneuraminic acid (NANA) as the molecular weight of these materials is unknown.

TABLE IV

Percent Distribution of Phospholipid in Fractions
Isolated from Salivary Glands

| | Phospholipids | | | |
|---|---|---|---|---|
| | PE | PC | Sph. | PI + PS |
| Whole Homogenate | 23.5 ± 1.7 | 48.8 ± 3.7 | 17.5 ± 2.2 | 4.5 ± 0.2 |
| $F_1$ | 29.7 ± 4.0 | 33.2 ± 4.1 | 11.3 ± 3.6 | 20.9 ± 3.7 |
| $F_2$ | 28.6 ± 1.7 | 29.8 ± 3.5 | 23.8 ± 1.3 | 16.6 ± 3.6 |
| $F_3$ | 24.2 ± 1.0 | 29.7 ± 2.8 | 27.8 ± 3.9 | 14.3 ± 4.6 |
| $F_4$ | 34.5 ± 2.4 | 31.2 ± 4.7 | 23.3 ± 1.3 | 9.3 ± 4.2 |
| $F_5$ | 26.3 ± 2.9 | 47.8 ± 6.0 | 14.3 ± 3.2 | 4.2 ± 0.8 |
| $F_6$ | 36.1 ± 8.0 | 39.8 ± 10.1 | 13.8 ± 5.5 | 6.4 ± 3.1 |
| $F_7$ | 26.0 ± 1.4 | 51.2 ± 3.4 | 14.8 ± 1.6 | 8.0 ± 1.8 |

The figures represent the distribution of lipid phosphorus within a given fraction and can be directly interpreted in the horizontal direction only. An estimation of the relative amounts of phospholipid between fractions can be obtained using Table III. A small percent of unidentified phospholipid is not reported in this table.

Abbreviations: PE = phosphatidylethanolamines; PC, phosphatidylcholines; Sph., sphingomyelins; PI, phosphoinositide and PS, phosphatidylserines

TABLE V

Comparison of Lipid Composition of Plasma Membranes
Isolated from Different Tissues

| Source | Phospholipids | | | | Reference |
|---|---|---|---|---|---|
| | PC | PE | SPH. | PS + PI | |
| Liver | 26.0** | 18.0 | 8.8 | 5.0 | 44 |
| Liver | 34.8 | 22.4 | 20.5 | 17.4 | 73 |
| Liver | 19.3 | 10.3 | 9.8 | traces | 71 |
| Liver | 40.0 | 20.0 | 18.0 | 13.0 | 70 |
| Liver | 41.0 | 11.0 | 33.0 | 12.0 | 72 |
| Pancreas | 24.5 | 34.4 | 19.2 | 4.7 | 140 |
| Brain | 33.9 | 28.2 | 3.0 | 11.8 | 74 |
| Stellar Nerves | 45.9 | 34.4 | 10.1 | 10.4 | 75 |
| L-Cells | 30.6 | 11.3 | 23.1 | 9.8 | 77 |
| SMSG* | 29.8 | 28.6 | 23.8 | 16.6 | Present Findings |

*Submandibular salivary glands

**% of total phosphatides

Abbreviations as explained in Table IV.

ethanolamine, sphingomyelin and phosphatidylserine and phosphatidylinositide are found in the decreasing order of concentrations respectively. However, in pancreatic plasma membranes (76), phosphatidylethanolamine formed a larger percentage of total phospholipids than phosphatidylcholine. In SMSG plasma membranes, phosphatidylinositide and phosphatidylserine were markedly increased (Table IV) over the whole homogenate. Ray et al. (71) also noted an increase in phosphatidylserine in liver plasma membranes over the whole homogenate, but Meldosi et al. (76) did not find any change in phosphatidylserine and phosphatidylinositide in pancreatic plasma membranes compared to the rough endoplasmic membranes. These differences in the phospholipid composition of SMSG plasma membranes from plasma membranes isolated from other tissues could not be due to variable recoveries of phospholipids from the TLC plates because the recovery of lipid phosphorus was always close to 100%.

In pancreatic plasma membranes, Meldosi et al. (76) noted a high content of free fatty acids and of lysolecithin (7 - 10% of total lipids) and they demonstrated that, during the isolation and homogenization procedures, the structural phospholipids were broken down by lipases and phospholipases to produce high levels of free fatty acids as well as lyso-lecithin. Ray et al. (71) also noted a large percentage of free fatty acids in liver plasma membranes. The use of inhibitors of hydrolases in the homogenizing medium did not reduce free fatty acid level. In the present study, free fatty acids, cholesterol esters and lysolecithin appeared to constitute a very small percentage of total lipids as they could not be found with the techniques used in this study. The presence of lysolipids in SMSG plasma membranes was only suggested by the incorporation studies. The formation of phosphatidylethanolamine and phosphatidylcholine from added (1-$^{14}$C)-palmitoyl CoA was increased several fold by the addition of lysophosphatidylethanolamine and lysophosphatidylcholine respectively suggesting that lysolipids were probably the endogenous acceptors. EDTA has been shown to inhibit phospholipases (167) and the breakdown of structural phospholipids in the present study would be minimal as EDTA was present in the homogenization medium. This was not the case in either of the above mentioned investigations.

The overall fatty acid composition of the major lipids of whole homogenate and plasma membrane was not greatly different (Table VI). The fatty acid profile of the rat SMSG plasma membrane lipids was found to be very similar to those published for rat liver plasma membranes (70, 73) and somewhat similar to brain synaptosomes (74). In rat SMSG plasma membranes, both stearic acid (18:0) and oleic acid (18:1) contributed almost equally and formed about 38% of total fatty acids in phosphatidylcholine and about 54% in phosphatidylethanolamine. In liver plasma membranes (70), although the total contribution by these fatty acids was comparable to that found in SMSG plasma membranes, stearic acid was predominent over oleic acid in both phosphatidylcholine (30% stearic acid versus 10% oleic acid) and phosphatidylethanolamine (32% stearic acid versus 7% oleic acid).

Sphingomyelin in plasma membranes contained less palmitic acid (16:0) and oleic acid (18:1) than that in the whole homogenate while stearic acid (18:0) and fatty acids longer than 20 carbon atoms of sphingomyelin in plasma membranes were higher than that in whole homogenate (Table VI). Other fatty acids of sphingomyelin were similar in both the plasma membranes and the whole homogenates.

The fatty acid composition of phosphatidylcholine was not much different in plasma membranes and the whole homogenate (Table VI). Palmitic acid in phosphatidylethanolamine was less than that in whole homogenate while stearic acid was more in plasma membranes.

The relative contribution of some fatty acids of plasma membrane triglycerides and whole homogenate was different. The level of palmitic acid, oleic acid in TG of plasma membranes was considerably lower than that in the whole homogenate while fatty acids with hydrocarbon chains longer than 20 carbon atoms were slightly less in plasma membranes than that in the whole homogenate. On the other hand, palmitoleic acid (16:1) and linolenic acid (18:2) and arachidonic acid (20:4) in TG of plasma membranes were markedly higher than that in whole homogenate. Other fatty acids were similar.

In plasma membranes, sphingomyelin contained very high levels of long

TABLE VI

Fatty Acid Composition of Lipids Isolated From Salivary Gland Plasma Membranes

| Chain length of fatty acid | Sph. | PC | PE | TG | FA |
|---|---|---|---|---|---|
| Less than 16 | 0.7 - 0.8 (1.1) | 1.2 - 1.7 (1.1) | 2.9 - 3.1 (2.5) | 9.2 - 13.2 (8.2) | 1.3 - 6.0 |
| 16:0 | 23.7 - 27.5 (35.5) | 43.8 - 56.6 (40.6) | 13.1 - 15.9 (24.6) | 10.5 - 12.6 (26.4) | 38.3 - 46.4 |
| 16:1 | 0.0 - 1.1 (1.0) | 1.2 - 1.4 (1.3) | 2.9 - 3.2 (3.6) | 22.5 - 37.4 (10.0) | 1.4 - 3.0 |
| 18:0 | 27.4 - 33.2 (23.5) | 15.3 - 18.7 (16.7) | 23.4 - 29.6 (13.7) | 6.8 - 7.2 (7.4) | 30.5 - 37.3 |
| 18:1 | 5.5 - 7.1 (14.6) | 12.9 - 19.7 (23.0) | 27.9 - 30.4 (27.2) | 7.5 - 8.5 (28.0) | - |
| 18:2 | 0.7 - 0.8 - | 0.4 - 0.6 (0.6) | 1.3 - 3.3 (3.5) | 12.8 - 17.7 (5.8) | - |
| 20:4 | 1.6 - 3.4 (4.8) | 3.5 - 6.7 (7.9) | 9.7 - 15.3 (16.0) | 4.8 - 5.5 (2.2) | - |
| Greater than 20 | 23.4 - 33.4 (17.6) | 1.7 - 9.4 (7.9) | 4.5 - 10.9 (9.4) | 4.1 - 5.5 (8.8) | 5.5 - 10.6 |

The results are expressed as mole % (moles/100 moles) x 100 and are directly comparable only in the vertical direction. Approximate comparison among the lipid classes can be made by reference to the data of Table IV. The results represent the range of values found in the three plasma membrane fractions ($F_1$ - $F_3$). Figures in brackets below the main values are the levels of the same fatty acid found in the original whole gland homogenate. Fatty acids present in low, or trace, amounts are not reported. Abbreviations as noted in Table IV except FA refers to free fatty acid.

chain fatty acids, a trend which was apparent in most of the other plasma membrane preparations (70, 75, 77). Triglycerides, on the other hand, contained the highest proportion of very short chain fatty acids.

Polyacrylamide gel electrophoresis showed some differences in the banding patterns of $F_1$, $F_2$ and $F_3$. However, the amino acid composition of these fractions was not markedly different from one another nor from the amino acid composition of the whole homogenate (Table VII). Acidic, basic and hydrophobic amino acids formed 20 - 24%, 22 - 27% and 26 - 29% of total amino acids respectively. Cysteine (2%), serine and threonine (7 - 11%) were also present. Glycine, alanine and proline together amounted to 16% of total amino acids. The amino acid composition of rat SMSG plasma membranes was similar to that reported for plasma membranes isolated from liver (69, 73, 165), red blood cells (166) and HeLa cells (43).

These data demonstrate the similarity in enzymic and chemical composition of SMSG plasma membranes and plasma membranes of other tissues isolated by different techniques. Furthermore, the data provide basic information for future investigations where one might study the changes in the enzymic and chemical composition of plasma membranes isolated from salivary glands of animals which have been injected with a drug promoting secretion. By this approach, it might be possible to relate the observed changes, if there are any, to the process of secretion.

A bimodal distribution of adenyl cyclase and glucose 6-phosphatase activities suggests that plasma membranes might be continuous with the endoplasmic reticulum at certain regions of cell surface, a view consistent with many others (41).

TABLE VII

Comparison of Amino Acid Composition of Proteins
Isolated from Salivary Gland

| Amino Acid | Tissue Preparation | | | | |
|---|---|---|---|---|---|
| | Whole homogenate | $F_1$ | $F_2$ | $F_3$ | $F_4$ |
| Basic | | | | | |
| Lysine | 10.4 | 8.7 | 7.8 | 9.1 | 8.5 |
| Histidine | 4.2 | 4.7 | 4.0 | 5.8 | 2.1 |
| Arginine | 5.5 | 7.2 | 7.2 | 7.7 | 6.2 |
| | 20.1 | 20.6 | 19.0 | 23.8 | 16.8 |
| Acidic | | | | | |
| Aspartic | 11.7 | 10.5 | 10.7 | 11.0 | 10.5 |
| Glutamic | 15.1 | 13.5 | 12.9 | 10.5 | 12.5 |
| | 26.8 | 24.0 | 23.6 | 21.5 | 23.0 |
| Hydrophobic | | | | | |
| Valine | 7.0 | 6.9 | 6.4 | 7.4 | 5.5 |
| Isoleucine | 4.9 | 4.7 | 5.1 | 4.7 | 5.3 |
| Leucine | 9.5 | 10.1 | 10.4 | 11.1 | 11.4 |
| Tyrosine | 1.4 | 0.8 | 0.9 | 2.1 | 1.7 |
| Phenylalanine | 4.0 | 5.0 | 5.0 | 0.5 | 4.8 |
| | 26.8 | 27.5 | 27.8 | 25.8 | 28.7 |
| Cysteine (1/2) | 1.6 | 1.4 | 1.2 | 3.9 | 1.7 |
| Serine | 2.9 | 4.9 | 4.7 | 5.9 | 3.7 |
| Threonine | 4.1 | 4.0 | 4.7 | 5.4 | 4.0 |
| Glycine | 11.9 | 8.2 | 7.9 | 7.8 | 8.1 |
| Alanine | - | 7.4 | 7.6 | 7.7 | 7.4 |
| Proline | 5.0 | 1.9 | 3.0 | tr. | 4.5 |

Results are expressed as moles %. Tryptophane and methionine were not estimated. See Table I for total protein levels in each fraction.

# SECTION III

## A. LIPIDS

### 1. Introduction

The early work of Hokin and his colleagues (97, 128) with salivary gland slices, and the perfusion studies of Burford and Huggins (26) with pancreas have indicated that phosphoinositides and phosphatidic acid undergo metabolic alterations during the process of exocrine secretion. The exact subcellular site(s) of such activity has not been firmly established. Van Deenen has emphasized the importance of fatty acid types for the integrity, as well as the function, for biological membranes (4, 5). Previous studies from our laboratory with slice, homogenate and isolated subcellular particulate systems (15 - 19) have shown that rat SMSG exhibit a very active overall lipid metabolism. Since the secretion of macromolecules reportedly involves the migration of secretory vesicles towards the periphery of the cell, fusion of these vesicles with the outer cell membrane (plasma membrane), rupture of cell membranes at the point of fusion and ultimately the discharge of secretory material into the acinar lumen (20, 27), the outer cell membranes must, therefore, undergo some changes during this process. Some of the features of lipid metabolism in plasma membranes have been described in three reports (165, 168, 170). In the present investigation, having reasonably well characterized the SMSG plasma membrane enzymically and chemically, some aspects of its lipid metabolism were then studied particularly that of fatty acid uptake. Since acetylcholine and epinephrine are known to promote salivary secretion both *in vivo* (26) and *in vitro* (15, 97, 126, 128), simultaneously increasing the incorporation of $^{32}$P-orthophosphate into certain phosphatides (15, 97, 126, 128), their effects on the incorporation of (1-$^{14}$C)-palmitoyl CoA into plasma membrane lipids *in vitro* were examined.

### 2. Methods

Fresh plasma membrane fractions (about 50 µg plasma membrane protein) were incubated with labelled lipid precursors in 50 mM Tris-HCl buffer (pH 7.4) for 10

min at 37°C in glass-stoppered centrifuge tubes. Total volume of the reaction mixture was 0.1 ml. Other additions are given in the legends to appropriate tables and figures. Controls, containing an equivalent amount of tissue boiled for 10 min at 100°C, were always performed. The reaction was terminated by the addition of 3.75 volumes of a mixture containing chloroform and methanol (1:2, v/v).

The lipids were extracted and separated by procedures described under Section II, 3. Chemical Methods, d (i) on page 30. Labelled lipids were located by radioautography with Ilflex 25 EP X-ray film (Ilford Limited, Ilford, Essex, England). The silica gel containing radioactive lipid was scraped off and transferred to counting vials. Scintillation fluid (10 ml) containing PPO (0.4%, w/v) and POPOP (0.005%, w/v) in toluene was added and mixed well before measuring the radioactivity in a Unilux II (Nuclear Chicago, Inc., Illinois, U.S.A.). Counts were corrected for quenching and calculated to 100% efficiency. The results are expressed as nmoles of the precursor incorporated/mg plasma membrane protein/10 min, unless otherwise noted.

### 3. Results

#### a) Incorporation of Labelled Lipid Precursors into Plasma Membrane Lipids

##### (i) (1-$^{14}$C)-palmitic acid

The results in Table VIII show that palmitic acid in the presence of ATP, Co-enzyme A and $Mg^{++}$ was readily incorporated into phosphatidylcholine and triglycerides of the pooled supernatant ($P_s$) and smooth membranes ($F_4$) but not into the lipids of the plasma membrane fractions ($F_1$, $F_2$ and $F_3$). When L-carnitine was added, palmitoylcarnitine was formed by $P_s$ and $F_4$ but not by the plasma membrane fractions. These results indicate that plasma membranes lack palmitoyl-CoA synthetase, a conclusion consistent with other authors (165, 171, 172). However, they do not agree with those of Stein et al. (170). These authors reported that isolated liver plasma membranes could form phosphatidylcholine from oleate

TABLE VIII

Measurement of Fatty Acid Activation by Salivary Gland Preparations

| Tissue Preparation | L-carnitine Added | pmoles ($^{14}$C)-palmitic acid incorporated / mg protein/h | | |
|---|---|---|---|---|
| | | Acyl-carnitine | PC | TG |
| 4000 x g supernatant | - | 0 | 450 | 490 |
| | + | 1900 | 420 | 330 |
| Plasma membranes ($F_1 - F_3$) | - | 0 | 0 | 0 |
| | + | 0 | 0 | 0 |
| Smooth membranes ($F_4$) | - | 0 | 1170 | 1800 |
| | + | 18,800 | 1220 | 800 |

Equivalent levels of tissue protein (50 - 60 µg) were incubated in 0.2 ml 50 mM TES buffer (pH 7.4) containing 13 mM ATP, 0.5 mM CoA, 12.5 mM $MgCl_2$, 5.21 nmoles (1-$^{14}$C)-palmitic acid and, when present, 10 mM L-carnitine for 60 min at 37°.

(Na salt), in the presence of ATP, CoA, $Mg^{++}$ and lysolecithin. It is possible that their acknowledged 20% microsomal contamination of the plasma membrane preparation was the source of the acyl-CoA synthetase. Pande and Mead (173) also reported that fatty acid-CoA synthetase activity was associated with liver plasma membranes, however, their plasma membrane preparation was a very crude one.

(ii) (U-C-14)-inositol

Since phosphatidylinositol has been reported to be intimately associated with active transport and secretion (174), the capacity of plasma membranes to synthesize phosphatidylinositol from ($^{14}$C)-myo-inositol was examined under the condition established by Pritchard (17). The results in Table IX show that plasma membrane fractions ($F_1$ - $F_3$) formed phosphatidylinositol, however, the formation of this lipid was about 66% less than that of the smooth membranes ($F_4$ - $F_7$). It is possible that the biosynthesis of phosphatidylinositol by plasma membranes was due to microsomal contamination but the comparison of relative rates of phosphatidylinositol formation by microsomal fractions ($F_4$ - $F_7$) and by plasma membranes would suggest that SMSG plasma membrane does possess a small innate capacity for phosphatidylinositol formation.

(iii) (1-$^{14}$C)-palmitoyl CoA

The suggestion that SMSG plasma membranes lack acyl-CoA synthetase activity was supported by studies on the incorporation of palmitoyl CoA into plasma membrane lipids. The results in Table X show that plasma membrane fractions ($F_1$ - $F_3$) readily incorporated this precursor into phosphatidylcholine, phosphatidylethanolamine and triglycerides and that phosphatidylcholine received the largest portion of the label from palmitoyl CoA. The lack of acyl-CoA synthetase in plasma membranes suggest that for plasma membranes to utilize fatty acids in situ for the synthesis of lipids, they must be activated at subcellular site(s) other than plasma membranes, presumably at the microsomal membranes.

When L-carnitine was added to the incubation flask, plasma membranes

TABLE IX

Formation of Lipid from (U-$^{14}$C)-inositol and ($^{14}$C-methyl)-carnitine by Submandibular Salivary Gland Membrane Preparations

| | pmoles of precursor incorporated into lipid / mg protein/30 min | |
|---|---|---|
| Tissue Fraction | (a) Phosphatidyl Inositol | (b) Acylcarnitine |
| $F_1 - F_3$ (pooled) | 58 | 5447 |
| $F_4$ | 142 | 10557 |
| $F_5$ | 188 | 8320 |
| $F_6$ | 175 | 298 |
| $F_7$ | 65 | 3692 |

Since the incubation conditions for the two studies were different, a direct comparison of (a) and (b) is not warranted. The incubation medium for inositol contained 50 mM Tris buffer (pH 7.4), 3 mM $MgCl_2$, 13 mM ATP, 35 mM KF, 1.3 mM CTP, 0.03 mM palmitoyl CoA and 615 pmoles (U-$^{14}$C)-myo-inositol in a total volume of 0.2 ml. Incubation was for 30 min at 37°. The medium for carnitine studies contained 50 mM TES buffer (pH 7.4), 0.043 mM palmitoyl CoA, 15 mM ATP and 14.3 nmoles ($^{14}$C-methyl)-DL-carnitine in a total volume of 0.07 ml. Incubation as for (a).

TABLE X

Incorporation of (1-$^{14}$C)-palmitoyl CoA into Lipids of Isolated Submandibular Gland Plasma Membrane Preparations

| Experiment No. | Plasma Membrane Fraction | Added to Basic Medium | nmoles ($^{14}$C)-palmitate incorporated into lipid / 10 mg protein / 5 min | | | |
|---|---|---|---|---|---|---|
| | | | Acyl-carnitine | PC | PE | TG |
| A. | | | | | | |
| | $F_1$ | Nil | 0 | 102 | 140 | 155 |
| | | L-carnitine | 198 | 93 | 129 | 154 |
| | | lyso-PC | 0 | 532 | 113 | 119 |
| | $F_2$ | Nil | 0 | 46 | 22 | 18 |
| | | L-carnitine | 53 | 33 | 25 | 13 |
| | | lyso-PC | 0 | 229 | 16 | 19 |
| | $F_3$ | Nil | 0 | 46 | 6 | 9 |
| | | L-carnitine | 19 | 43 | 5 | 10 |
| | | lyso-PC | 0 | 115 | 4 | 7 |
| B. | | | | | | |
| | $F_1$ | Nil | 0 | 2.2 | 0.8 | - |
| | | lyso-PE | 0 | 2.2 | 5.8 | - |
| | $F_2$ | Nil | 0 | 2.4 | 0.7 | - |
| | | lyso-PE | 0 | 2.3 | 7.1 | - |
| | $F_3$ | Nil | 0 | 1.8 | 20.2 | - |

In each experiment the basic medium consisted of 50 mM TES buffer (pH 7.4) with 15 - 20 µg tissue protein and, when added, lysolecithin 50 µM, lyso-phosphatidylethanolamine 1.7 mM and L-carnitine 10 mM. Palmitoyl CoA content was (A) 6.4 nmoles, (B) 0.5 nmoles. Abbreviations as in Table IV.

formed palmitoyl carnitine with a concomitant decrease in the formation of most other lipids (Table X). These findings were confirmed in experiments using ($^{14}$C-methyl)-carnitine and unlabelled palmitoyl CoA (Table IX). These results demonstrate the presence of palmitoyl-carnitine transferase activity in plasma membranes. Smooth membranes ($F_4$), however, showed much higher activity (Table IX). Pritchard et al. (18) also reported the formation of palmitoyl-carnitine by plasma membranes isolated from rat SMSG by another procedure.

Palmitoyl-carnitine transferase has been considered a characteristic mitochondrial enzyme and its role in the transport of fatty acids across mitochondrial membranes has been established (221). The result of the present study (Table IX and Table X) and those of Pritchard et al. (18) demonstrate that the activity of this enzyme was also present in smooth membranes (Table IX) and plasma membranes (Table X). The functions of this enzyme in these latter subcellular particulates is not yet known. However, it is possible that this enzyme system might function in the transport of fatty acids across the outer cell membrane.

The addition of lysophosphatidylcholine and lysophosphatidylethanolamine increased the incorporation of palmitoyl CoA into phosphatidylcholine and phosphatidylethanolamine respectively by plasma membrane fractions with a small decrease in the formation of other lipids (Table X). These results strongly indicate the presence of acyl transferase activity in SMSG plasma membrane and are in agreement with the reports of Stahl and Trams (165) and Stein et al. (170) for liver plasma membrane; but not with those of Eibl et al. (176), of van den Busch (169) and of Kaulen et al. (168) who reported that acyl transferase activity was largely confined to microsomes. Eibl et al. (176) and Kaulen et al. (168) noted some activity in plasma membranes but attributed this activity to the contamination of plasma membranes by microsomes.

The present findings also indicate that lysophosphatides were probably the major endogenous acceptors for acyl groups in transferase reactions forming phosphatidylcholine and phosphatidylethanolamine, a suggestion consistent with the reports of other authors (165, 170).

Whether palmitic acid from palmitoyl CoA was incorporated into 1 or 2 position of lecithin was not ascertained in the present study. The lysophosphatidylcholine and lysophosphatidylethanolamine used in this study contained a mixture of 1-acyl and 2-acyl isomers (Applied Science Laboratories, personal communication). Since it is known that fatty acids from saturated acyl CoA are preferentially transferred to 1-acyl position by rat liver microsomes (169), it is possible that palmitic acid in the present study was predominately incorporated into 1-acyl isomer of both lysolipids. However, hydrolytic studies have not yet been done to establish this point.

b) Effects of Various Incubation Conditions on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane Lipids

It has been suggested by Van Deenen and his co-workers (4, 5) that fatty acids of cell membrane phospholipids might regulate the structure and permeability of plasma membranes. The enzymes which are concerned with the turnover of fatty acid residues in the plasma membrane lipids might have a significant role in the dynamic nature of plasma membranes. As part of this study, the lysolecithin-palmitoyl transferase system of plasma membranes was examined in some detail.

(i) Tissue Concentration

Palmitoyl CoA incorporation into phosphatidylcholine increased with increasing protein concentration up to 204 µg protein/0.1 ml (Figure 3). In the presence of lysolecithin, this incorporation was markedly increased reaching maximum by 64 µg plasma membrane protein/0.1 ml. These results suggest that a lysolecithin concentration at 64 µg plasma membrane protein/0.1 ml saturated the enzyme system completely under the present conditions. The incorporation of palmitoyl CoA into phosphatidylethanolamine and sphingomyelin was reduced in the presence of lysolecithin indicating that the incorporation into most lipids probably took place from a common pool of palmitoyl CoA.

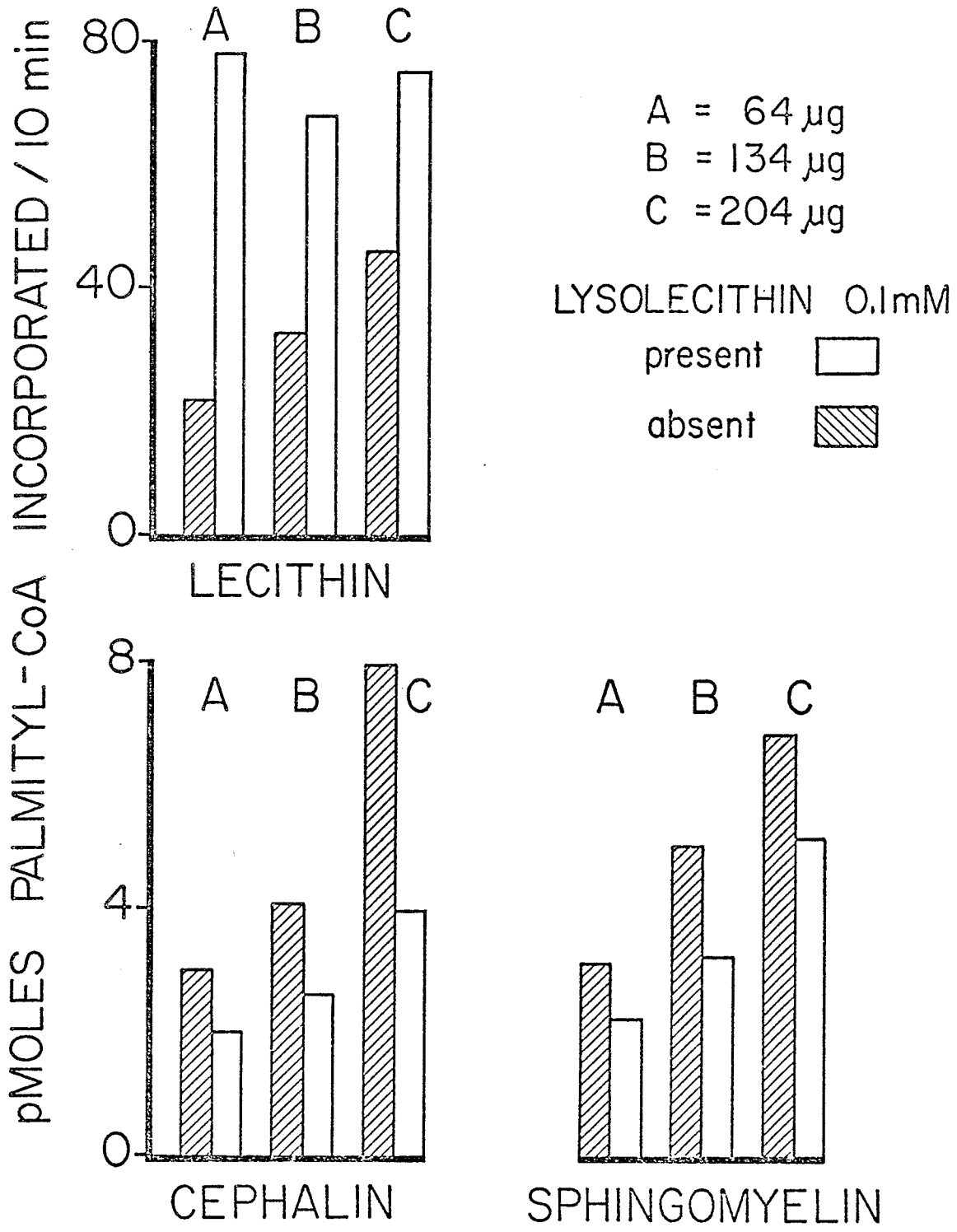



Figure 3. Incorporation of (1-$^{14}$C)-palmitoyl CoA into phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (Sph.). The incubation medium contained 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM lysolecithin whenever present, 1.5 µM palmitoyl CoA (101,010 dpm/nmole) and different concentrations of plasma membrane ($F_3$) protein in a final volume of 0.1 ml. The incubation was carried out for 10 min at 37°C.

(ii) Time of Incubation

In two earlier studies with isolated liver plasma membranes (165, 170), the incubation of the lipid precursor was carried out for 30 min or longer. In the present study, the incorporation of palmitoyl CoA into phosphatidylcholine and phosphatidylethanolamine by $F_2$ and $F_3$ was complete within 10 min and there was no further change up to 30 min of incubation (Figure 4). Therefore, the incubation was routinely carried out for 10 min. While $F_3$ was more active than $F_2$ in the formation of phosphatidylcholine, $F_3$ incorporated less palmitoyl CoA into phosphatidylethanolamine than $F_2$ (Figure 4).

In $F_2$, the appearance of radioactivity in free fatty acids was slow for the first 5 min, gradually increasing to a maximum at 10 min with no further change up to 30 min (Figure 5). The appearance of label in free fatty acids could result from either the hydrolysis of palmitoyl CoA or phospholipid, or both.

The slow formation of free fatty acids from palmitoyl CoA for the first 5 min of incubation (Figure 5) might suggest that palmitoyl CoA might have been coverted to some intermediate before yielding fatty acids. Stahl and Trams reported the formation of s-acyl pantetheine from palmitoyl CoA when incubated with plasma membranes isolated from either the liver (165), or adipose tissue or electroplax tissue (222) and the formation of s-acyl pantetheine was inversely related to the formation of fatty acids. Further work of these authors has shown that s-acyl pantetheine was an intermediate in the formation of fatty acid from acyl CoA by plasma membranes (223).

(iii) pH

The pH of the medium was varied from 4.0 to 5.0 with MES buffer, from 6.0 to 8.0 with TES buffer and from 8.0 to 10.0 with Tricine buffer. The incorporation of palmitoyl CoA into phosphatidylcholine was maximum between pH 7.0 and 9.0 (Figure 6) and this finding disagrees with Stahl and Trams (165) who reported that the formation of phosphatidylcholine from palmitoyl CoA by liver plasma membranes was greatest at pH 5.6.

(iv) Lysolecithin Concentration

The incorporation of palmitoyl CoA into phosphatidylcholine by $F_2$

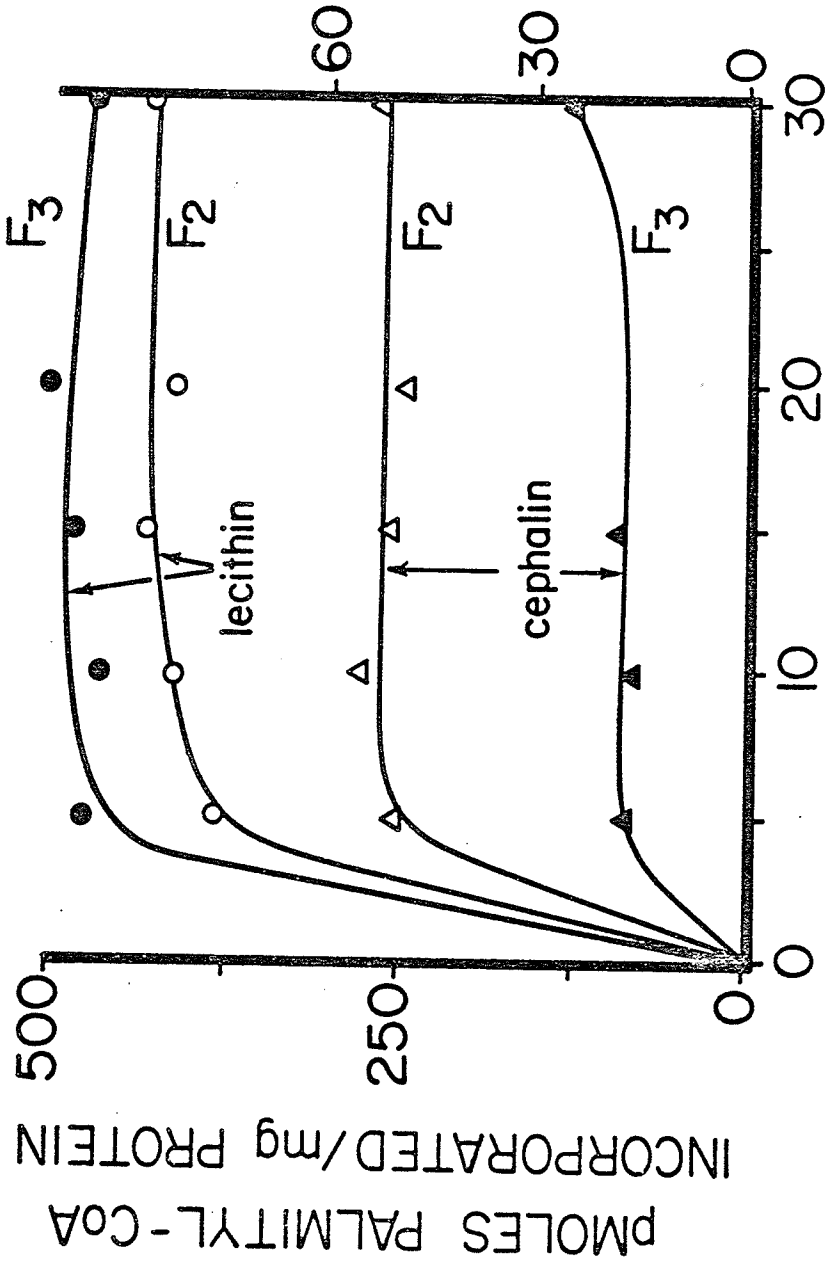



Figure 4. Incorporation of (1-$^{14}$C)-palmitoyl CoA into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by plasma membranes ($F_2$, $F_3$) as a function of incubation time. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM lysolecithin, 1.5 - 1.6 μM (1-$^{14}$C)-palmitoyl CoA (101,010 dpm/nmole) and about 50 μg of protein in a total volume of 0.1 ml. The incubation was carried out for 10 min at 37°C. The lipids were extracted, separated and the radioactivity measured according to the procedure described under Methods.

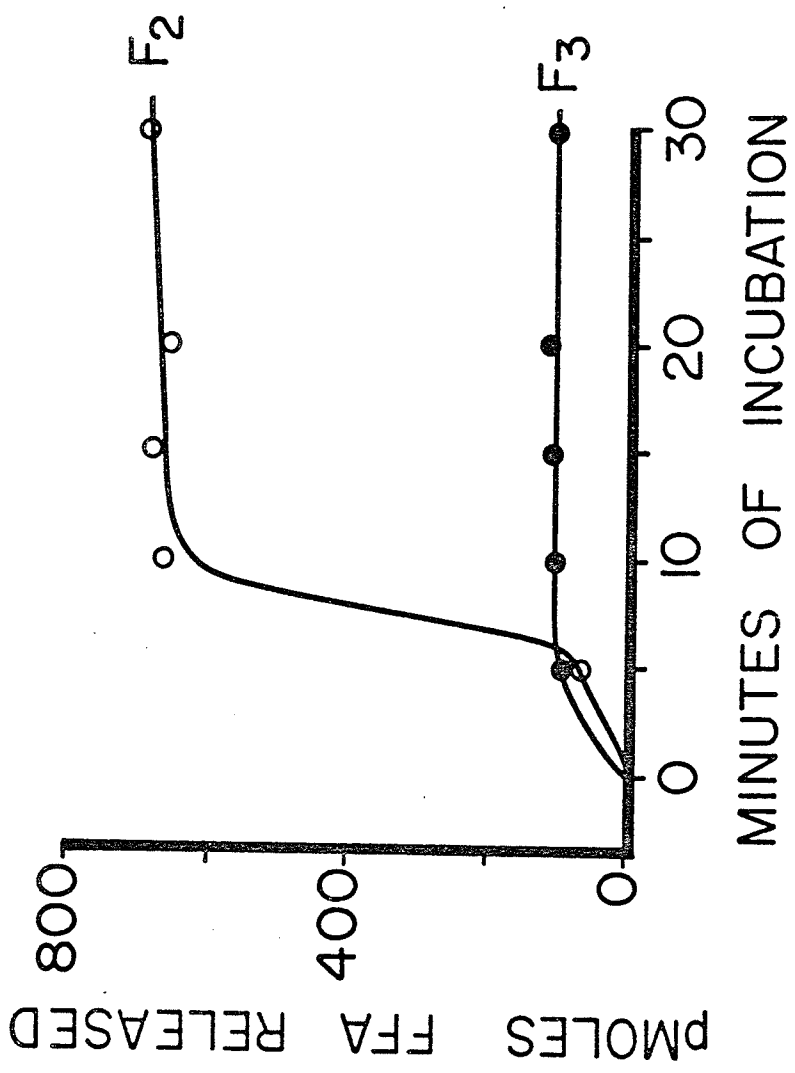



Figure 5. The appearance of free fatty acids in the medium during the incubation of plasma membranes ($F_2$, $F_3$) with (1-$^{14}$C)-palmitoyl CoA in the presence of 0.1 mM lysolecithin. Other incubation conditions and analytical procedures were exactly as described in the legend to Figure 4.

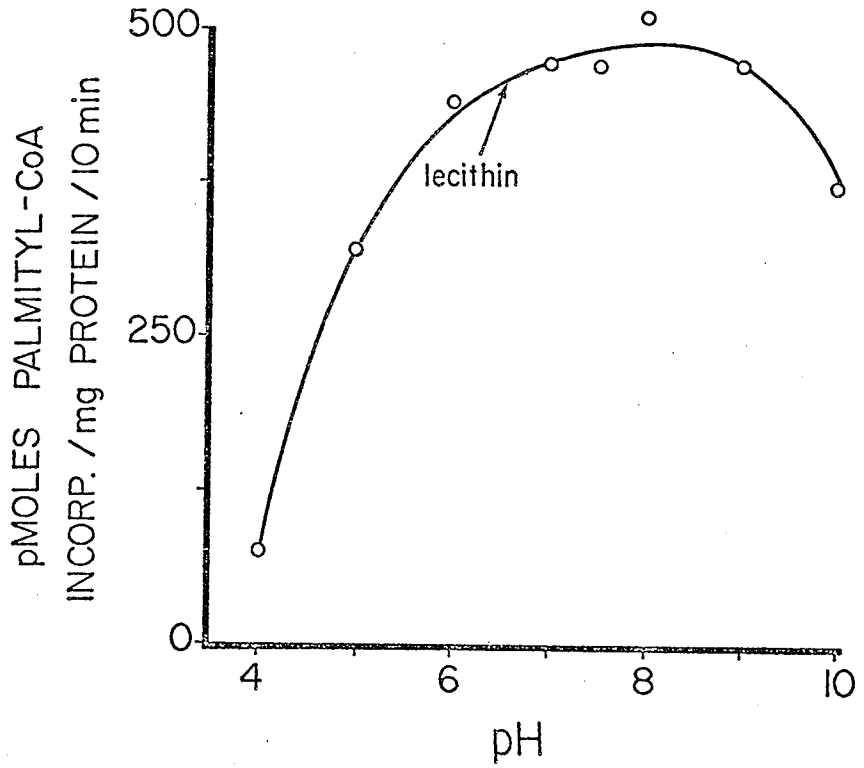



Figure 6. Effect of pH on the incorporation of (1-$^{14}$C)-palmitoyl CoA into phosphatidylcholine (PC) by a plasma membrane ($F_3$) preparation in the presence of 0.1 mM lysolecithin. The pH was varied from 4.0 - 6.0 with MES buffer, 6.0 - 8.0 with TES buffer and from 8.0 - 10.0 with Tricine buffer. Other incubation conditions and analytical procedures were as noted in the legend to Figure 4.

increased with increasing concentrations of lysolecithin up to 1.0 mM. At 4.5 mM, lysolecithin inhibited phosphatidylcholine formation. Phosphatidylcholine formation by $F_3$ reached a maximum at a lower level of lysolecithin, i.e. 0.5 mM (Figure 7).

Lysolecithin probably increases the formation of phosphatidylcholine by providing more substrate for acylation by palmitoyl CoA. The possibility that lysophosphatidylcholine altered the membrane structure by its detergent action so as to enhance acylation activity, seems unlikely as Stein et al. (170) have shown that the treatment of liver plasma membranes with lysophosphatidylcholine (0.05 mM) did not alter the structure of plasma membranes as evidenced by electron microscopy. Further support is given by the unreported results of Pritchard which showed that several detergents inhibited the synthesis of all lipids.

(v) Palmitoyl CoA Concentration

Incorporation into phosphatidylcholine and phosphatidylethanolamine was maximum at 0.1 mM palmitoyl CoA, decreasing at higher concentrations (Figure 8). The formation of triglycerides was optimal at 0.2 mM palmitoyl CoA and was inhibited at higher levels. Similar findings have been reported by Pritchard (15) in a SMSG whole homogenate system.

The inhibitory effect of palmitoyl CoA at high levels could be due to its detergent properties. Pritchard et al. (18) have shown that Lubrol W inhibited the incorporation of palmitoyl CoA into all lipids of different subcellular particulates except the formation of palmitoylcarnitine from carnitine and palmitoyl CoA. It was suggested that the operation of acylcarnitine transferase did not require the presence of intact membranes while the other enzymes concerned with the lipid metabolism did.

(vi) Ions

$Na^+$ (50 mM) and $K^+$ (50 mM), either alone or together did not alter the incorporation of palmitoyl CoA into phosphatidylcholine whereas $Mg^{++}$ (50 mM)

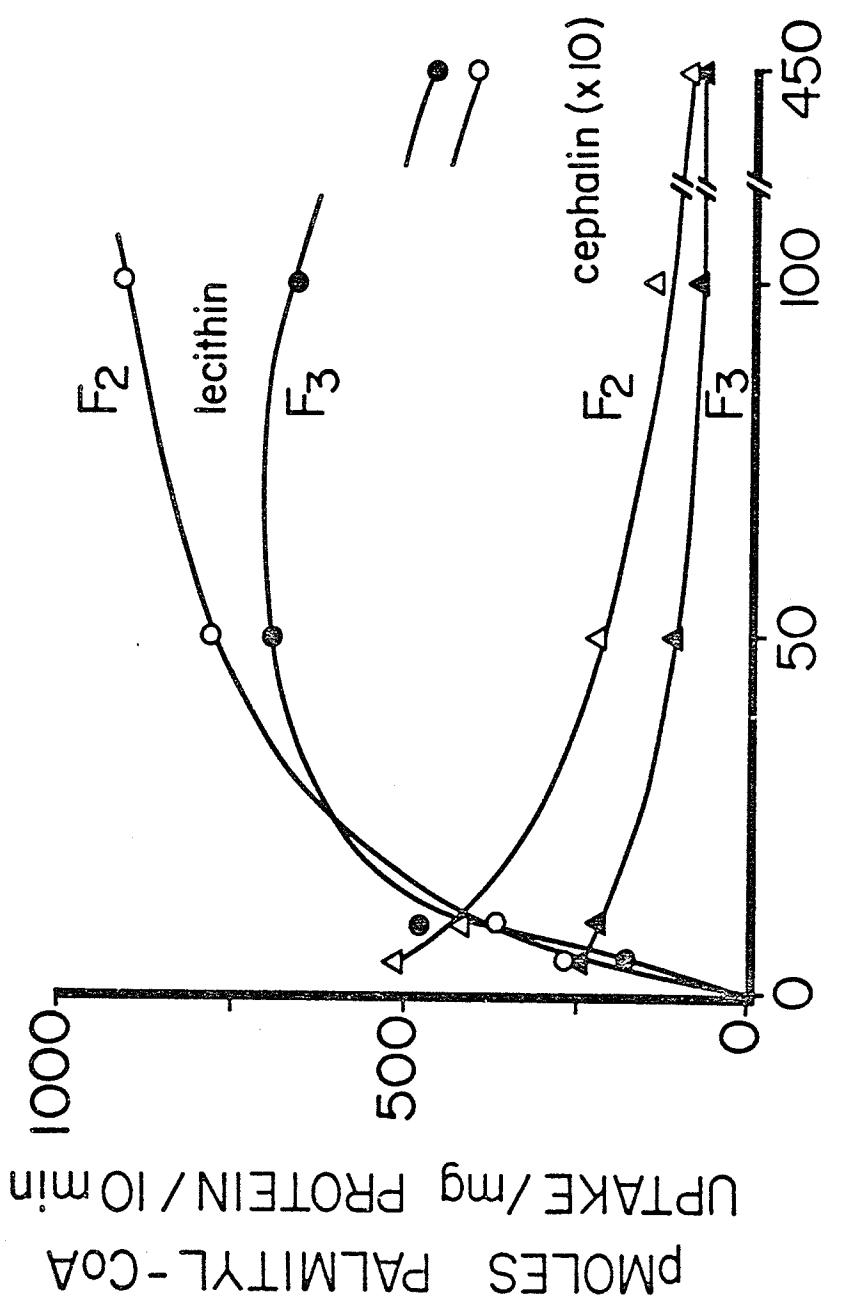



Figure 7. Influence of lysolecithin concentration on the incorporation of (1-$^{14}$C)-palmitoyl CoA into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by plasma membranes ($F_2$, $F_3$). Incubation conditions as described in the legend to Figure 4.

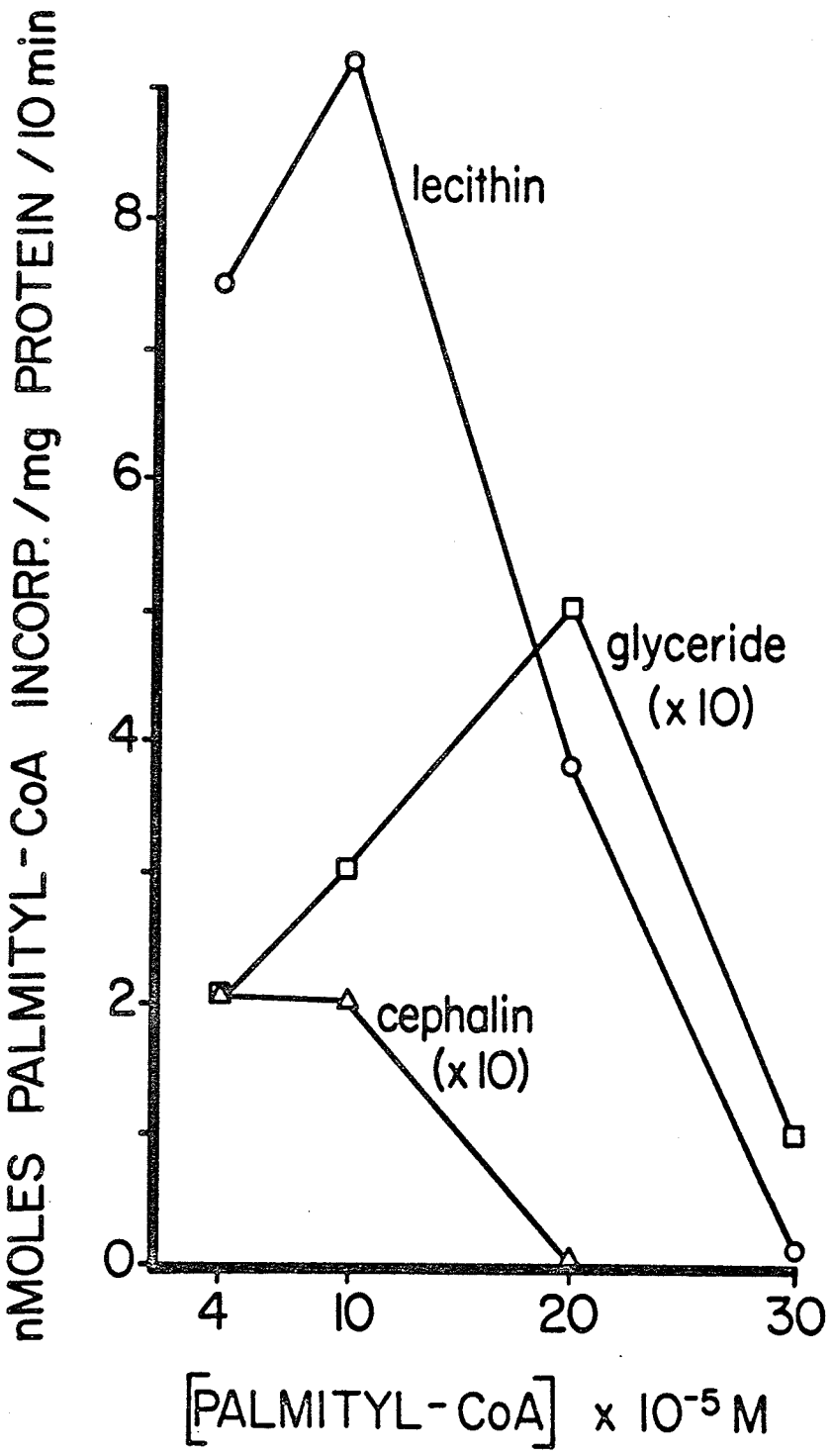



Figure 8. Incorporation of (1-$^{14}$C)-palmitoyl CoA into plasma membrane ($F_2$) lipids in the presence of 0.1 mM lysolecithin as a function of palmitoyl CoA concentration. The incubation conditions and analytical procedures were similar to those described in the legend to Figure 4.

and $Ca^{++}$ (50 mM) decreased the incorporation to 33 and 55% respectively of the control, and $F^-$ (45 mM) was slightly stimulatory (Table XI and Table XII). The incorporation of palmitoyl CoA into sphingomyelin was enhanced by $Na^+$, $K^+$, $Ca^{++}$ and $Mg^{++}$ to 167, 135, 125 and 280% of the control while $F^-$ had no effect (Table XI and Table XII). When $Na^+$ (50 mM) and $K^+$ (50 mM) were present simultaneously, there was a slight further increase in the formation of sphingomyelin (195% of the control) over that which occurred in the presence of each of these ions, however, the effect was not additive. The synthesis of phosphatidylcholine from palmitoyl CoA was uninfluenced by $Na^+$, $K^+$ and $F^-$ but was diminished to 18% of control by $Ca^{++}$, 24% by $Mg^{++}$ (Table XI and Table XII). The formation of triglycerides was reduced to 67% of control by $Na^+$, 38% by $K^+$ and to somewhat less extent, i.e. 85% of control by $Mg^{++}$ and to 65% by $Ca^{++}$. $F^-$ had no effect. The results of experiments, where the effects of increasing concentration of $Na^+$ (Table XIII) and $F^-$ (Table XIV) on the incorporation of palmitoyl CoA into different plasma membrane lipids were studied, were similar to those described above.

These results demonstrate that the effect of ions on the lipogenesis was not specific to the valency of cations as all the cations either inhibited or stimulated the incorporation of palmitoyl CoA into lipids to a variable degree. Yoshida et al. (177) also noted the stimulatory effects of KCl on phospholipogenesis in brain slices from acetate-2-$^{14}C$. However, it was observed that the stimulation of phospholipogenesis by KCl required NaCl in the medium and the effects were evident only in slices and not in the whole homogenate. Since the turnover of phosphatidic acid was most affected, it was suggested that probably this lipid might have a role in the transport of cations in the brain.

The present findings that $F^-$ enhanced the incorporation of palmitoyl CoA into phosphatidylcholine in plasma membranes (Table XI and Table XIV) are consistent with those of Pritchard (17) in a whole homogenate system of rat SMSG. However, they differ from those of Stahl and Trams (165) who reported no effect of NaF (15 mM) on the synthesis of phosphatidylcholine by the liver plasma membranes. It could be that the $F^-$ effect is a characteristic of SMSG and $F^-$

TABLE XI

Effects of Ions and Theophylline on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane Lipids

| | Lipids | | | |
|---|---|---|---|---|
| | Sphingomyelins | Phosphatidyl-choline | Phosphatidyl-ethanolamine | Triglycerides |
| Control | *36.0 (30.0 - 42.0) | 680.9 (673.6 - 688.3) | 80.2 (79.1 - 81.0) | 46.6 (44.5 - 48.8) |
| NaCl (50 mM) | 60.5 | 675.0 (649.0 - 700.9) | 87.0 (84.5 - 90.5) | 31.1 (27.8 - 34.4) |
| KCl (50 mM) | 48.0 (43.1 - 52.9) | 701.9 (677.4 - 726.2) | 88.6 (85.4 - 91.6) | 17.7 (12.8 - 22.6) |
| NaCl (50 mM) + KCl (50 mM) | 70.1 (67.6 - 72.5) | 664.6 (651.8 - 676.9) | 82.4 (78.0 - 86.5) | 14.7 (0.0 - 14.7) |
| NaF (45 mM) | 43.6 (32.7 - 54.5) | 747.5 (712.0 - 782.9) | 85.4 (83.7 - 87.0) | 42.8 (41.5 - 44.5) |
| Theophylline (5 mM) + ATP (2 mM) | 147.0 | 533.1 | 73.1 | 49.1 |

*nanomoles of palmitoyl CoA incorporated /mg PM protein /10 min

The numbers in parenthesis show the range of values.

The incubation conditions and analytical procedures were similar to those described in the legend to Table XII.

TABLE XII

Effects of Ions and Deoxycholate on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane Lipids

| Conditions | Lipids | | | |
|---|---|---|---|---|
| | Sphingomyelins | Phosphatidyl-choline | Phosphatidyl-ethanolamine | Triglycerides |
| Control + LYSO-PC | *75.9 | 382.4 | 142.4 | 42.6 |
| $MgCl_2$ (50 mM) | 211.6 | 124.0 | 34.3 | 36.1 |
| $CaCl_2$ (50 mM) | 94.5 | 209.4 | 25.6 | 27.6 |
| DOC (.05%) | 78.7 | 345.9 | 149.3 | 26.6 |

*nmoles palmitoyl CoA incorporated/mg protein/10 min

The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.4), 1.5 - 1.6 μM (1-$^{14}$C)-palmitoyl CoA (101,010 dpm/nmole), 25 - 50 μg of plasma membrane protein and an effector to a final concentration as shown in the table. The incubation was carried out for 10 min at 37°C. Lipids were extracted, separated and the radioactivity measured according to the procedure described under Methods of this section.

TABLE XIII

Effects of NaCl on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane ($F_2$) Lipids

| Concentration (mM) | Lipids | | |
|---|---|---|---|
| | Phosphotidyl-choline | Phosphatidyl-ethanolamine | Sphingomyelins |
| 0.0 | *680.9 (673.6 - 688.3) | 80.2 (79.1 - 81.0) | 36.0 (30.0 - 42.0) |
| 12.5 | 715.6 (707.1 - 728.8) | 92.2 (77.7 - 106.4) | 48.5 (40.9 - 55.9) |
| 25.0 | 729.2 (711.5 - 746.9) | 93.8 (87.8 - 99.5) | 51.3 (47.2 - 55.1) |
| 50.0 | 700.9 (649.0 - 700.9) | 90.5 (84.5 - 90.5) | 100.4 (60.5 - 140.2) |
| 75.0 | 688.3 (683.1 - 693.5) | 89.7 (85.1 - 94.1) | 66.3 (64.1 - 68.5) |
| 100.0 | 655.6 (637.3 - 673.6) | 93.0 (83.5 - 102.3) | - |

*nanomoles of palmitoyl CoA incorporated /mg protein /10 min

The numbers in parenthesis show the range of values.

Incubation conditions and analytical procedures were exactly as described in the legend to Table XII.

TABLE XIV

Effects of NaF on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane ($F_2$) Lipids

| Concentration (mM) | Lipids | | |
|---|---|---|---|
| | Phosphatidyl-choline | Phosphatidyl-ethanolamine | Sphingomyelins |
| 0 | *680.9 (673.6 - 688.3) | 80.2 (79.1 - 81.0) | 36.0 (30.0 - 42.0) |
| 5 | 792.2 (772.6 - 811.6) | 90.8 (89.2 - 92.2) | 66.5 (55.6 - 77.2) |
| 10 | 785.4 (781.8 - 788.9) | 84.0 (81.5 - 86.5) | 63.8 (55.9 - 71.7) |
| 15 | 783.2 (771.5 - 794.9) | 88.6 (87.3 - 90.0) | 54.5 (45.3 - 63.8) |
| 20 | 751.6 (730.8 - 772.0) | 84.8 (81.3 - 88.1) | 57.8 (45.3 - 70.1) |
| 30 | 773.7 (758.7 - 789.2) | 87.0 (85.6 - 88.4) | 45.3 (43.6 - 46.6) |
| 40 | 747.5 (712.0 - 782.9) | 85.4 (83.7 - 87.0) | 43.6 (32.7 - 54.5) |

*nanomoles of palmitoyl CoA incorporated /mg PM protein /10 min

The numbers in parenthesis show the range of values.

Other incubation conditions and analytical procedures were similar to those described in the legend to Table XII.

might have inhibited the breakdown of phosphatidylcholine by lipases in the plasma membranes. The stimulatory effect of NaF could not be due to the $Na^+$ component since $Na^+$ itself had a little or no effect on the formation of phosphatidylcholine (Table XI and Table XIII).

(vii) Deoxycholate

The incorporation of palmitoyl CoA into plasma membrane lipids was not altered by 0.05% deoxycholate (Table XII). Stahl and Trams (165) observed complete inhibition of phosphatidylcholine synthesis by deoxycholate in isolated liver plasma membranes, however, these authors used deoxycholate concentration 10 times higher than that in the present investigation.

The results of the present study show that phosphatidylethanolamine received less label than phosphatidylcholine from (1-$^{14}$C)-palmitoyl CoA and were consistent with the observations of Stahl and Trams (165) with liver plasma membrane. These results were also consistent with the recent findings of Wright and Green (224) with liver parenchymal cells in vitro. These authors found that after incubating the cells, with $^{14}$C-palmitic acid, most of the incorporation of palmitic acid occurred into phosphatidylcholine which was recovered in the plasma membrane fraction. Their in vivo studies also showed that after 1 min of $^{14}$C-palmitic acid infusion into the femoral vein of rat, radioactive phosphatidylcholine concentrated in the plasma membrane fraction prepared from liver but the proportion of radioactivity present in this fraction declined rapidly indicating the very rapid turnover of the acyl moieties of phosphatidylcholine in plasma membranes. It was also found that maximum incorporation of palmitic acid into phosphatidylcholine occurred before the peak incorporation into phosphatidic acid or diglyceride. It was proposed that the rapid formation of phospholipids in the plasma membranes is by acylation of their lysoderivatives.

The results of preliminary experiments (Table XV) showed that both

TABLE XV

Effects of Secretagogues on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane Lipids ($F_2$)

| | Lipids | | | |
|---|---|---|---|---|
| | Sphingomyelins | Phosphatidyl-choline | Phosphatidyl-ethanolamine | Triglycerides |
| Control | *55.3 (25.7 - 84.9) | 523.0 (480.3 - 565.7) | 175.0 (159.5 - 190.5) | 151.5 (132.2 - 170.7) |
| ATP (2 mM) | 42.6 (33.3 - 51.9) | 538.2 (526.0 - 550.3) | 130.2 (126.0 - 134.3) | 10.0 (6.5 - 13.4) |
| Epinephrine (1 mM) | 35.1 (16.1 - 54.0) | 425.6 (406.6 - 444.5) | 165.4 (151.3 - 179.5) | 77.1 (70.2 - 84.0) |
| ATP (2 mM) + Epinephrine (1 mM) | 51.9 | 385.0 (361.2 - 408.7) | 143.0 (133.4 - 152.6) | 66.1 (63.3 - 68.8) |
| Eserine (1.5 mM) | 131.8 (104.9 - 158.6) | 392.8 (365.6 - 419.9) | 101.6 (68.2 - 135.0) | 57.5 (43.7 - 71.2) |
| Acetylcholine (1.5 mM) + Eserine (1.5 mM) | 179.3 (105.6 - 252.9) | 486.5 (451.6 - 521.4) | 129.5 (122.6 - 136.3) | 46.9 (45.2 - 48.5) |

*nanomoles of palmitoyl CoA incorporated /mg PM protein /10 min

The numbers in parenthesis show the range of values.

Other incubation conditions and analytical procedures were exactly as noted in the legend to Table XII.

epinephrine (1 mM), and acetylcholine (1.5 mM) in the presence of eserine (1.5 mM) slightly decreased the incorporation of palmitoyl CoA into phosphatidylcholine to 80 and 90% of the control respectively whereas the incorporation of this precursor into sphingomyelin was increased to 325% of the control by acetylcholine. The incorporation of palmitoyl CoA into phosphatidylethanolamine was not affected by epinephrine but was reduced to 74% of the control by acetylcholine.

Further experiments were then carried out with several concentrations of these agents. The results are shown in Table XVI a, b, c and were consistent with the preliminary observations (Table XV) in that the incorporation of palmitoyl CoA into phosphatidylcholine was decreased as the level of epinephrine and acetylcholine was increased (Table XVI a). The incorporation of this precursor into sphingomyelin was increased by acetylcholine but was unaffected by epinephrine (Table XVI c). The incorporation into phosphatidylethanolamine was little altered by either epinephrine or acetylcholine (Table XVI b).

The findings that epinephrine and acetylcholine decreased the incorporation of palmitoyl CoA into phosphatidylcholine of plasma membranes were similar to those reported by other authors for SMSG slices (15), nerve endings (180), adrenal medulla (127) and for liver slices (179).

In other studies with isolated rabbit pancreas (212) and with parotid gland slices (115, 225), it has been demonstrated that cyclic 3',5'-adenosine monophosphate was an intermediate in the process of secretion induced by epinephrine (115, 225) and pancreozymin (212). The effects of several concentrations of cyclic 3',5'-adenosine monophosphate on the incorporation of palmitoyl CoA into plasma membrane lipids were examined.

Cyclic 3',5'-adenosine monophosphate affected the incorporation of palmitoyl CoA into plasma membrane lipids (Table XVI a, b, c) in a similar manner to epinephrine or acetylcholine. Cyclic 3',5'-adenosine monophosphate

TABLE XVI a

Effects of Secretagogues on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane Phosphatidyl Choline ($F_2$)

| Concentration (mM) | Secretagogues | | |
|---|---|---|---|
| | Dibutyryl-cyclic AMP | Epinephrine | Acetylcholine + Eserine (1.5 mM) |
| 0.0 | *680.9[1] (673.6 - 688.3) | 523.0[2] (480.3 - 565.7) | 523.0[2] (480.3 - 565.7) |
| 0.5 | 649.2 (649.0 - 649.3) | 482.4 (480.3 - 484.4) | - |
| 1.0 | 638.9 (635.7 - 641.7) | 425.6 (406.6 - 444.5) | - |
| 1.5 | - | - | 486.5 (451.6 - 521.4) |
| 2.0 | 569.7 (555.2 - 582.2) | - | - |
| 3.0 | 501.5 (498.0 - 504.8) | 357.4 (304.7 - 410.1) | 400.7 (361.4 - 439.9) |
| 4.5 | 440.1 (435.8 - 444.2) | - | - |

[1, 2] indicate PM preparation

*nanomoles of palmitoyl CoA incorporated /mg protein /10 min

The numbers in parenthesis show the range of values.

Other incubation conditions and analytical procedures were the same as described in the legend to Table XII.

TABLE XVI b

Effects of Secretagogues on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane Phosphatidyl Ethanolamine ($F_2$)

| Concentration (mM) | Secretagogues | | |
|---|---|---|---|
| | Dibutyryl-cyclic AMP | Epinephrine | Acetylcholine + Eserine (1.5 mM) |
| 0.0 | *80.2[1] (79.1 - 81.0) | 175.0[2] (159.5 - 190.5) | 175.0[2] (159.5 - 190.5) |
| 0.5 | 80.7 (76.9 - 84.3) | 125.1 (120.3 - 129.9) | 227.2 |
| 1.0 | 89.2 (86.5 - 91.9) | 165.4 (151.3 - 179.5) | - |
| 1.5 | - | - | 129.5 (122.6 - 136.3) |
| 2.0 | 82.6 (80.7 - 84.5) | - | - |
| 3.0 | 85.6 (85.1 - 86.2) | 134.4 (120.3 - 148.5) | 68.5 (61.3 - 75.7) |
| 4.5 | 68.7 (65.2 - 72.0) | - | - |

[1, 2] indicate PM preparation

*nanomoles of palmitoyl CoA incorporated /mg PM protein /10 min

The numbers in parenthesis show the range of values.

Other incubation conditions and analytical procedures were the same as described in the legend to Table XII.

TABLE XVI c

Effects of Secretagogues on the Incorporation of (1-$^{14}$C)-palmitoyl CoA into Plasma Membrane Sphingomyelins ($F_2$)

| Concentration (mM) | Secretagogues | | |
|---|---|---|---|
| | Dibutyryl-cyclic AMP | Epinephrine | Acetylcholine + Eserine (1.5 mM) |
| 0.0 | *36.0[1] (30.0 - 42.0) | 55.3[2] (25.7 - 84.9) | 55.3[2] (25.7 - 84.9) |
| 0.5 | 37.4 (35.5 - 39.0) | 76.4 (64.3 - 88.4) | 317.7 |
| 1.0 | 51.8 (50.2 - 53.2) | 35.1 (16.1 - 54.0) | - |
| 1.5 | - | - | 179.3 (105.6 - 252.9) |
| 2.0 | 49.4 (43.4 - 55.4) | - | - |
| 3.0 | 56.2 (51.8 - 60.3) | 47.8 (38.1 - 57.4) | 153.8 (117.0 - 190.0) |
| 4.5 | 53.5 (53.5 - 55.4) | - | - |

[1], [2] indicate PM preparation

*nanomoles of palmitoyl CoA incorporated /mg PM protein /10 min at 37°C

The numbers in parenthesis show the range of values.

Other incubation conditions and analytical procedures were the same as described in the legend to Table XII.

inhibited the incorporation of palmitoyl CoA into phosphatidylcholine more strongly than epinephrine. Theophylline (5 mM) which prevents the degradation of cyclic 3',5'-adenosine monophosphate by cyclic 3',5'-adenosine monophosphate phosphodiesterase, produced effects similar to cyclic 3',5'- adenosine monophosphate or epinephrine (Table XI).

4. Discussion

The mechanism by which epinephrine and acetylcholine affect lipogenesis in plasma membranes from palmitoyl CoA is not clear because of the complex nature of the system including the presence of many enzymes competing for the same substrate (palmitoyl CoA). However, some possibilities may be considered.

All results of this laboratory strongly suggest that the incorporation of palmitic acid into lipids occurred from a common pool of palmitoyl CoA. An agent which could inhibit one or more enzymes leading to the synthesis of one lipid would likely increase the available palmitoyl CoA for the formation of other lipids. Many of the effects of stimulants of secretion tested in the present study could be explained on this basis since the total incorporation of palmitoyl CoA into various lipids was not significantly altered by any one of these agents.

Previously, Pritchard (17) noted that epinephrine and acetylcholine decreased the incorporation of palmitate into triglycerides and increased $CO_2$ production in slices of rat SMSG and suggested that these agents might have activated a lipase hydrolyzing triglyceride to free fatty acids which were subsequently oxidized. Similarly, it is possible in this study that these agents might have activated nonspecific lipases in plasma membranes which could deacylate phosphatidylcholine to produce lysophosphatidylcholine and free fatty acids. However, in view of the findings that lysophosphatidylcholine enhanced the synthesis of phosphatidylcholine from palmitoyl CoA (Table X), enhanced synthesis of phosphatidylcholine should occur. No such increase in the formation of phosphatidylcholine was observed.

Epinephrine activates adenyl cyclase in a variety of tissues subsequently elevating the tissue level of cyclic 3',5'-adenosine monophosphate (178). The finding that plasma membranes had adenyl cyclase activity (Table I) and that the effects of epinephrine, cyclic 3',5'-adenosine monophosphate and theophylline on the synthesis of phosphatidylcholine were similar suggest that epinephrine might have acted through the adenyl cyclase system in plasma membranes. The mechanism by which cyclic 3',5'-adenosine monophosphate influences plasma membrane lipid metabolism remains unknown.

## B. PROPERTIES OF PLASMA MEMBRANE ENZYMES

### 1. 5'-Nucleotidase

#### a) Introduction

It is generally accepted that 5'-nucleotidase activy is predominently found in the outer cell membranes (41 -43, 55, 133, 135). The function(s) of this enzyme in relation to plasma membrane metabolism is not yet known. Franklin and Trams (223) reported that 5'-nucleotidase is one of the enzymes concerned with the metabolism of nucleotides in liver plasma membranes. These authors suggested that since adenine nucleotides are involved in maintaining membrane structure, function and permeability, 5'-nucleotidase might be a regulatory enzyme. Similar function(s) for this enzyme were proposed by other investigators in brain (246) and in Acanthamoeba plasma membranes (188). However, no direct evidence is yet available to either substantiate or reject this hypothesis. In the present study, some of the properties of 5'-nucleotidase of SMSG plasma membranes ($F_3$) were studied. The effects of cyclic 3',5'-adenosine monophosphate, a stimulant of salivary (115, 225) and pancreatic (212) secretion; ouabain, an inhibitor of cation transport in a variety of tissues (130) and NaF, both a metabolic inhibitor (214, 215, 243) and activator of adenyl cyclase (163), on 5'-nucleotidase activity were determined.

#### b) Methods

Plasma membranes in $F_3$ were used to study the properties of 5'-nucleotidase. This fraction was chosen for this study because of the higher yield of plasma membrane protein. In the earlier experiments (Table I) the activity of 5'-nucleotidase was 29.8 $\pm$ 4.4 µmoles of Pi released/mg plasma membrane protein/h which was 24 fold greater than activity in the whole homogenate.

The analytical techniques and assay conditions have been fully described in Section II, 2.a) page 24. Any change in the incubation conditions are given

in legends to appropriate tables and graphs.

c) Results

Effects of incubation conditions on the activity of 5'-nucleotidase:

(i) Time of incubation and protein concentration

The rate of Pi liberation from adenosine-5'-monophosphate (AMP) was linear up to 30 min (Figure 9 a) and the activity was directly proportional to the concentration of protein up to 150 µg/ml (Figure 9 b).

(ii) Adenosine-5'-monophosphate concentration

The activity was highest between 1.5 and 2.0 mM AMP. Higher levels of AMP decreased the activity of 5'-nucleotidase (Figure 11 a).

(iii) KCl concentration

No change in activity was observed by the addition of KCl up to 200 mM (Figure 10 a).

The assay mixture described by Michell and Hawthorne (148) for estimating the activity of 5'-nucleotidase contained 5 mM AMP and 100 mM KCl. However, the present results show that AMP at this level inhibited the enzyme activity (Figure 11 a), and KCl had no effect upon the activity of this enzyme over a wide range of concentrations (Figure 10 a). In the present study 2 mM AMP was used in the assay mixture but KCl was retained at 100 mM so that the results of this study could be compared with published data.

(iv) $MgCl_2$ concentration

5'-nucleotidase exhibited an activity of 30 µmoles of Pi released/mg plasma membrane protein/h in the absence of $MgCl_2$ which was increased by $MgCl_2$ reaching a maximum (55 µmoles Pi/mg/h) at about 10 mM $Mg^{++}$ (Figure 10 b). This was about a 50% increase in activity. Further increasing the level of $MgCl_2$ to 50 mM did not change the activity.

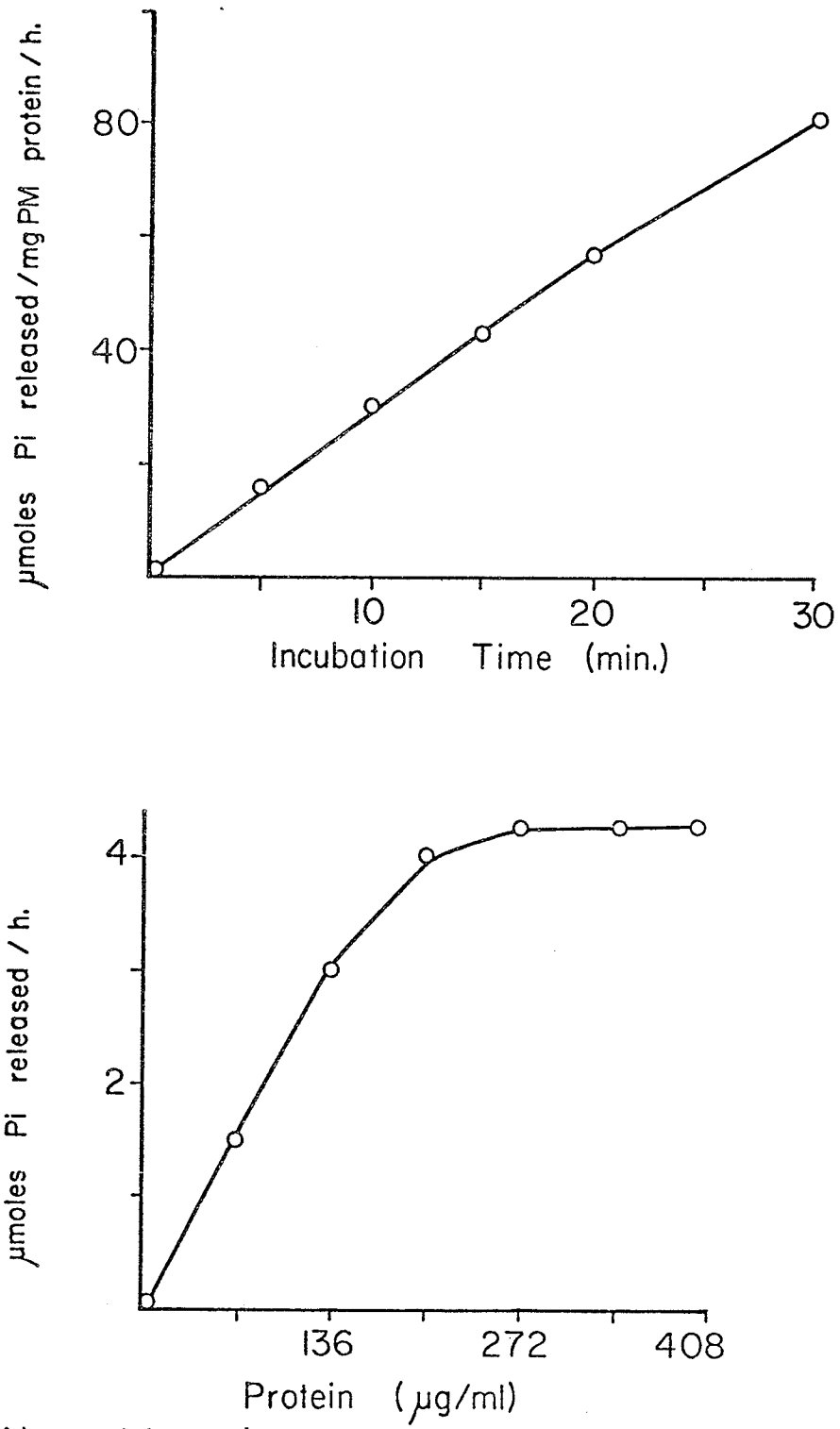



Figure 9. 5'-Nucleotidase activity of plasma membranes as a function of incubation time (a) and protein concentration (b). The incubation mixture contained 50 mM Tris-HCl buffer (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 10 mM sodium potassium tartrate 2 mM AMP and about 50 µg of plasma membrane protein in a final volume of 0.5 ml. The reaction was carried out for 15 min at 37°C. Liberated Pi was analysed on a Technicon Autoanalyzer by a slightly modified procedure of Fisk and Subbarow (149) and protein was measured according to the procedure of Lowry et al. (156).

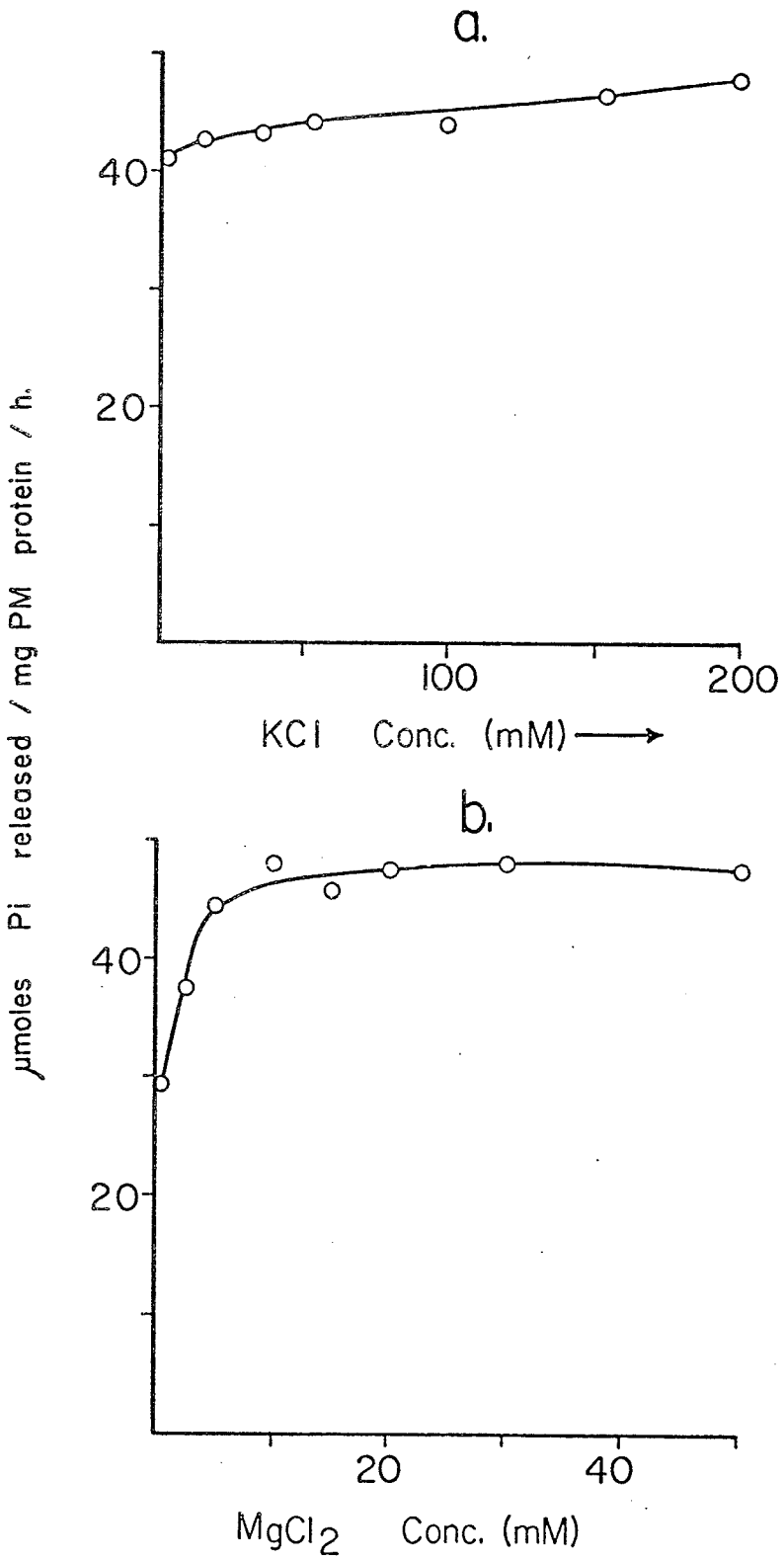



Figure 10. Influence of KCl (a) and $MgCl_2$ (b) on 5'-nucleotidase activity of plasma membranes. The incubation conditions and analytical procedures were exactly as described in the legend of Figure 9.

The finding that 5'-nucleotidase exhibited activity in the absence of $MgCl_2$ did not agree with reports on 5'-nucleotidase of cerebral cortical tissue (181) or rat liver (186) as the enzyme prepared from both these sources exhibited complete dependence on the presence of $Mg^{++}$. It is possible that $F_3$, as prepared in this laboratory, contained a small amount of endogenous $Mg^{++}$. It may also well be that the SMSG enzyme was not entirely dependent upon $MgCl_2$. 5'-nucleotidase activity was maximum at 10 mM $Mg^{++}$, a finding consistent with the report of Bosmann and Pike (181) for cerebral cortical tissue. However, the enzyme prepared from rat heart showed an inhibition by low levels of $MgCl_2$ (182) as compared to the results of present investigation.

(v) Effect of pH

The activity of 5'-nucleotidase was low at pH 4.0 and, as the pH was increased, the activity increased reaching a maximum around pH 7.5. Further increasing the pH up to 10.0 did not alter the activity (Figure 11 b).

The pH optimum for 5'-nucleotidase of rat cerebellum was 6.8 (181) and of chicken liver 6.5 (186). It varied from 6.5 - 7.5 in sheep brain (187). While Edwards and Maguire (182) reported a pH optimum of 7.5 with the enzyme preparation from rat heart, Baer et al. (183) noted the maximum of 5'-nucleotidase activity at pH 9.2 in the same tissue. The discrepancies in these various results has been attributed to the variation in level of $Mg^{++}$ in the incubation mixture of 5'-nucleotidase assay by Baer et al. (183). Song and Bodansky (185) have shown that in the absence of $Mg^{++}$, 5'-nucleotidase activity of liver plasma membranes was optimum at pH 7.5. However, when the incubation mixture contained $Mg^{++}$, there was an additional pH optimum between 9.1 - 9.3. In the present study, $MgCl_2$ was included in the assay mixture and this could be one of the reasons why the activity of 5'-nucleotidase was maximum over a wide range of pH, e.g. 7.5 - 10.0. $Mg^{++}$-free media were not tested.

(vi) Substrate specificity of 5'-nucleotidase

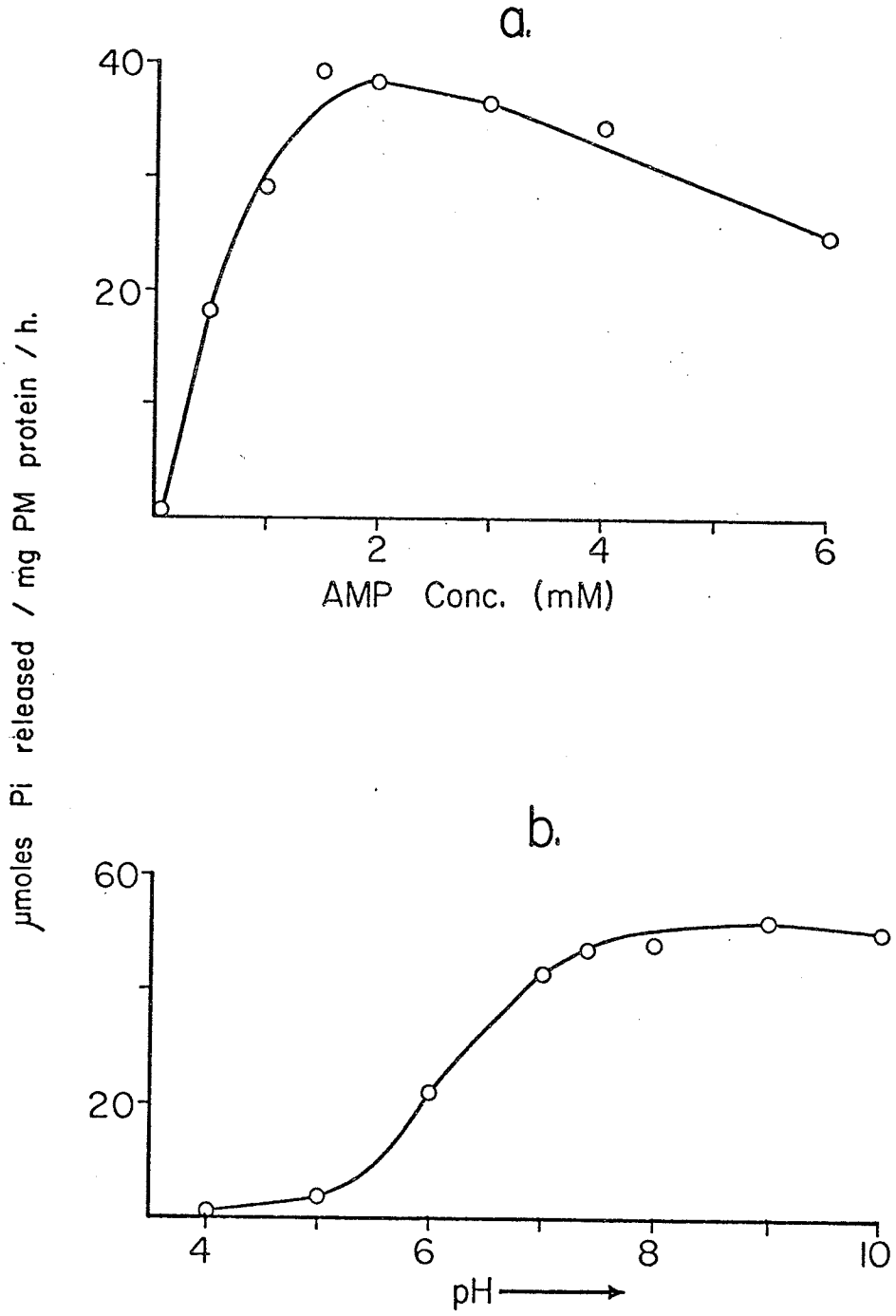



Figure 11. Effect of AMP concentration (a) and pH (b) on the activity of 5'-nucleotidase of plasma membranes. The pH of the incubation mixture was varied from 4.0 - 6.0 with MES buffer, 6.0 - 8.0 with TES buffer and 8.0 - 10.0 with Tricine buffer. Other incubation conditions and analytical procedures were similar to those described in the legend to Figure 9.

As shown in Table XVII, the enzyme hydrolysed UMP, AMP, CMP, GMP, and IMP at different rates. While UMP and AMP were hydrolysed at almost equal rates, CMP, GMP and IMP were hydrolysed much more slowly. The apparent Michaelis constants for AMP, UMP, GMP, CMP were 4,000, 4,000, 2,340 and 1,370 μM respectively. These values were different from those reported by Bosmann and Pike (181) for 5'-nucleotidase of cerebral cortex, and are most probably due to the degree of purification of these preparations. The enzyme preparation of Bosmann and Pike (181) was highly purified.

(vii) Effects of ouabain, cyclic 3',5'-adenosine monophosphate and NaF

The results in Table XVIII show that ouabain (1 mM) and cyclic 3',5'-adenosine monophosphate (2 mM) had no influence upon 5'-nucleotidase activity. However, NaF (10 mM) completely inhibited the activity which is consistent with the earlier findings of other investigators with preparations from cerebral cortex (181) and chicken liver (186).

d) Discussion

The fact that cyclic 3',5'-adenosine monophosphate and ouabain did not modify the activity of 5'-nucleotidase demonstrates that although the preparation of 5'-nucleotidase was rather crude in the present study, it did not exhibit any ($Na^+ - K^+$)-ATPase activity which is very strongly inhibited by both cyclic 3',5'-adenosine monophosphate and ouabain. A further discussion of ATPase will be found in the following section.

It has been demonstrated that the stimulatory effect of epinephrine (115, 225) and pancreozymin (212) on the exocrine secretion of proteins is mediated through cyclic 3',5'-adenosine monophosphate. In the present investigation, cyclic 3',5'-adenosine monophosphate did not influence 5'-nucleotidase activity in isolated plasma membranes nor did added $F^-$, a stimulator of adenyl cyclase, suggesting that the 5'-nucleotidase activity of plasma

TABLE XVII

Specificity of Plasma Membrane 5'-Nucleotidase Activity for Substrate

| Concentration (mM) | Substrates | | | | |
|---|---|---|---|---|---|
| | AMP | CMP | GMP | IMP | UMP |
| 0.25 | *12.2 (12.2 - 12.2) | 13.6 (13.6 - 13.6) | 12.3 (11.9 - 12.6) | 14.4 (14.0 - 14.7) | 14.4 (14.0 - 14.7) |
| 0.50 | 24.8 (24.8 - 24.8) | 25.3 24.4 - 26.2) | 20.6 (20.2 - 20.9) | 22.3 (22.3 - 22.3) | 26.2 (25.8 - 26.5) |
| 0.75 | 37.2 (36.3 - 38.0) | 34.9 (34.9 - 34.9) | 22.2 (21.6 - 22.7) | 23.1 (22.0 - 24.1) | 40.1 (38.4 - 41.8) |
| 1.00 | 42.7 (41.8 - 43.6) | 39.5 (38.4 - 40.5) | 23.0 (23.0 - 23.0) | 23.8 (23.4 - 24.1) | 48.5 (47.8 - 49.2) |
| 1.50 | 43.6 (40.1 - 47.1) | 43.1 (42.5 - 53.6) | 23.9 (23.4 - 24.4) | 24.3 (24.1 - 24.4) | 52.5 (52.3 - 52.7) |
| 2.00 | 47.1 (46.0 - 48.1) | 46.6 (46.0 - 47.1) | 25.1 (24.4 - 25.8) | 24.1 (23.0 - 25.1) | 55.8 (54.1 - 17.5) |

*μmoles of Pi released/mg protein/h

Incubation conditions and analytical procedures were similar to those described in the legend to Figure 9 except that AMP was replaced by other mononucleotides as substrate.

TABLE XVIII

Effects of Agents on Plasma Membrane 5'-Nucleotidase Activity

| AMP Concentration (mM) | Effectors | | | |
|---|---|---|---|---|
| | Control | Ouabain (1 mM) | NaF (10 mM) | cAMP (2 mM) |
| 0.25 | *12.2 (12.2 - 12.2) | 13.7 (13.3 - 14.0) | 3.5 (3.5 - 3.5) | 13.8 (13.6 - 14.0) |
| 0.50 | 24.8 (24.8 - 24.8) | 25.6 (25.1 - 26.2) | 3.5 (3.5 - 3.5) | 26.2 (26.2 - 26.2) |
| 0.75 | 37.2 (36.3 - 38.0) | 37.5 (36.6 - 38.4) | 3.5 (3.5 - 3.5) | 35.4 (34.9 - 35.9) |
| 1.00 | 42.7 (41.8 - 43.6) | 41.0 (40.1 - 41.8) | 3.5 (3.5 - 3.5) | 37.7 (36.6 - 38.7) |
| 1.50 | 43.6 (40.1 - 47.1) | 46.2 (45.7 - 46.7) | 3.5 (3.5 - 3.5) | 42.9 (42.2 - 43.6) |
| 2.00 | 47.1 (46.0 - 48.1) | | | |

*μmoles of Pi released/mg protein/h.

The incubation conditions and analytical procedures were exactly as described in the legend to Figure 9 except that the effectors were added to the noted final concentration.

membranes of rat SMSG might not be directly involved in the process of cyclic 3',5'-adenosine monophosphate mediated secretion. Furthermore, the present finding that 5'-nucleotidase activity was not influenced by ouabain or NaF, inhibitors of cation transport ATPase in many tissues (130) indicates the unlikelihood of this enzyme involved in the transport of cations. These conclusions, however, remain somewhat tentative as the present investigation was not sufficient to produce unequivocal data.

## 2. Sodium-, Potassium-Activated Adenosine Triphosphatase

### a) Introduction

The presence of sodium, potassium-activated adenosine triphosphatase ($(Na^+ - K^+)$-ATPase, E.C. 3.6.1.3.)) activity in both secretory (38, 189, 190) and non-secretory tissues (162, 191) is well known (see also 192). $(Na^+ - K^+)$-ATPase has been implicated in the transport of $Na^+$ and $K^+$ in a variety of tissues (8, 107, 130) and also in the absorption of amino acids and sugars from the small intestine (110). Until recently, most of the work was carried out with preparations from either whole tissue (7, 189, 192, 193) or microsomes (194, 195). However, recent reports (130, 138, 196, 197) indicate that plasma membrane is the site of much, if not all, $(Na^+ - K^+)$-ATPase activity. It would seem unlikely, however, that the enzyme would be exclusive to plasma membranes in all tissues (194, 196, 197). Hence an enzyme prepared from the whole tissue could be heterogenous in its subcellular origin and its overall properties might differ in many respects from the enzyme prepared from one particular subcellular fraction. Recently, the properties of $(Na^+ - K^+)$-ATPase in the plasma membranes of intestinal mucosal cells (198) and of rat myometrium (162) has been reported. In this section, some properties of $(Na^+ - K^+)$-ATPase and $(Mg^{++})$-ATPase extracted from plasma membrane of rat submandibular salivary glands as well as the effects of some secretagogues on this activity are described.

(i) Purification of $(Na^+ - K^+)$-ATPase from plasma membranes

After density gradient centrifugation of 4,000 x g supernatant, three plasma membrane fractions ($F_1$, $F_2$ and $F_3$) exhibiting different levels of $(Na^+ - K^+)$-ATPase and $(Mg^{++})$-ATPase activities were obtained (Table I). $(Na^+ - K^+)$-ATPase activity was highest in $F_1$ while $F_2$ and $F_3$ showed similar activities. As the yield of plasma membranes was highest in $F_3$ of the three plasma membrane fractions, this fraction was utilized to prepare $(Na^+ - K^+)$-ATPase.

Deoxycholate Treatment: The tissue suspension was treated with 9 volumes of 0.1% deoxycholate (w/v) in Tris-EDTA buffer (pH 7.0) and homogenized by 15 - 25 hand-driven strokes in a Potter-Elvejhem homogenizer. The suspension was then centrifuged at 12,000 x g for 15 min. The supernatant was retained and the pellet was rehomogenized in 0.05% deoxycholate (w/v) in Tris-EDTA buffer and centrifuged as before. The supernatants of both spins were pooled andspun at 100,000 x g for 45 min. The resulting pellet was washed once with Tris-EDTA buffer and finally made to an appropriate volume with the same buffer.

Sodium iodide treatment: A portion of the above preparation was treated with 0.5 volumes of ice-cold 6 M sodium iodide solution containing 150 mM Tris-HCl buffer (pH 8.4), 15 mM EDTA and 7.5 mM $MgCl_2$, allowed to stand for 30 min in an ice-bath. It was then diluted 2.5 times the original volume with Tris-EDTA buffer and allowed to stand for a few min. It was then centrifuged at 35,000 x g for 20 min. The resulting pellet was washed twice with the Tris-EDTA buffer before suspending it to an appropriate volume with the same buffer. The final preparation was divided into small portions and frozen at -75°C. Required portions were thawed at 2 to 4°C overnight prior to use.

(ii) Assay of ATPase activity

The assay method used in this study was that described by Schwartz and Moore (9). Total ATPase activity ($Na^+ - K^+ - Mg^{++}$)-ATPase was measured in a mixture containing 50 mM Tris-HCl buffer (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 5 - 10 µg of enzyme protein and 5 mM Tris-ATP, whenever a cation-free medium was required; otherwise, the disodium salt of ATP was used. To estimate the activity of ($Mg^{++}$)-ATPase (ouabain-insensitive ATPase), 1.5 mM ouabain was added to the above medium. Total volume of the reaction mixture was 0.2 ml.

The reactants were pre-equiliberated for 5 min at 37°C before initiating the reaction with either ATP or the enzyme. The reaction was continued for 15 min and stopped by the addition of an equal volume of ice-cold TCA (12.5% final concentration). The amount of Pi released was estimated by the method of Taussky and Shorr (155). Proteins were measured by the procedure of Lowry et al. (156).

The ($Na^+ - K^+$)-ATPase activity was obtained by subtracting the activity of ($Mg^{++}$)-ATPase from the total ATPase activity. Both enzymic activities were corrected for nonenzymic hydrolysis of ATP under similar conditions. The activity of both the enzymes was expressed as µmoles of Pi released/mg enzyme protein/h.

b) Results

The treatment of plasma membranes twice with deoxycholate and once with sodium iodide yielded a preparation of ($Na^+ - K^+$)-ATPase which exhibited an activity of 109 µmoles of Pi released/mg/h and thus showed a purification of about 28 fold over the whole homogenate (Figure 12). The activity of this final preparation was lower than that reported for preparations from adrenal medulla (200) and kidney (201). Schwartz and Moore (9) prepared an enzyme from whole homogenates of rat SMSG which exhibited a specific activity of 137.3 - 300.2 µmoles of Pi released/mg protein/h which was higher than the specific activity

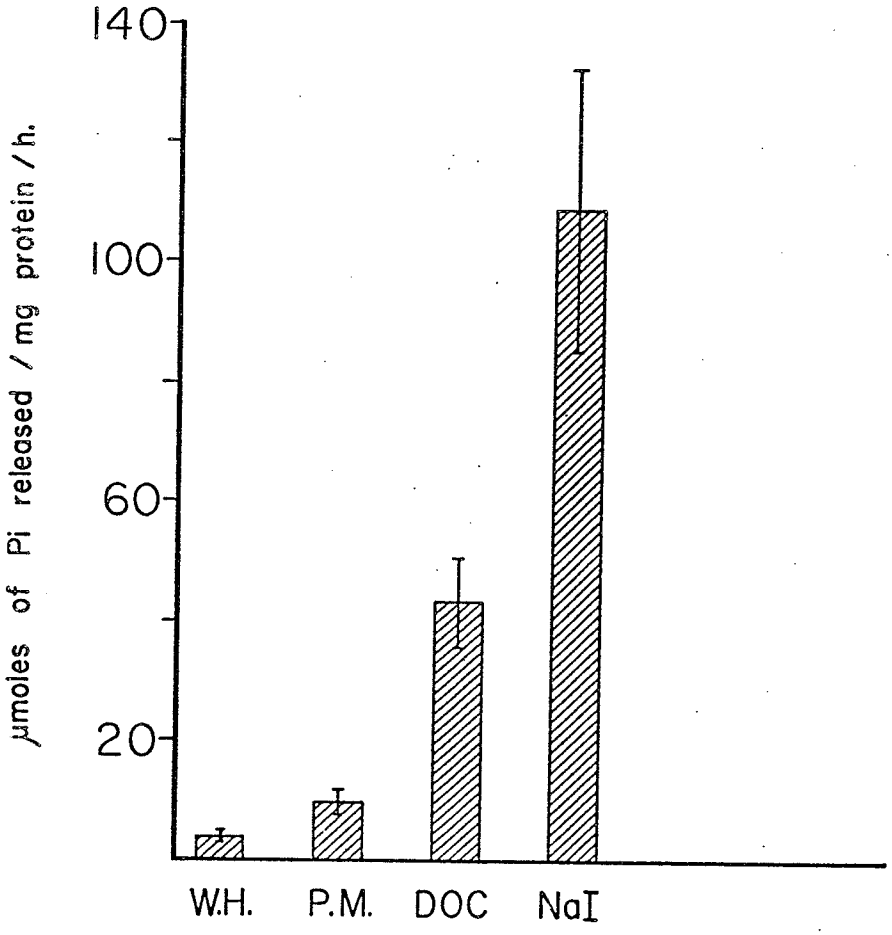



Figure 12. Specific activity of $(Na^+ - K^+)$-ATPase at various stages of purification of the enzyme from plasma membranes ($F_3$). The values represent mean ± the range of values.

of final preparation in this study. The reason for this difference in activity is not clear but it might be that if all the three plasma membrane fractions ($F_1$, $F_2$ and $F_3$) were used to extract the enzyme in the present study, a preparation with activity as high as reported by these authors could have been achieved. It is pertinent to point out that the insoluble residue after the treatment of plasma membranes twice with deoxycholate retained about 16% of the original protein and exhibited a relatively high specific activity of $(Na^+ - K^+)$-ATPase (unreported observations).

(i) Effects of various incubation conditions on the activity of $(Na^+ - K^+)$-ATPase and $(Mg^{++})$-ATPase

Time of Incubation and Protein Concentration: $(Na^+ - K^+)$-ATPase activity increased linearly with the time of incubation up to 15 min and appeared to be reaching a plateau thereafter (Figure 13 a). The activity was also directly proportional to the enzyme concentration in the incubation mixture up to 75 µg/ml and reached a plateau at 175 µg protein/ml (Figure 13 b).

Cations: The activity of $(Na^+ - K^+)$-ATPase was not influenced by $Na^+$ up to 5 mM, however, as the concentration of $Na^+$ was increased further, the activity was stimulated and continued to increase in a hyperbolic manner up to 300 mM $Na^+$ still not having reached a plateau at this concentration (Figure 14).

$(Mg^{++})$-ATPase activity was also stimulated by low levels of $Na^+$ reaching a maximum level at 50 mM $Na^+$. However, unlike $(Na^+ - K^+)$-ATPase, the activity of $(Mg^{++})$-ATPase tended to decrease at higher levels of $Na^+$ and was lowered to 64% of the maximum activity at 300 mM $Na^+$ (Figure 14).

$(Na^+ - K^+)$-ATPase activity was stimulated by low levels of $K^+$ reaching a maximum level at 10 mM KCl. However, higher levels of $K^+$ were inhibitory as the activity was decreased to 43% of the maximum activity at 150 mM KCl (Figure 15).

$K^+$ also stimulated the activity of $(Mg^{++})$-ATPase but the maximal stimulation

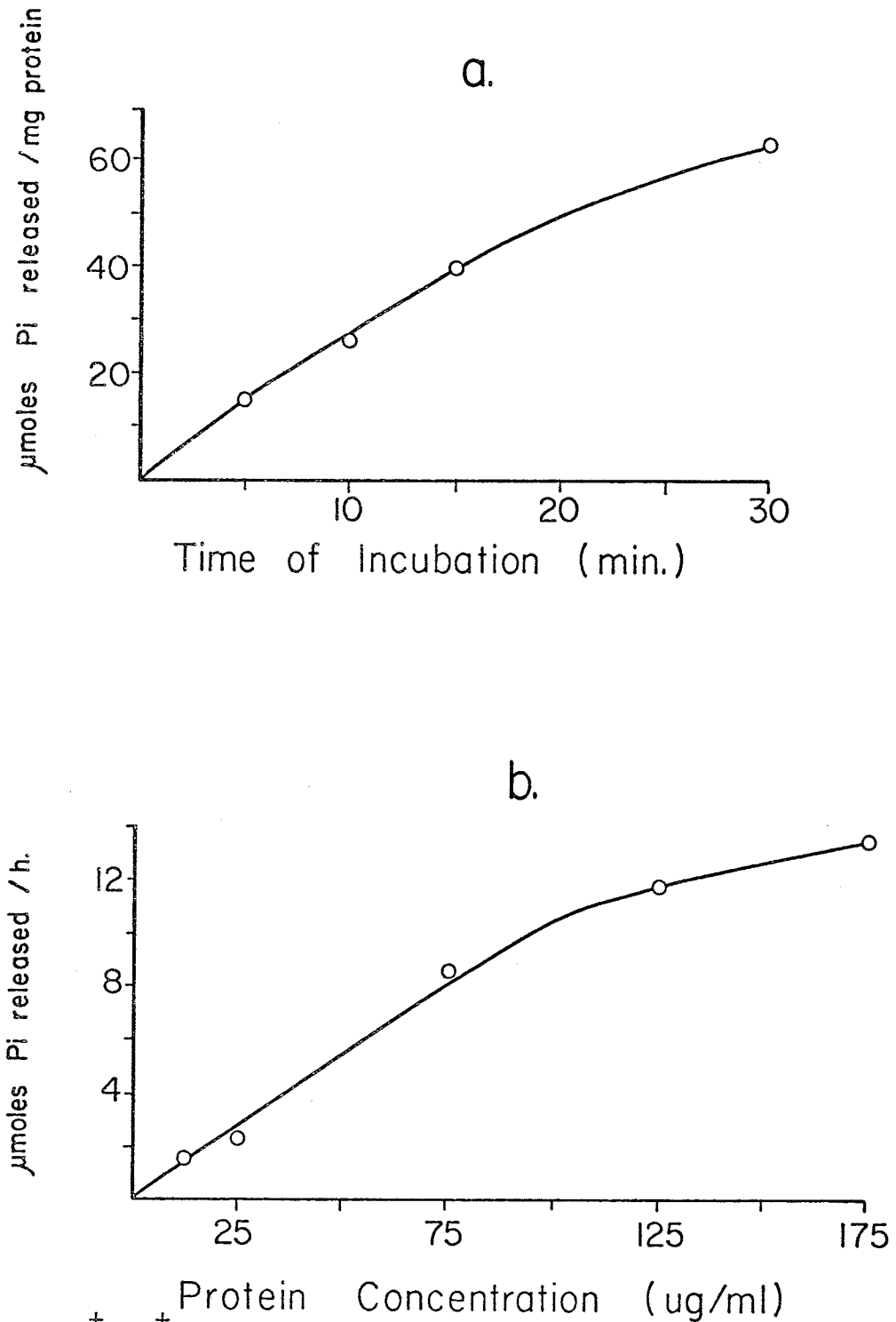



Figure 13. $(Na^+ - K^+)$-ATPase activity as a function of incubation time at 37°C (a) and enzyme concentration (b). Other incubation conditions and analytical procedures were similar to those described under Methods of this section.

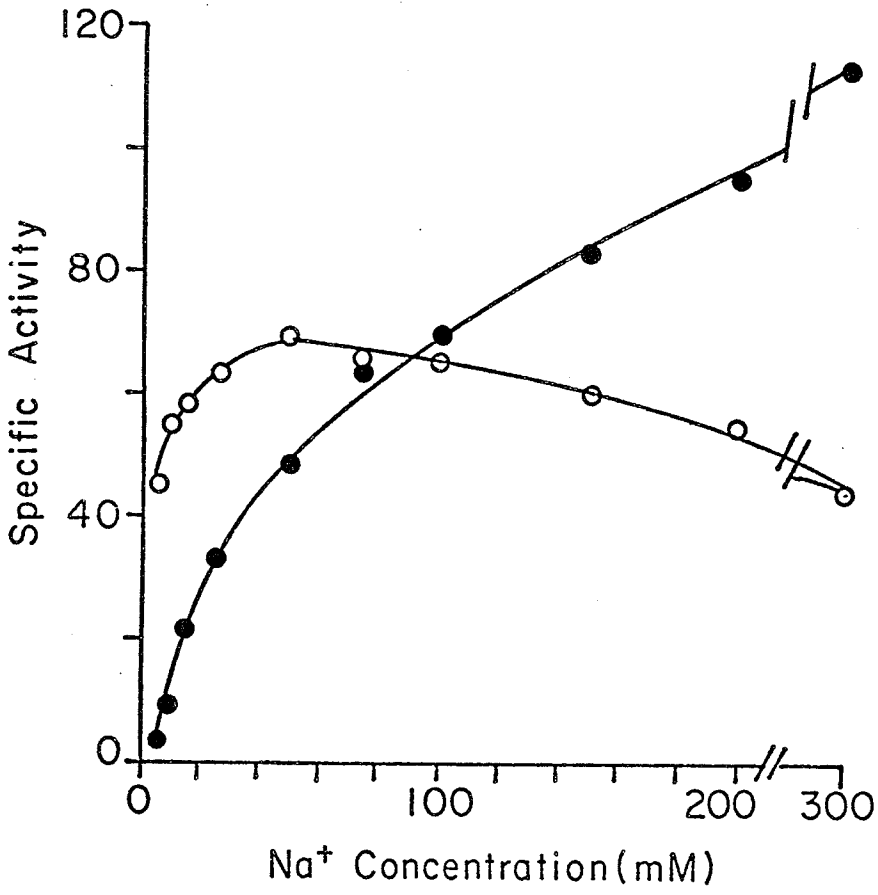



Figure 14. Effect of NaCl on the activity of $(Na^+ - K^+)$-ATPase and $(Mg^{++})$-ATPase of plasma membranes. The incubation conditions and analytical procedures were the same as noted under Methods of this section. Specific activity of $(Na^+ - K^+)$-ATPase (●—●) and $(Mg^{++})$-ATPase (○—○) is expressed as μmoles of Pi released/mg protein/h.

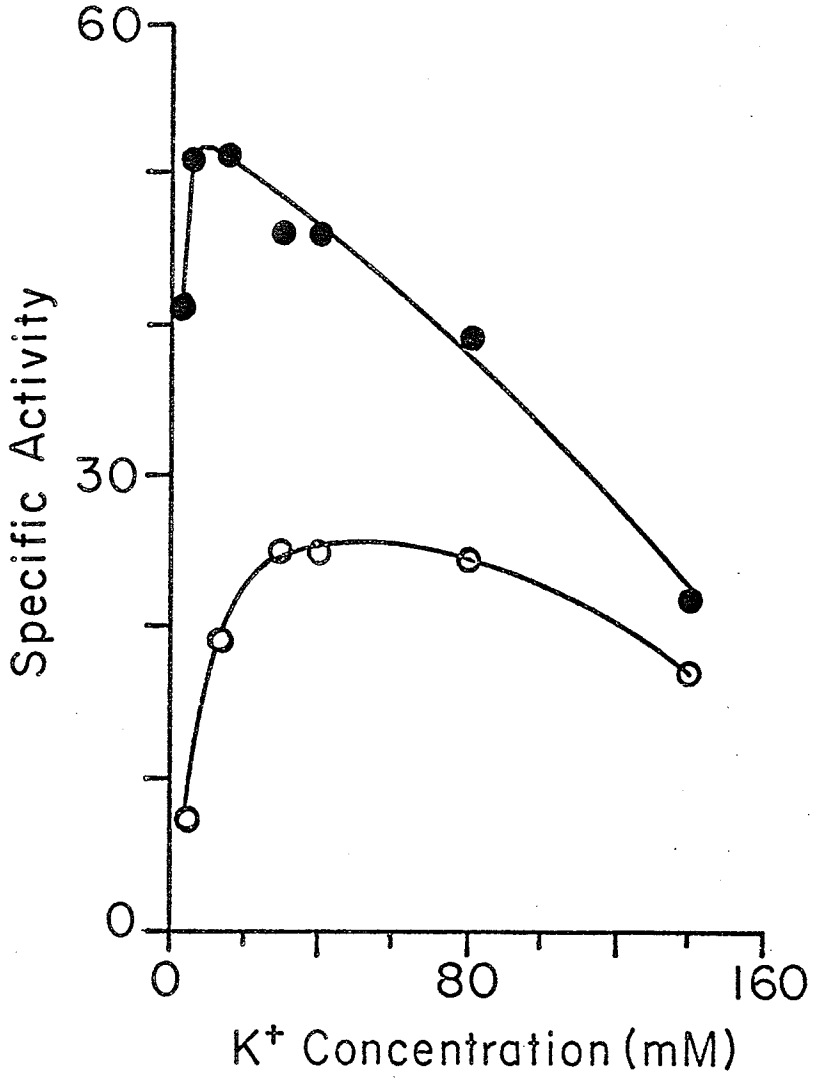



Figure 15. Effect of KCl on the activity of $(Na^{+} - K^{+})$-ATPase and $(Mg^{++})$-ATPase of plasma membranes. The incubation conditions and analytical procedures were the same as noted in Methods of this section. Specific activity of $(Na^{+} - K^{+})$-ATPase (●—●) and $(Mg^{++})$-ATPase (o—o) is expressed as μmoles of Pi released/ mg protein/h.

of the enzyme occurred at 40 mM KCl slightly higher than the level of KCl which maximally stimulated ($Na^+ - K^+$)-ATPase (Figure 15). Moreover, ($Mg^{++}$)-ATPase activity was somewhat less affected by higher levels of KCl as ($Mg^{++}$)-ATPase activity was decreased to 68% of its maximum activity at 150 mM KCl.

$Mg^{++}$ affected both ($Na^+ - K^+$)-ATPase and ($Mg^{++}$)-ATPase similarly, gradually increasing their activities to a maximum level around 5 mM $MgCl_2$ (Figure 16). However, 10 mM $Mg^{++}$ decreased the activity of ($Na^+ - K^+$)-ATPase to 49% of the maximum activity whereas the activity of ($Mg^{++}$)-ATPase was only reduced to 71% of the maximum activity (Figure 16). The concentration of $MgCl_2$ necessary to fully activate ($Na^+ - K^+$)-ATPase and ($Mg^{++}$)-ATPase seems to be directly related to the level of ATP in the assay system as was noted by Skou (202) for the enzyme of nerve membranes.

Somewhat similar effects of these cations on the activity of ($Na^+ - K^+$)-ATPase prepared from avian salt gland (189), red blood cells (107), peripheral nerves (202), kidney and brain (203), intestinal mucosa (198) and parotid glands (7) have been reported. Some differences as to the concentration of a cation at which maximal activity of the enzyme was observed were evident and it appears that the optimal concentration of a cation for maximal enzymic activity depends upon the concentration of other ions present in the test medium (107, 108, 189, 203).

Tris-ATP concentration: ($Na^+ - K^+$)-ATPase activity was increased as the concentration of Tris-ATP was increased reaching a maximum level at 5 mM ATP (Figure 17). Higher levels of ATP were inhibitory as the activity was completely abolished by 10 mM ATP.

($Mg^{++}$)-ATPase activity was also increased by low levels of Tris-ATP. However, the enzyme was maximally stimulated by 1 mM ATP as compared with 5 mM ATP required for the maximal stimulation of ($Na^+ - K^+$)-ATPase (Figure 17). Tris-ATP beyond 1 mM inhibited the activity of ($Mg^{++}$)-ATPase completely

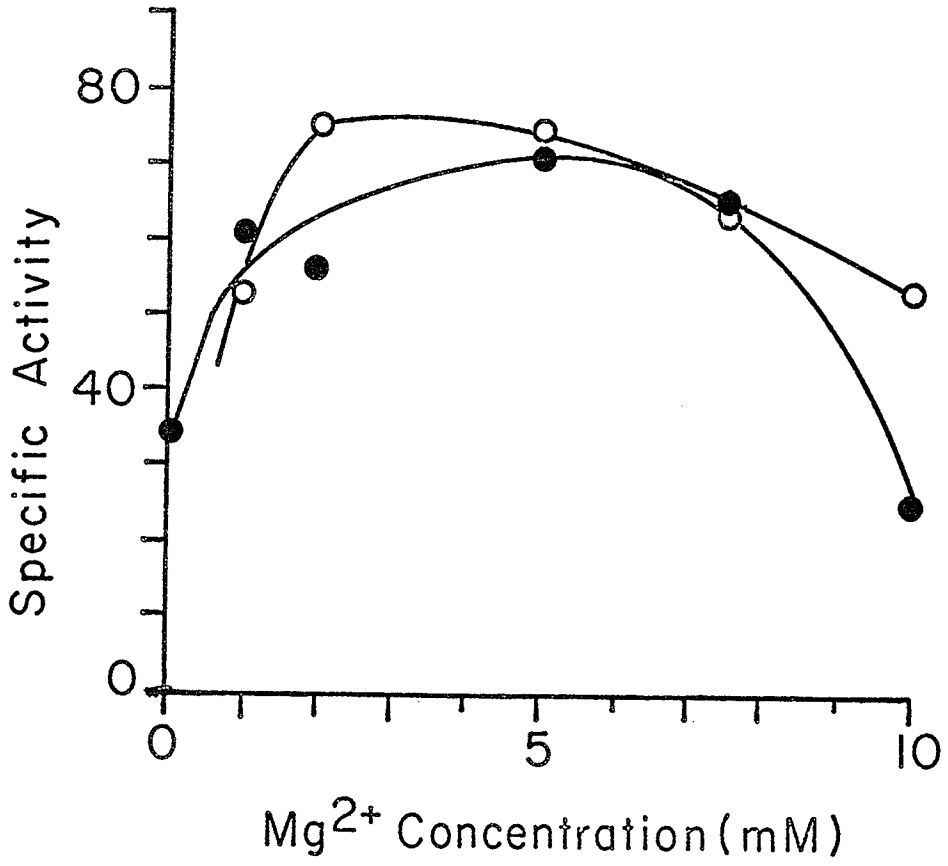



Figure 16. Influence of $MgCl_2$ on the activity of $(Na^+ - K^+)$-ATPase and $(Mg^{++})$-ATPase of plasma membranes. Other incubation conditions and analytical procedures were as noted under Methods of this section. Specific activity of $(Na^+ - K^+)$-ATPase (●—●) and $(Mg^{++})$-ATPase (o—o) is expressed as μmoles of Pi released/ mg protein/h.

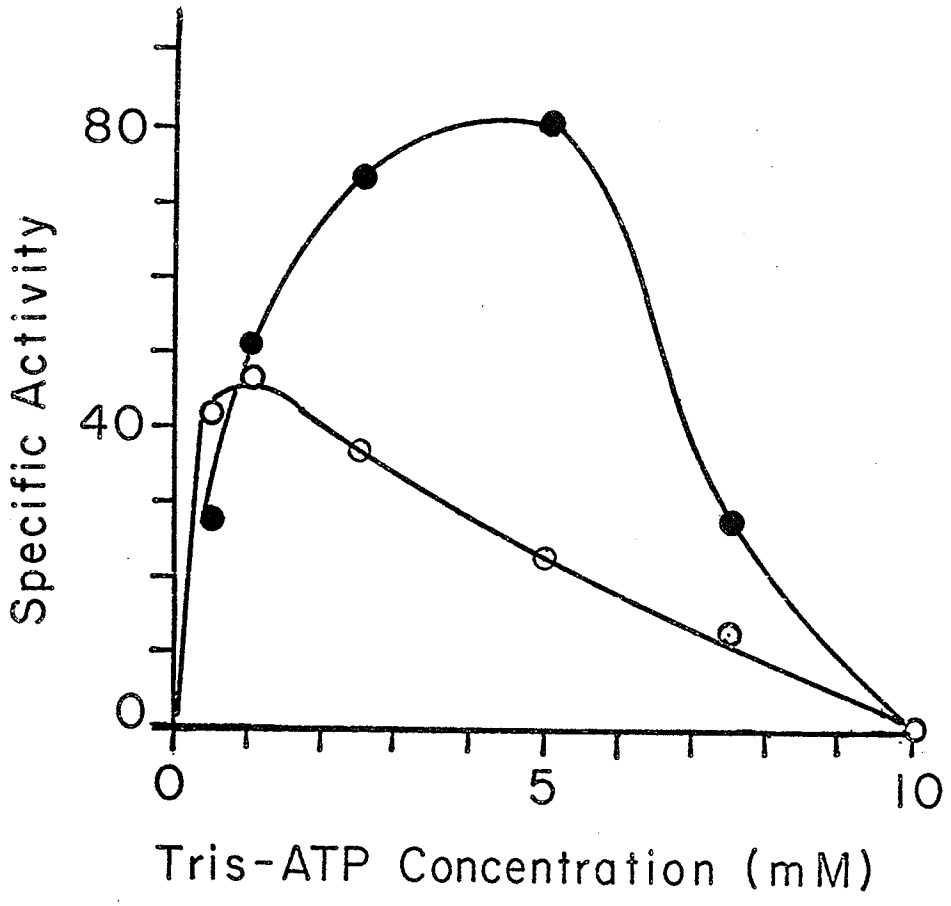



Figure 17. Activity of $(Na^{+} - K^{+})$-ATPase and $(Mg^{++})$-ATPase of plasma membranes as a function of Tris-ATP concentration in the reaction mixture. Other incubation conditions and analytical procedures were the same as described under Methods of this section. Specific activity of $(Na^{+} - K^{+})$-ATPase (●—●) and $(Mg^{++})$-ATPase (○—○) is expressed as μmoles of Pi released/mg protein/h.

abolishing it at 10 mM ATP.

Effect of pH: $(Na^+ - K^+)$-ATPase exhibited highest activity at pH 7.5 (Figure 18). $(Mg^{++})$-ATPase activity also showed a maximum around pH 7.5, however, this enzyme showed fewer changes with variations in pH. A pH optimum of around 7.5 has been reported for $(Na^+ - K^+)$-ATPase prepared from intestinal mucosal cells (198), red blood cell ghosts (206) and from crab nerves (202). These findings show the similarity in pH optimum among enzymes prepared from different tissues.

Ouabain concentration: Total ATPase activity was reduced rather rapidly by low concentrations (0.05 - 0.10 mM) of ouabain (Figure 19). At 1 mM, ouabain inhibited about 85% of the original activity and 1.5 mM or higher level of ouabain completely abolished the $Na^+$ and $K^+$ -stimulated activity. The degree of inhibition of $(Na^+ - K^+)$-ATPase by ouabain seems to depend upon the source of enzyme. The present findings that 1.5mM ouabain was required to completely inhibit $(Na^+ - K^+)$-ATPase activity is consistent with earlier observations wherein it was demonstrated that $(Na^+ - K^+)$-ATPase prepared from rat tissues showed more resistance to ouabain inhibition than the enzyme prepared from tissues of other animal species (193, 207).

(ii) Substrate specificity of $(Na^+ - K^+)$-ATPase and $(Mg^{++})$-ATPase

The ability of the $(Na^+ - K^+)$-ATPase preparation to hydrolyze various nucleotides and other substrates was examined (Table XIX). The results showed that the enzyme hydrolyzed ATP at the highest rate(131.7 µmoles of Pi released/mg protein/h) while CTP was hydrolyzed to a slight extent (18 µmoles of Pi released/mg protein/h). Other nucleotides such as GTP, ITP, UTP, AMP and ADP were not hydrolyzed. Some hydrolysis of glucose 6-phosphate was observed but none with p-nitrophenylphosphate. Similar results of $(Na^+ - K^+)$-ATPase prepared from red blood cell ghosts (107) and intestinal mucosal cells (198) have been reported.

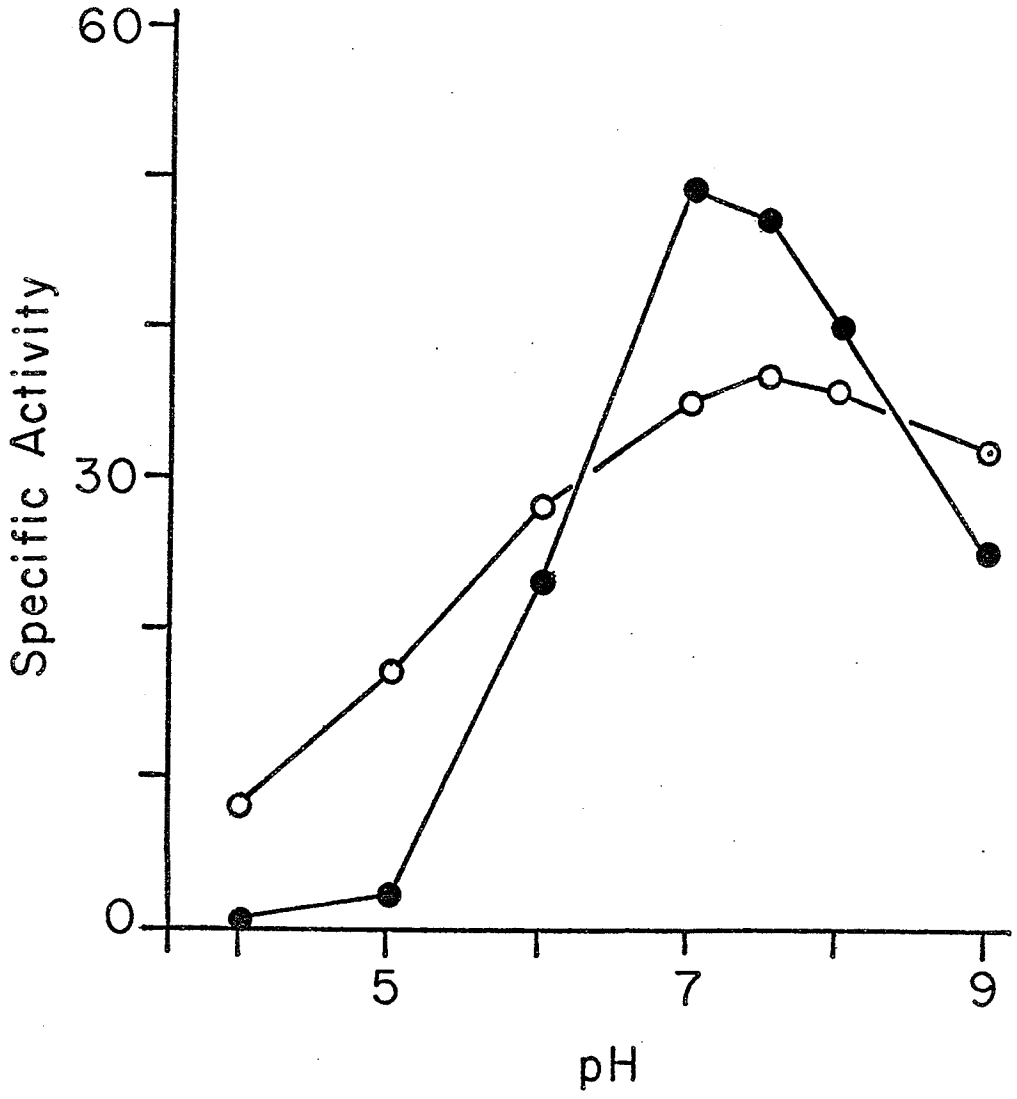



Figure 18. Effect of pH of the incubation medium on the activity of $(Na^{+} - K^{+})$-ATPase and $(Mg^{++})$-ATPase of plasma membranes. The pH of the incubation medium was varied from 4.0 - 6.0 with MES buffer, 6.0 - 8.0 with TES buffer and from 8.0 - 10.0 with Tricine buffer. Other incubation conditions and analytical procedures were the same as noted under Methods of this section. Specific activity of $(Na^{+} - K^{+})$-ATPase (●—●) and $(Mg^{++})$-ATPase (○—○) is expressed as μmoles of Pi released/mg protein/h.

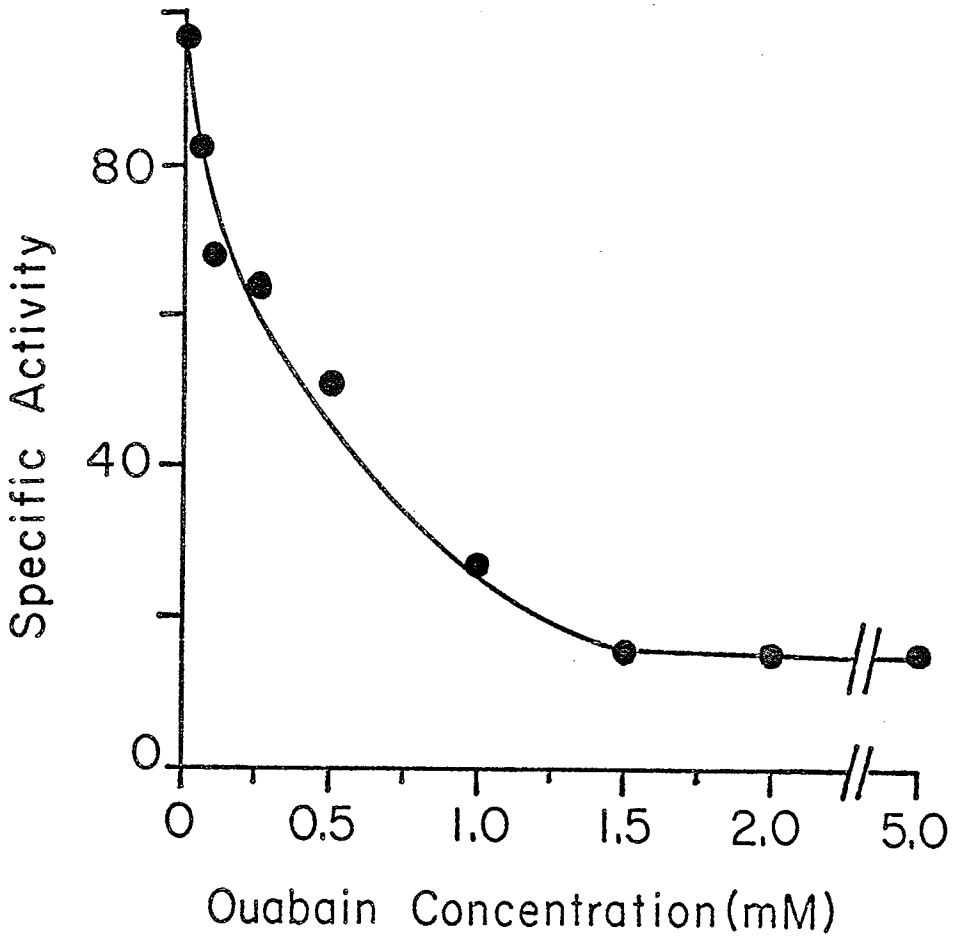



Figure 19. Inhibition of $(Na^+ - K^+)$-ATPase activity of plasma membranes by ouabain. Other conditions were exactly as noted in Methods of this section.

$(Mg^{++})$-ATPase hydrolyzed ATP, GTP, ITP, UTP and ADP at decreasing rates in that order (Table XIX). AMP and glucose 6-phosphate were not utilized as substrates while p-nitrophenylphosphate was slightly hydrolyzed (Table XIX). These findings indicate that while $(Na^{+} - K^{+})$-ATPase was very specific for ATP, $(Mg^{++})$-ATPase was much less so.

(iii) Effects of secretagogues on the activity of $(Na^{+} - K^{+})$-ATPase and $(Mg^{++})$-ATPase

At low concentration, acetylcholine (0.2 mM), epinephrine (0.2 mM) or cyclic 3',5'-adenosine monophosphate (0.25 mM) did not influence the activities of either enzyme (Table XX). However, at higher levels, they all inhibited $(Na^{+} - K^{+})$-ATPase activity, with epinephrine being the most effective.

$(Mg^{++})$-ATPase activity was slightly inhibited by epinephrine (0.2 and 1.0 mM) and acetylcholine (0.2 and 1.0 mM) but was much reduced by cyclic 3',5'-adenosine monophosphate (1.25 mM) (Table XX).

Inconsistent effects of these agents on the activity of $(Na^{+} - K^{+})$-ATPase have been reported. While epinephrine has been shown to inhibit the activity of this enzyme prepared from rat gastric mucosa (208), no such inhibitory effect was noted in the preparation from cat gray matter and retina (192) and rat brain (197). Acetylcholine did not influence the activity of $(Na^{+} - K^{+})$-ATPase prepared from rat gastric mucosa (208), cat gray matter and retina (192), rat brain (197) and from avian salt glands (189). Consistent with the present findings cyclic 3',5'-adenosine monophosphate inhibited $(Na^{+} - K^{+})$-ATPase of rat gastric mucosa (209). Inconsistent effects of these agents on the activity of $(Na^{+} - K^{+})$-ATPase could be due to differences in the tissue sources or due to differences in the levels of these agents used in various investigations. The present findings in rat SMSG preparation show that these agents did not alter the activity of this enzyme at low concentration but they all inhibited the enzyme at higher concentration.

TABLE XIX

Nucleotide Specificity of Plasma Membrane ($Na^+ + K^+$)-ATPase and $Mg^{++}$-ATPase

| | Specific Activity (μmoles $P_i$ released /mg protein/ h) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ($Na^+ + K^+$)-ATPase | | | | | $Mg^{++}$-ATPase | | | | |
| | Concentration Substrate (mM) | | | | | | | | | |
| Added Substrate | 0.25 | 0.50 | 1.00 | 1.50 | 2.00 | 0.25 | 0.50 | 1.00 | 1.50 | 2.00 |
| ATP | 35.7 | 65.4 | 102.9 | 115.5 | 131.7 | 32.2 | 34.7 | 22.9 | 21.3 | 9.3 |
| CTP | 3.3 | 4.3 | 12.0 | 17.7 | 17.0 | 11.1 | 16.2 | 9.3 | 3.4 | 0.0 |
| GTP | 0.0 | 0.0 | 0.0 | 5.1 | 0.0 | 13.6 | 11.0 | 4.3 | 0.0 | 0.0 |
| ITP | 1.2 | 0.8 | 0.0 | 4.3 | 5.1 | 14.9 | 11.9 | 4.2 | 0.0 | 0.0 |
| UTP | 0.0 | 0.0 | 0.0 | 5.9 | 0.0 | 7.7 | 10.2 | 4.3 | 0.0 | 0.0 |
| ADP | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.0 | 7.4 | 3.9 | 1.7 | 0.0 |
| AMP | 0.9 | 0.0 | 0.9 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Glucose-6-phosphate | 1.7 | 0.4 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| p-nitrophenyl-phosphate | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.7 | 1.7 | 3.9 |

The incubation conditions as described in Methods except that various substrates were substituted for ATP.

TABLE XX

ATPase Activity in the Presence of some Metabolically Active Materials

| Additions to Medium | | Specific Activity (μmoles $P_i$ /mg protein /h) | | | | |
|---|---|---|---|---|---|---|
| Conc. tris-ATP (mM) added to medium | | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 |
| $(Na^+ + K^+)$-ATPase | | | | | | |
| None | | 43 | 71 | 85 | 83 | 84 |
| Acetylcholine | (0.2 mM) | 42 | 89 | 84 | 76 | 82 |
| | (1.0 mM) | 35 | 59 | 74 | 71 | 65 |
| Epinephrine | (0.2 mM) | 51 | 75 | 71 | - | 74 |
| | (1.0 mM) | 35 | 47 | 57 | 51 | 55 |
| cAMP | (0.25 mM) | 39 | 73 | 75 | 81 | 71 |
| | (1.25 mM) | 37 | 61 | 65 | - | 67 |
| NaF | (1.0 mM) | 41 | 69 | 82 | 71 | 67 |
| | (5.0 mM) | 7 | 15 | 18 | 24 | 21 |
| $CaCl_2$ | (1.0 mM) | 15 | 31 | 39 | 32 | 23 |
| | (5.0 mM) | 0 | 13 | 33 | 29 | 0 |
| $Mg^{++}$-ATPase | | | | | | |
| None | | 37 | 61 | 49 | 39 | 20 |
| Acetylcholine | (0.2 mM) | 51 | 53 | 43 | 26 | 15 |
| | (1.0 mM) | 48 | 42 | 35 | 23 | 15 |
| Epinephrine | (0.2 mM) | 48 | 41 | 31 | 23 | 10 |
| | (1.0 mM) | 46 | 45 | 33 | - | 11 |
| cAMP | (0.25 mM) | 53 | 49 | 41 | 25 | 19 |
| | (1.25 mM) | 40 | 33 | 17 | - | 1 |
| NaF | (1.0 mM) | 47 | 43 | 28 | 23 | 19 |
| | (5.0 mM) | 33 | 29 | 26 | 18 | 9 |
| $CaCl_2$ | (1.0 mM) | 31 | 49 | 20 | 3 | 11 |
| | (5.0 mM) | 27 | 7 | 0 | 0 | 0 |

Medium as described in Methods except that ATP concentration was varied as shown and appropriate additions were made as shown.

(iv) Effects of inhibitors on ($Na^+ - K^+$)-ATPase and ($Mg^{++}$)-ATPase

NaF at 1 mM concentration did not affect the activity of either enzyme, however, 5 mM $F^-$ inhibited ($Mg^{++}$)-ATPase considerably and ($Na^+ - K^+$)-ATPase very strongly (Table XX). Inhibition of ($Na^+ - K^+$)-ATPase (204, 205) and ($Mg^{++}$)-ATPase (205) by $F^-$ has been reported.

When compared with all the other agents tested in this study, $Ca^{++}$ (1 mM) was found to be one of the most potent inhibitors. This inhibition, however, was not further increased by increasing $Ca^{++}$ concentration indicating a basic level $Ca^{++}$-insensitive activity.

($Mg^{++}$)-ATPase activity was also considerably reduced by 1 mM $CaCl_2$ but 5 mM $CaCl_2$ completely abolished this activity.

These findings were in agreement with the observations made by other authors (7, 107, 108, 192). $Ca^{++}$ might have inhibited both these enzymes by competing with $Mg^{++}$ ions which have been shown to be necessary for the activities of both the enzymes (107, 108). It appears that the $Mg^{++}$ site of ($Mg^{++}$)-ATPase is more sensitive to $Ca^{++}$ than the $Na^+$ and $K^+$ sites of ($Na^+ - K^+$)-ATPase.

c) Discussion

Throughout this investigation, differences in the properties of ($Mg^{++}$)-ATPase and ($Na^+ - K^+$)-ATPase were observed. Moreover, the activities of both the enzymes were affected differently by the same agent, i.e. epinephrine, acetylcholine, cyclic 3',5'-adenosine monophosphate (Table XX). These findings suggest that either ($Na^+ - K^+$)-ATPase and ($Mg^{++}$)-ATPase are separate enzymes or that the $Mg^{++}$ catalytic site is much different from that for $Na^+$ and for $K^+$. The differential loss of ($Na^+ - K^+$)-ATPase and ($Mg^{++}$)-ATPase during storage supports the two enzyme postulate (162, 217).

Ridderstrap and Bonting (211) showed by perfusion studies of isolated rabbit pancreas using various inhibitors that enzyme secretion was not coupled to the sodium pump which might be responsible for fluid and electrolyte secretion.

Their further work with perfused pancreas showed that pancreozymin or acetylcholine enhanced the secretion of proteins and this effect could be elicited by cyclic 3',5'-adenosine monophosphate or by theophylline (212). Consistent with the above studies were the findings of Pritchard (15) with salivary gland slices where ouabain had no effect on the secretion of proteins in vitro. The results of this study with purified $(Na^+ - K^+)$-ATPase preparation from plasma membranes of rat SMSG provide evidence in support of the above conclusion. It was demonstrated that the potent stimulants of salivary secretion did not activate but rather inhibited the activity of $(Na^+ - K^+)$-ATPase of plasma membranes. These findings demonstrate that epinephrine, acetylcholine and cyclic 3',5'-adenosine monophosphate promote the exocrine secretion from rat submandibular salivary glands by mechanism(s) other than the activation of $(Na^+ - K^+)$-ATPase of plasma membranes.

The inhibition of $(Na^+ - K^+)$-ATPase by epinephrine, acetylcholine and cyclic 3',5'-adenosine monophosphate at higher levels could serve as a protective measure against the depletion of cell ATP because in the presence of epinephrine, and acetylcholine, adenyl cyclase would be strongly activated to utilize ATP. If both ATPase and adenyl cyclase were activated simultaneously, ATP would be rapidly utilized and the cell would be depleted of energy. Consistent with this view are the findings of Moszik who noted that epinephrine (208) and cyclic 3',5'-adenosine monophosphate (209) inhibited $(Na^+ - K^+)$-ATPase activity of a preparation from the rat gastric mucosa. He proposed a scheme depicting an inverse relationship between the activity of $(Na^+ - K^+)$-ATPase and adenyl cyclase. Accordingly, any substance activating adenyl cyclase would inhibit $(Na^+ - K^+)$-ATPase not only by its direct action but also through a feedback inhibition by cyclic 3',5'-adenosine monophosphate. The present findings (Table XX) support the above scheme.

## C. CALCIUM BINDING PROPERTIES OF PLASMA MEMBRANES

### 1. Introduction

The requirement for calcium in the secretion of amylase from parotid glands (14, 184, 216, 226), insulin from pancreas (210, 227), catecholamines from adrenal medullary granules (228, 229), and in the release of neurotransmitters from synaptic junctions (230, 231) is well known. Calcium could alter the permeability of cell membranes by binding to the membranes or by stimulating one or more enzymes concerned with the secretory process. Both these possibilities have been discussed in a recent paper (215).

Although the uptake of calcium by microsomes (232, 233) and by mitochondria (234) is well documented, much less is known about the binding of calcium to the outer cell membranes (235, 236, 237). Gent et al. (235) reported that the cell membranes of human erythrocytes can bind calcium and this binding was not energy dependent. Contrary to this report are the findings of Cha et al. (236) who reported that this process was closely associated with the activity of a $Ca^{++}$-stimulated ATPase in red blood cell ghosts. Recently, Thorpe and Seeman (237) reported that cell membranes of skeletal muscle have the ability to bind calcium, and this binding was increased by ATP. However, they attributed the ATP-augmented calcium binding to microsomal contamination of their plasma membrane preparation. The data reported in this section show that plasma membrane preparations isolated from rat submandibular salivary glands bind calcium and this process is influenced by the ionic environment, ATP and several secretagogues.

Since $F_2$ appeared to be the plasma membrane fraction with the least contamination by other subcellular material according to enzymic, chemical and morphological indicators (see Section II), it was used for examination of the $Ca^{++}$ binding properties of SMSG plasma membranes.

2. Methods

The method of estimating calcium binding described by Sulakhe and Dhalla (238) was used in the present study with slight modification. The incubation medium contained 100 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM $^{45}CaCl_2$ mixed with $CaCl_2$ (1,000 - 2,000 dpm/nmole of $CaCl_2$) and approximately 25 µg of plasma membrane protein in a final volume of 0.2 ml. Whenever noted, Tris-ATP at various concentrations was added to the above medium. The samples were incubated at 25°C in a shaking water bath for 10 min. The reaction was terminated by filtering the total reaction mixture through a 0.45 µ Millipore filter (HAWP 013 00, Millipore Filter Corp., Boston, Mass., U.S.A.). A set of controls containing all the reactants except tissue was always employed. A 50 µl aliquot of the clear filtrate was mixed with 10 ml of Bray's solution and the radioactivity measured in a Unilux II scintillation counter. The amount of calcium bound to plasma membranes was calculated as the difference in the radioactivity between the filtrates from test sample and controls.

dpm bound $Ca^{++}$ = (dpm in control) - (dpm in test)

The results are expressed as nmole $Ca^{++}$ bound/mg plasma membrane protein/10 min. Controls containing either the equivalent amount of boiled tissue or bovine serum albumin were employed. $Ca^{++}$ binding in both these controls was less than that to fresh plasma membranes.

The amount of Pi liberated when ATP was present in the incubation medium was measured in the filtrates by the method of Taussky and Shorr (155) (Section II, Chemical Methods,3.a)(ii) on page 28. Correction for non-enzymic hydrolysis of ATP was always made. The results are expressed as µmoles of Pi released/mg plasma membrane protein/h unless otherwise noted.

3. Results

Effects of various conditions on calcium binding to plasma membranes:

a) Time of Incubation

The binding of calcium to plasma membranes was rather rapid both in the absence and presence of ATP (4 mM), reaching a value of about 60 and 68 nmoles, respectively, of $Ca^{++}$ bound/mg plasma membrane protein within 5 min of incubation. No further change up to 30 min (Figure 20) was observed. Plasma membrane appeared to bind slightly more calcium in the presence of ATP. In samples containing ATP, the release of Pi from added ATP was evident only after 3 min of incubation but increased linearly thereafter.

Rapid equilibration between calcium and microsomes of adrenal medulla (229) and red blood cell membranes (235, 236) has been reported.

b) Calcium Concentration

Calcium binding increased linearly with an increase in calcium concentration up to 150 μM both in the absence and presence of ATP (80 μM) (Figure 21). However, ATP seems to slightly enhance the rate of calcium binding without under-going hydrolysis as the amount of Pi released was not affected by an increase in calcium concentration.

c) KCl Concentration

In a medium containing 10 mM $MgCl_2$, 80 μM Tris-ATP and 0.1 mM $CaCl_2$, 20 nmoles of $Ca^{++}$ were bound to plasma membrane (Figure 22). On addition of KCl, there was a rapid increase in $Ca^{++}$ binding reaching a maximum at about 25 mM $K^+$. However, 150 mM KCl slightly reduced (87% of maximum binding) $Ca^{++}$ binding. The amount of Pi released was uninfluenced by KCl up to 150 mM.

d) $MgCl_2$ Concentration

$Ca^{++}$ binding to plasma membrane in a medium containing 100 mM KCl, 80 μM Tris-ATP and 0.1 mM $CaCl_2$ was about 100 nmoles (Figure 23) and was 5 times more than in (c) where 100 mM KCl was replaced by 10 mM $MgCl_2$

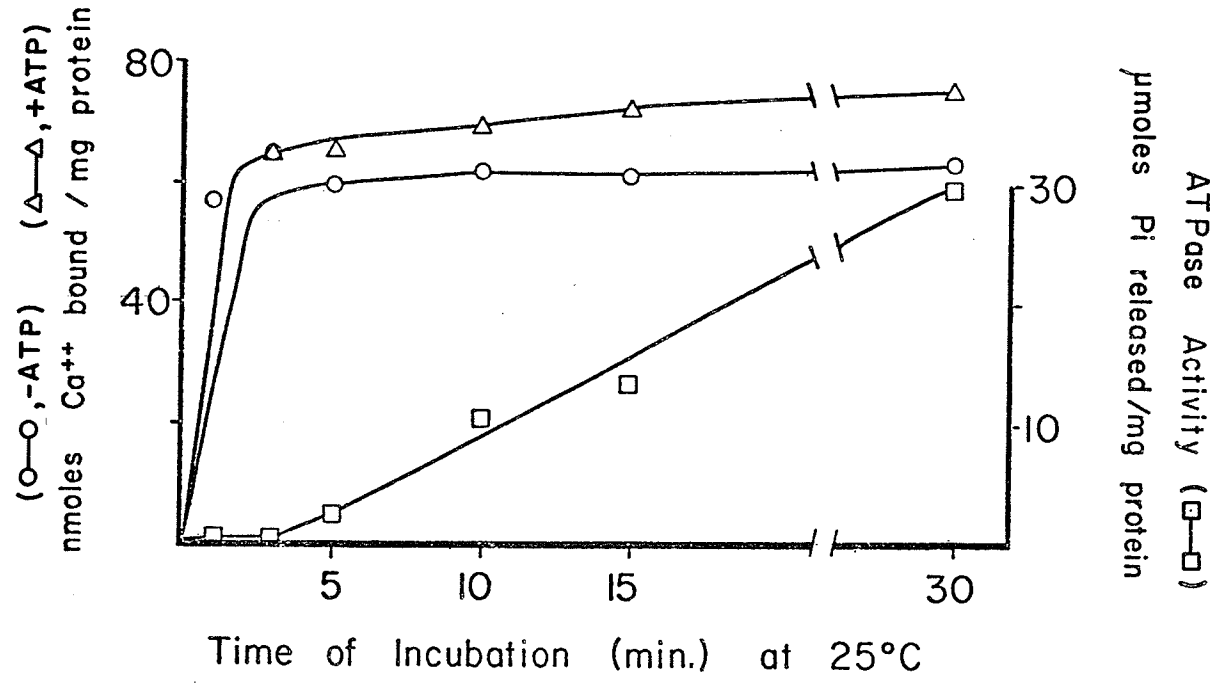



Figure 20. $Ca^{++}$ binding to plasma membranes in the absence and presence of 4 mM ATP (disodium salt) as a function of incubation time at 25°C. The incubation conditions were similar to those described under Methods of this section. The release of Pi during incubation is also shown.

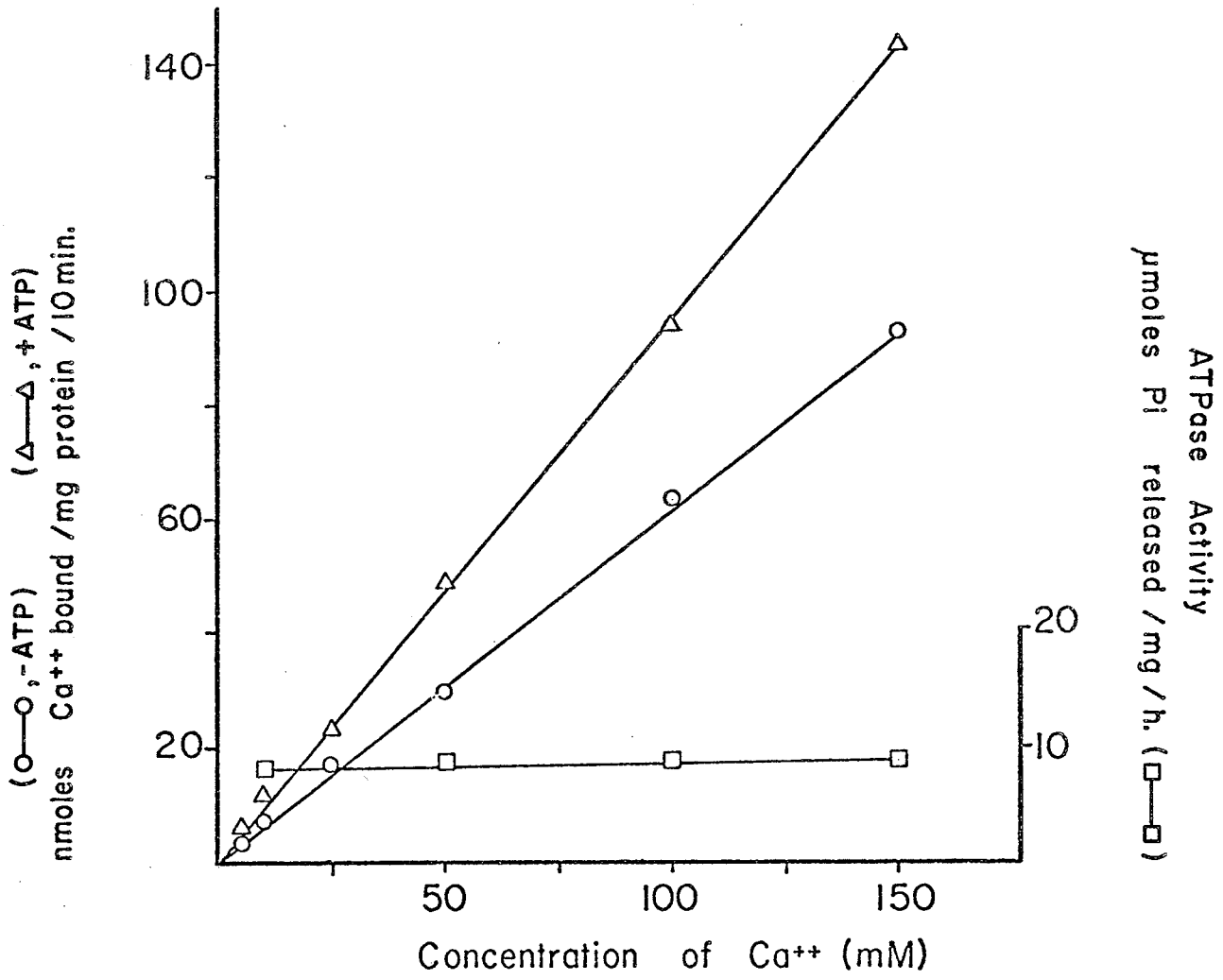



Figure 21. $Ca^{++}$ binding to plasma membranes in the absence and presence of 80 μM Tris-ATP and the release of Pi in samples containing ATP as influenced by $Ca^{++}$ concentration. Other incubation conditions were exactly as noted under Methods of this section.

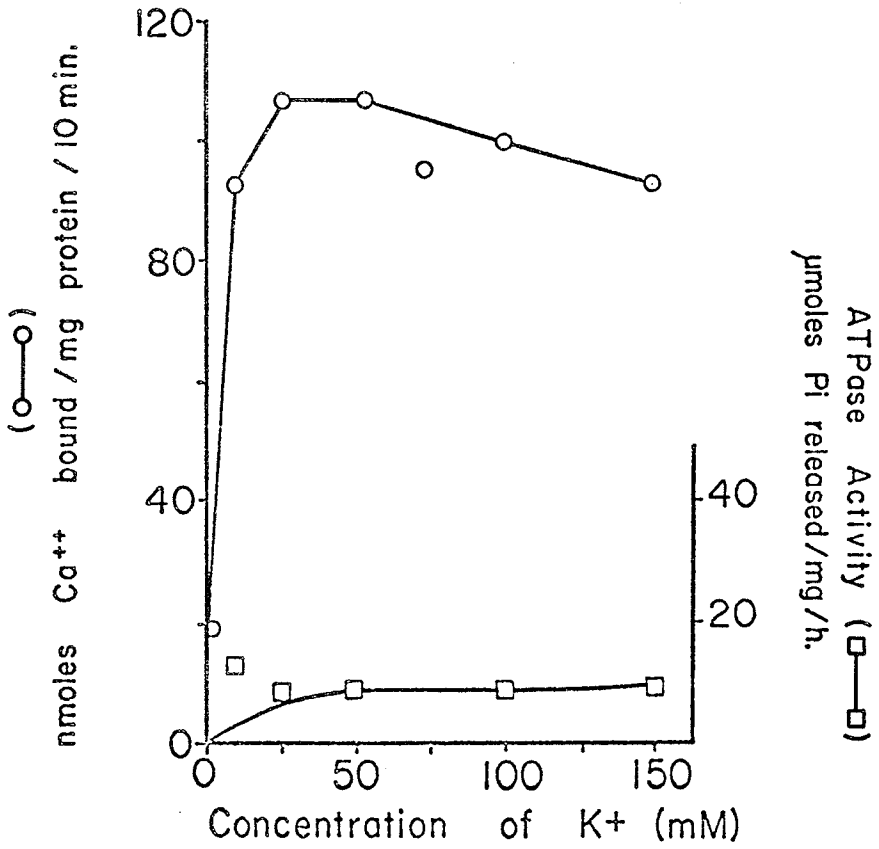



Figure 22. Influence of KCl on $Ca^{++}$ binding to plasma membranes in the presence of 10 mM $MgCl_2$ and 80 μM Tris-ATP. The amount of Pi released is also shown. Other incubation conditions were exactly as noted under Methods of this section.

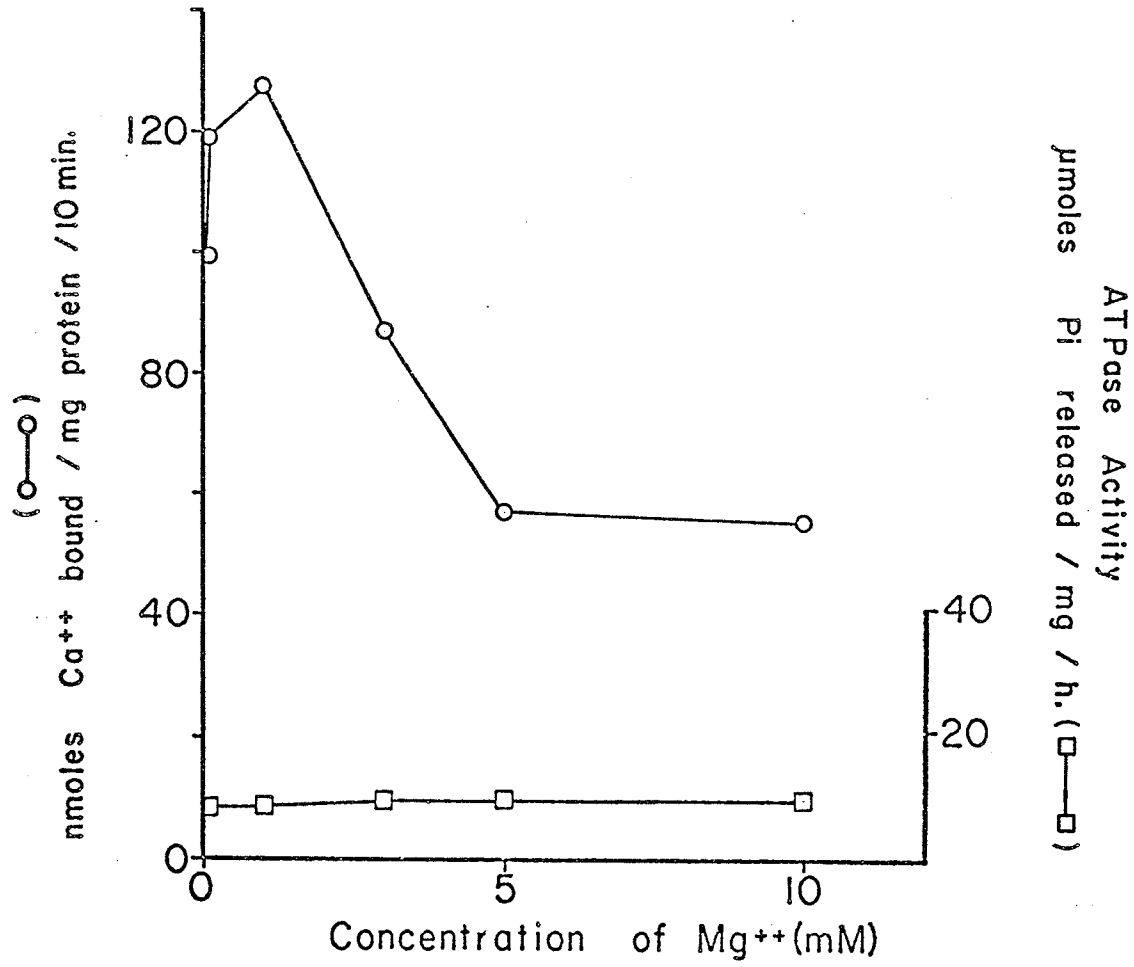



Figure 23. Influence of $MgCl_2$ on $Ca^{++}$ binding to plasma membranes and Pi released in the presence of 100 mM KCl and 80 μM Tris-ATP. Other incubation conditions were the same as described in Methods of this section.

(Figure 22). The addition of $MgCl_2$ to a medium containing 150 mM KCl and ATP increased $Ca^{++}$ binding reaching a maximum at 1 mM, however, at 5 mM, it reduced the binding to a level well below that seen in its absence. Further addition of $MgCl_2$ to 10 mM did not alter the binding (Figure 23). The failure of $Mg^{++}$ to completely abolish $Ca^{++}$ binding suggests that there were two types of $Ca^{++}$ binding sites, ones which were affected by $Mg^{++}$ and others which were not. These findings also indicate that $Mg^{++}$ competed with $Ca^{++}$ more effectively than $K^+$. Similar inhibitory effects of $Mg^{++}$ and $K^+$ on calcium binding have been reported with microsomes (236, 239, 240). The mechanism by which low levels of $Mg^{++}$ and $K^+$ enhanced calcium binding in the presence of ATP is not known.

e) Effects of Ions on Calcium Binding to Plasma Membranes in the Absence of ATP

To elucidate the mechanism of the effect of cations on calcium binding, further experiments were carried out to evaluate the effects of these ions on calcium binding in a reaction mixture lacking ATP and containing 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM $CaCl_2$ and approximately 25 µg of plasma membrane protein. Both $Na^+$ and $K^+$ at 10 mM decreased calcium binding at different levels of $Ca^{++}$. $Mg^{++}$ (2.5 mM) did not influence $Ca^{++}$ binding at 10 and 20 µM $Ca^{++}$, however, reduced it considerably (35% of control) at 50 µM $Ca^{++}$ (Figure 24). At a level of 5 mM, $Mg^{++}$ inhibited calcium binding at 20 µM $Ca^{++}$ and completely abolished it at 50 µM $Ca^{++}$. The results of these experiments are in complete agreement with those of Cohen and Selinger (239) with endoplasmic reticulum of rabbit skeletal muscle and of Carvalho (240) with microsomes of liver.

Whether these ions inhibited calcium binding by increasing the ionic strength of the medium or by competing with $Ca^{++}$ for the same binding site(s) is not certain. The finding that $Mg^{++}$ reduced calcium binding more than $K^+$ or $Na^+$ indicates that probably all these ions bind to the same binding site(s) but $Mg^{++}$

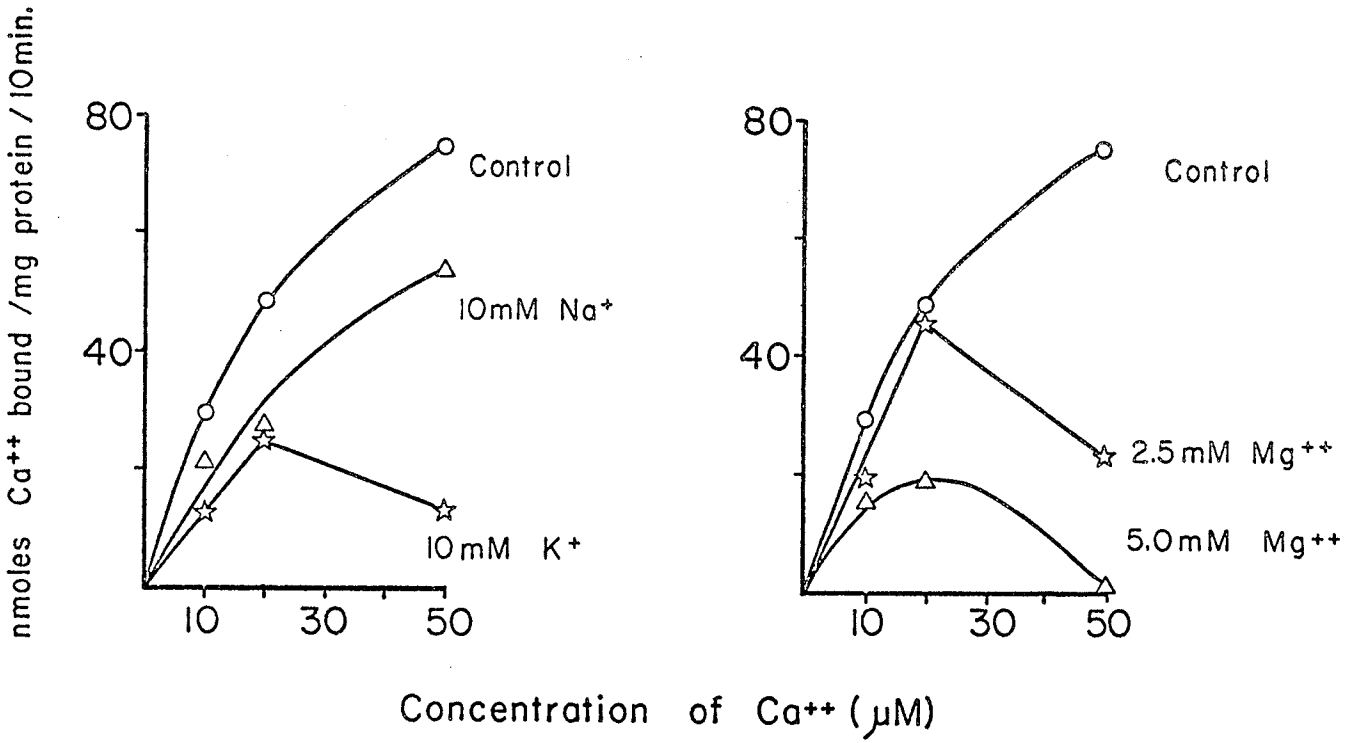



Figure 24. Influece of NaCl, KCl and $MgCl_2$ on $Ca^{++}$ binding to isolated plasma membranes in an incubation medium containing 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM $CaCl_2$ (1000 - 2000 dpm/nmole of $CaCl_2$) and about 25 μg of plasma membrane protein in a final volume of 0.2 ml. Other incubation conditions were similar to those noted under Methods of this section.

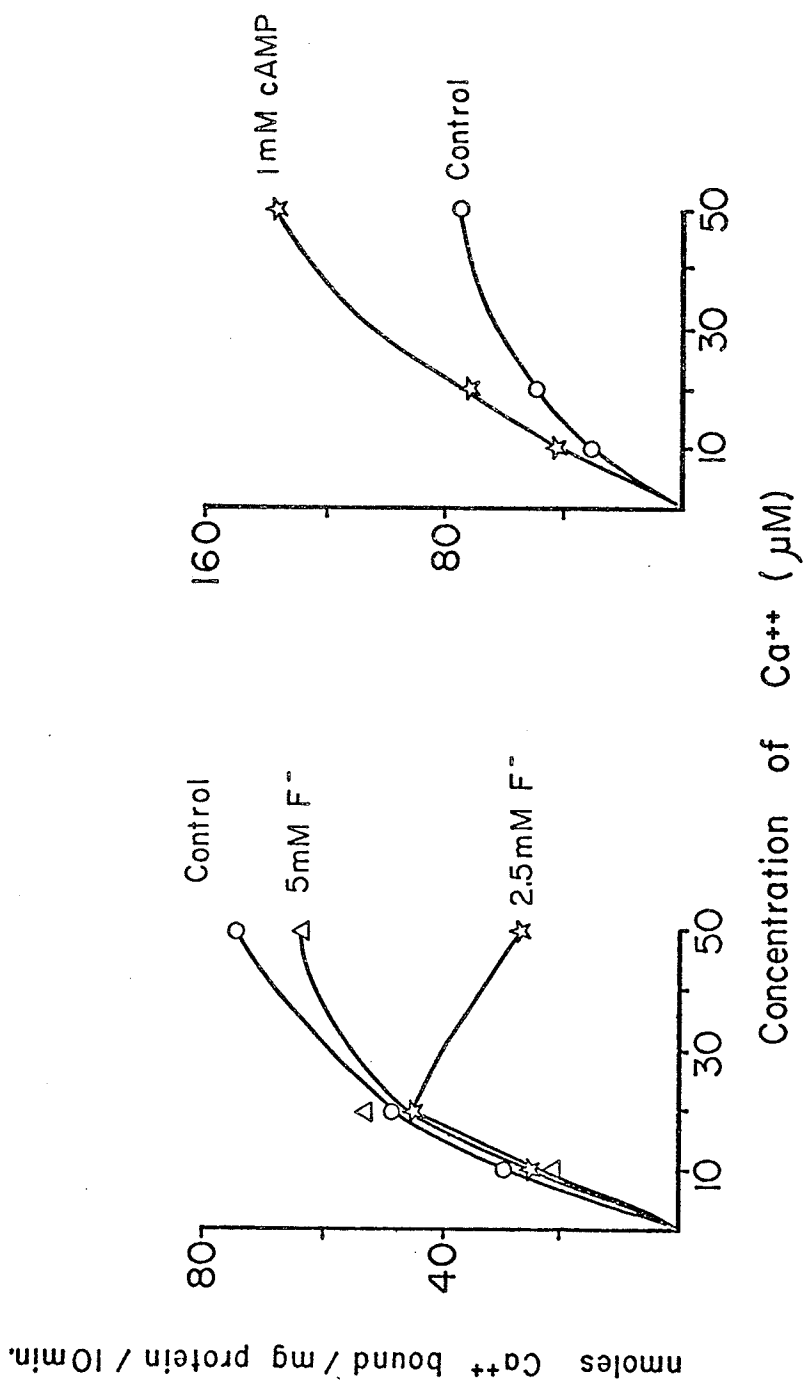



Figure 25. Effect of NaF and cAMP on $Ca^{++}$ binding to isolated plasma membranes in a medium exactly as described in the legend to Figure 24. Other incubation conditions were exactly as described under Methods of this section.

can compete with $Ca^{++}$ more effectively than $Na^{+}$ or $K^{+}$, a conclusion consistent with reports of others (239, 240). The $Ca^{++}$ binding sites do not seem to be specific for $Ca^{++}$ as $Na^{+}$, $K^{+}$, $Mg^{++}$ reduced calcium binding by competing with $Ca^{++}$ for the same binding sites. The degree of calcium binding inhibition displayed by $Na^{+}$, $K^{+}$, and $Mg^{++}$ seems to depend upon the $Ca^{++}$ concentration, being higher at higher $Ca^{++}$ concentrations (Figure 24). These findings suggest that ionic strength might be partly responsible for the reduced calcium binding in the presence of these cations. Similar conclusions have been deduced by other workers from observations made on red blood cell membranes (235) and microsomes isolated from various tissues (239, 240).

f) pH

In a medium containing all the reactants except ATP, calcium binding was maximum at pH 8.0 and it was reduced at both acidic and alkaline pHs (Figure 26). A similar pH optimum for calcium binding to endoplasmic reticulum derived from rabbit skeletal muscle has been reported (239).

g) Epinephrine, Acetylcholine, Cyclic 3',5'-adenosine Monophosphate, Ouabain and Oxalate

The control samples contained 100 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM $CaCl_2$ (+ $^{45}CaCl_2$) and about 25 μg of plasma membrane proteins in a final volume of 0.2 ml. Acetylcholine (0.5 mM) did not influence calcium binding (Table XXI). The addition of epinephrine (250 μM) increased calcium binding only slightly, but cyclic 3',5'-adenosine monophosphate (1 mM), ouabain (1 mM) and oxalate (5 mM), all enhanced calcium binding considerably but the hydrolysis of ATP, as measured by the liberation of Pi, was unaffected (Table XXI).

Although Marinetti et al. (136, 241) have shown that liver plasma membrane binds epinephrine, our efforts to demonstrate epinephrine binding to SMSG plasma membrane have been unsuccessful thus far. The findings that cyclic 3',5'-adenosine monophosphate, ouabain and oxalate enhanced calcium binding equally are puzzling.

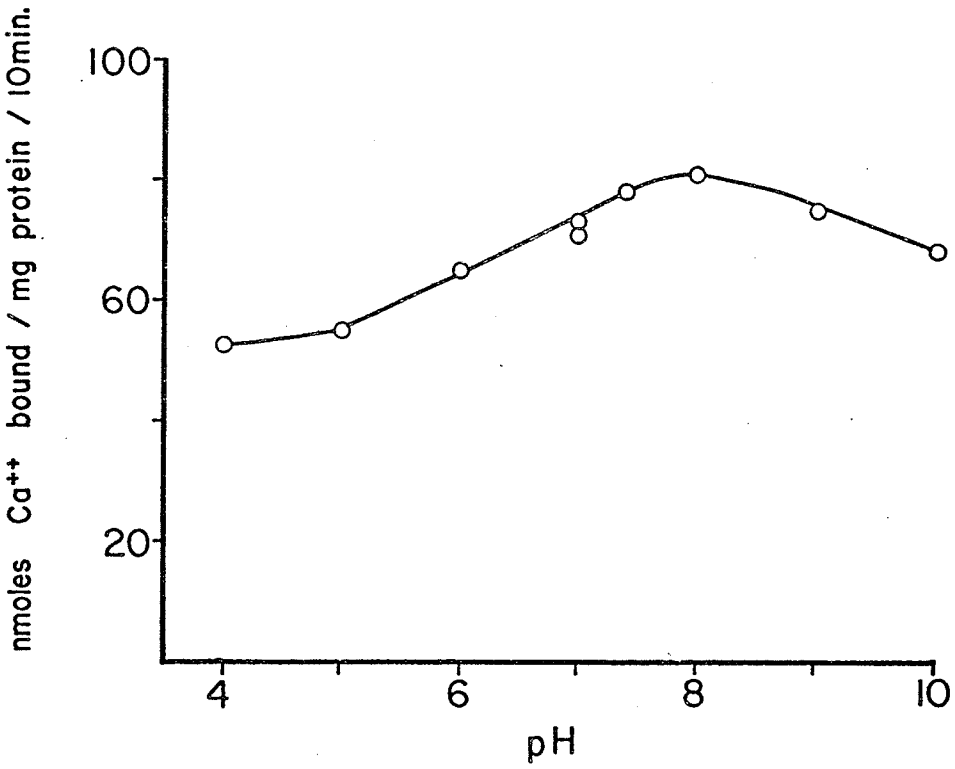



Figure 26. Effect of pH on $Ca^{++}$ binding to isolated plasma membranes in a medium containing 10 mM $MgCl_2$, 100 mM KCl and no ATP. Other conditions were similar to those described under Methods of this section.

TABLE XXI

Effects of Some Compounds on $Ca^{++}$ Binding to Plasma Membranes in the Presence of 100 mM $K^{+}$, 10 mM $Mg^{++}$ and 0.08 mM Tris-ATP

| | $Ca^{++}$ Concentration (μM) | | | |
|---|---|---|---|---|
| Additions | 5 | 10 | 25 | 50 |
| Ions + ATP | 5.9* | 11.7 | 22.5 | 48.1 |
| + Acetylcholine (0.5 mM) | 7.7 | 4.1 | 10.6 | 49.4 |
| + Epinephrine (0.25 mM) | 5.7 | 22.7 | 40.9 | 57.2 |
| + cAMP (1 mM) | 16.2 | 37.1 | 116.7 | 159.2 |
| + Ouabain (1 mM) | 18.8 | 39.3 | 114.5 | 159.0 |
| + Oxalate (5 mM) | 16.3 | 39.1 | 118.4 | 167.6 |
| + NaF (1.25 mM) | 2.1 | 14.9 | 31.5 | 58.5 |

*nmoles of $Ca^{++}$ bound/mg protein/10 min

Other incubation conditions were the same as described in Methods of this section.

Since there is no structural similarity between these compounds except negative surface charges, it is possible that they might have enhanced calcium binding by chelating $Mg^{++}$ or $K^{+}$ from the medium which compete with $Ca^{++}$ for the binding site(s). However, the finding that cyclic 3',5'-adenosine monophosphate increased $Ca^{++}$ binding in the absence of ions argues against this explanation (Figure 25).

h) Tris-ATP Concentration

In the absence of ATP, 53 nmoles of $Ca^{++}$/mg protein were bound to plasma membranes. On addition of ATP to 20 μM, the binding was decreased (Figure 27). On further increasing the ATP concentration, there was an increase in calcium binding to a maximum value between 100 to 600 μM ATP. At ATP levels higher than 1 mM, calcium binding was decreased. The release of Pi from ATP was negligible upto 500 μM ATP, thereafter, Pi release increased reaching a maximum at 2 mM ATP.

i) Sodium Fluoride

In the complete medium containing ions and ATP, NaF (1.25 mM) did not influence calcium binding at several concentrations of calcium (Table XXI).

4. Discussion

The results of the present study show that ATP augmented calcium binding slightly but consistently. The findings that calcium binding was greatly influenced by many conditions without parallel changes in the liberation of Pi from ATP indicate that the effect of ATP on calcium binding was not linked to ATP hydrolysis, i.e. an ATPase system. Further support for the non-involvement of an ATPase system is given by the findings with NaF, a known inhibitor of ATPases which did not alter $Ca^{++}$ binding. MacLennan and Wong (242) have recently reported a calcium binding protein (Calsequestrin) from sarcoplasmic reticulum of rabbit skeletal muscle which was distinct from ATPase suggesting that $Ca^{++}$ binding process is separate from ATPase system.

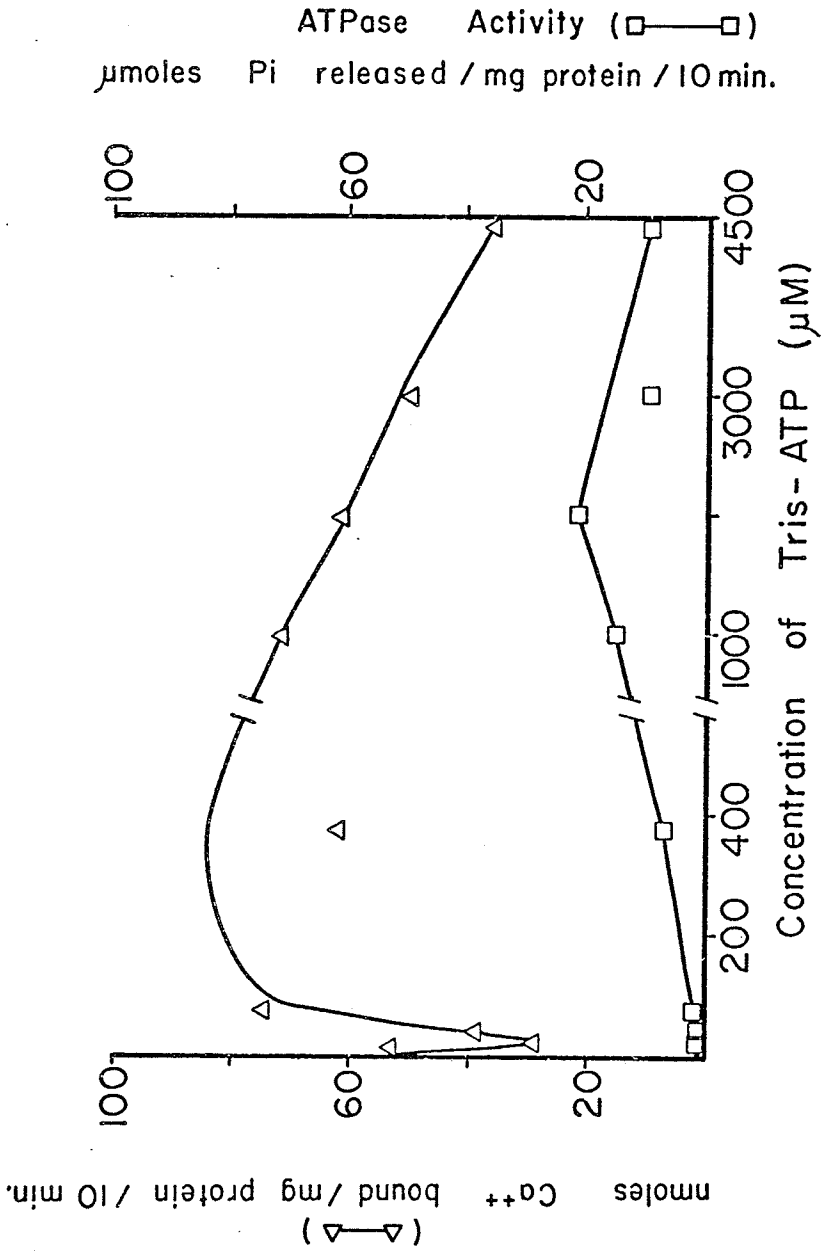



Figure 27. $Ca^{++}$ binding to isolated plasma membranes and Pi release as influenced by Tris-ATP concentration in the presence of 100 mM KCl and 10 mM $MgCl_2$. Other incubation conditions were exactly as described in Methods of this section.

The effects of ions, oxalate and ouabain on calcium binding to SMSG plasma membranes were similar in some respects to those reported for microsomes. It is possible that $Ca^{++}$ binding to plasma membranes noted in this study was due at least in part to microsomal contamination of the preparation. Chaudry et al. (245) have advocated that microsomes can be distinguished from plasma membranes on the basis of ATP-dependent $Ca^{++}$ binding which is absent from the plasma membranes. The present findings that ATP slightly enhanced $Ca^{++}$ binding but this effect was not mediated through a Ca-ATPase system argues against the microsomal participation if SMSG plasma membranes and microsomes are comparable to those of liver and muscle tissue.

Acyl CoA synthetase activity is considered to be present rather exclusively in microsomes (171, 172), and the present findings that SMSG plasma membrane preparations lacked measurable activity of this enzyme indicate that plasma membrane preparations were reasonably free from microsomes. Moreover, the enzymic and chemical composition of SMSG plasma membrane was comparable to plasma membranes prepared from other tissues for which the investigators claimed had little microsomal contamination.

Since many morphologists claim that plasma membranes are continuous with the endoplasmic reticulum at certain regions, the possiblity that the present plasma membrane preparations were slightly contaminated with contiguous smooth endoplasmic reticular membranes can not be completely excluded. The observed $Ca^{++}$ binding to plasma membranes might be partly due to this contamination.

According to Rasmussen (215), a secretory stimulant interacts with the cell surface leading to an increase in its calcium permeability and to adenyl cyclase activation. It was suggested that the subsequent increase in intracellular cyclic 3',5'-adenosine monophosphate prevents the uptake of $Ca^{++}$ into intracellular pools and activates a phosphorylase kinase in several tissues leading to the phosphorylation of the micro-tubular system and converting it from a calcium insensitive form to a calcium-sensitive state. It was further claimed that the increased

influx of calcium activates this complex and the secretory vesicle moves to the cell surface. Contact between vesicle membranes and plasma membranes leads to fusion with subsequent discharge of secretory material.

The findings i) that adenyl cyclase activity was present in SMSG plasma membranes (Table I), ii) that epinephrine activates adenyl cyclase in variety of tissues (178), iii) that epinephrine and cyclic 3',5'-adenosine monophosphate enhanced calcium binding to SMSG plasma membrane (Table XXI), and iv) that $Ca^{++}$ was required for epinephrine- and cyclic 3',5'-adenosine monophosphate-induced amylase secretion from salivary gland slices (184, 216) are completely consistent with Rasmussen's scheme for secretion.

The finding that the treatment of smooth membranes with phospholipase reduced the calcium binding (244) indicated the involvement of plasma membrane phospholipids at the binding sites. It has been suggested that $Ca^{++}$ forms bridges between adjacent phosphate groups of phosphatides especially phosphoinositides (230). The plasma membranes used in this study contained approximately 18% of total phospholipids as phosphoinositides and phosphatidyl-serine. It is possible that calcium binding to SMSG plasma membranes was associated with the phosphate groups of phosphoinositides as well as the carboxyl groups of sialic acid containing proteins. The findings that the binding of calcium to plasma membranes was influenced by pH and by ionic environment are not inconsistent with the above inference.

# SECTION IV

## GENERAL DISCUSSION AND CONCLUSIONS

Plasma membranes of rat submandibular salivary glands were localized in three fractions of variable density. The enzymic and chemical composition of plasma membranes in these fractions was different. These findings were not unique to SMSG as other investigators (218, 219, 22)) have also reported similar findings with liver plasma membranes. These authors also noted differences in the enzymic (218, 219, 220) and chemical composition (219) of plasma membranes in sub-fractions which were quite comparable to the results of present study. The light sub-fraction obtained by Evans (219) expressed high specific activity of 5'-nucleotidase, ($Mg^{++}$ - $K^{+}$)-ATPase and contained more phospholipids, cholesterol and NANA than the heavy sub-fraction. It seems that their light and heavy sub-fractions corresponded to $F_2$ and $F_3$ respectively of the present study. These authors regard liver parenchyma-cell membrane as a complex composed of three physiologically specialized surface areas. Certain parts of plasma membrane are in contact with lacunae and neighbouring parenchymal cells, whereas other parts form microvilli that surround bile canaliculi. It is likely that these liver cell surface areas have different enzymic and chemical composition. By analogy, one might conclude from the observations made with the salivary gland plasma membrane in the present study that probably $F_2$ represented a type of microvilli and $F_3$ contained that portion of the cell surface which was in contact with the other cells. One should note that the $F_1$, $F_2$ and $F_3$ fractions did not display the morphological differences noted by Evans (219) in liver plasma membrane sub-fractions. This could be due to the presence of EDTA in the homogenizing medium of the present preparation which reportedly disrupts the contacts between neighbouring cells and persumably the morphology of plasma membranes in these isolated fractions would not be quite the same as it might be in situ.

Plasma membranes isolated from rat SMSG were not much different in their enzymic and chemical composition from plasma membranes isolated from other

tissues by different isolation procedures. The present findings that plasma membranes of SMSG contained 60% or more saturated fatty acids and high levels of cholesterol and sphingomyelins were consistent with the findings of others (3) and support the view that the structure of plasma membranes is rather rigid and compact as befitting its role as an outer cell covering.

The findings that fatty acids were taken up by the isolated plasma membranes into various lipid species demonstrate the viability and dynamic nature of plasma membranes in view of the findings that the removal and replacement of fatty acids from lipids of cell membranes might determine not only the structure of membranes but might also change the permeability characteristics of cell membranes (4, 5). Factors which would influence the activities of plasma membrane enzymes, such as acyltransferases and phospholipases which are concerned with the turnover of fatty acid residues of plasma membrane lipids might regulate the structure, as well as the biological functions, of cell membranes. Many factors were shown to influence the uptake of palmitic acid into various lipids of plasma membranes isolated from SMSG. However, no precise mechanism could be proposed on the basis of the present data because of the complex nature of the system. Future studies on isolated enzyme systems from plasma membranes might be rewarding.

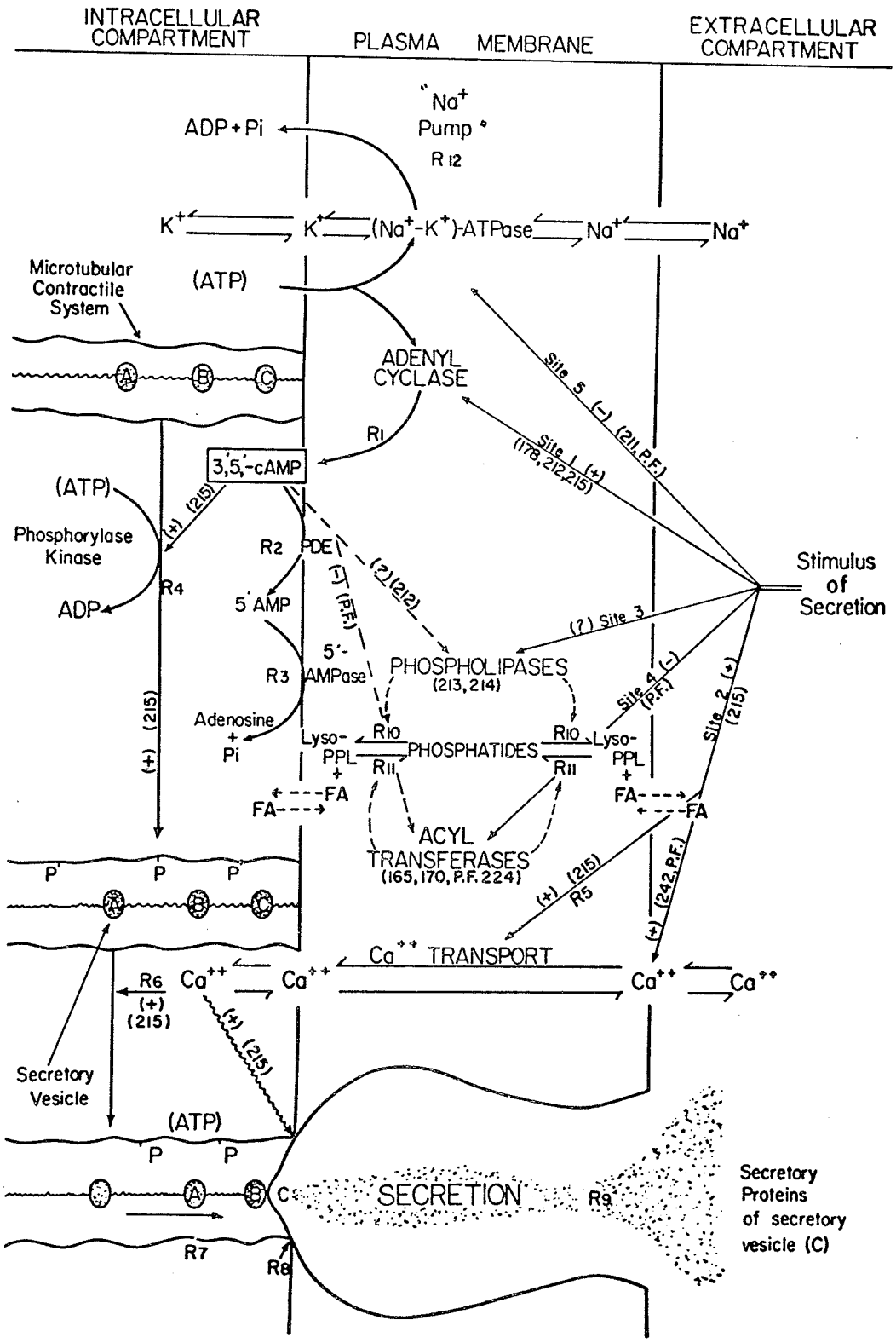
The present findings along with the reports of other investigators (15, 211) demonstrate the non-participation of $(Na^+ - K^+)$-ATPase of plasma membranes in the secretion of macromolecules from the salivary glands.

On the basis of published data and the results of the present investigation, a possible scheme for secretion has been constructed (Figure 28). According to this scheme, a stimulus of secretion may act simultaneously upon multiple enzyme and transport systems (Sites 1 - 5) located in the plasma membrane.

A stimulus of secretion may act upon the adenyl cyclase system of plasma membranes (Site 1) to produce cyclic 3',5'-adenosine monophosphate (Reaction 1)

Figure 28. This diagram shows the possible sites, i.e. 1 - 5 of action of a stimulus of secretion on the plasma membrane and illustrates the intracellular sequence of events and ultimate rupture of plasma membranes which reportedly occur during the exocrine secretion of macromolecules. The numbers in parenthesis refer to the number in the Reference Section. P.F. stands for present findings. The (+) symbol indicates that the process is stimulated and the (-) sign that it is inhibited by a stimulus of secretion. A question mark (?) indicates a possible point of reaction where there is no firm evidence yet available. Reaction numbers are denoted as $R_1 - R_{12}$.

Following abbreviations are used: PDE-, cAMP phosphodiesterase; 5'-AMPase, 5'-nucleotidase; Lyso-PPL, lysophosphatides and F.A., fatty acids.


INTRACELLULAR COMPARTMENT
PLASMA MEMBRANE
EXTRACELLULAR COMPARTMENT
"Na+ Pump" R12
ADP + Pi
K+
(Na+-K+)-ATPase
Na+
Microtubular Contractile System
(ATP)
ADENYL CYCLASE
R1
3,5,-cAMP
Site 5 (-) (211, P.F.)
Site 1 (+) (178, 212, 215)
(ATP)
Phosphorylase Kinase
ADP
(+) (215)
R2
PDE
(-) (P.F.)
(?) (212)
5' AMP
R4
Stimulus of Secretion
(?) Site 3
5'-AMPase
R3
PHOSPHOLIPASES (213, 214)
Site 4 (-) (P.F.)
Site 2 (+) (215)
Adenosine + Pi
(+) (215)
Lyso-PPL + FA
R10
R11
PHOSPHATIDES
ACYL TRANSFERASES (165, 170, P.F. 224)
P
A
B
C
(+) (215) R5
(+) (242, P.F.)
Ca++ TRANSPORT
R6 (+) (215)
(+) (215)
Secretory Vesicle
SECRETION
R9
Secretory Proteins of secretory vesicle (C)
R7
R8

(178, 212). Cyclic 3',5'-adenosine monophosphate can either be metabolized to adenosine and phosphate by cyclic 3',5'-adenosine monophosphate phosphodiesterase (Reaction 2) and 5'-nucleotidase (Reaction 3) of plasma membranes or can activate phosphorylase kinase (Reaction 4) which catalyzes the phosphorylation of a microtubular contractile system rendering this system sensitive to calcium (215).

The same stimulus of secretion simultaneously enhances the influx of calcium (Site 2) either by increasing the concentration of $Ca^{++}$ at the $Ca^{++}$ binding sites of plasma membrane (242) and/or by some other mechanism(s) (Reaction 5) (214). The increased level of intracellular $Ca^{++}$ then acts upon the microtubular system previously sensitized by cyclic 3',5'-adenosine monophosphate through phosphorylation (Reaction 6). This sequence of metabolic events causes the movement of secretory vesicle towards the periphery of a secretory cell (Reaction 7). Ultimately, the vesicle membrane fuses with the outer cell membrane (Reaction 8) and a rupture at the point of fusion releases the secretory material into the lumen of the acini duct system (Reaction 9) (215).

The same stimulus of secretion may also influence the activities of phospholipases (Site 3) (Reaction 10) and/or acyltransferases (Site 4) (Reaction 11) of plasma membranes either through cyclic 3',5'-adenosine monophosphate or directly (Figure 28), such that the level of lysophosphatides in the plasma membranes is increased. An increase in the level of lysophosphatides of plasma membranes could either rupture areas within the plasma membranes due to the lytic properties of lysophosphatides (5) or render the plasma membranes more permeable by changing the configuration of lipids in relation to other plasma membrane components (5). The transformation between lecithin and lysolecithin (Reaction 10 and Reaction 11) has also been implicated in the transport of fatty acids across the plasma membranes (Sites 3 and 4) (224). The present findings provide some support for the action of a stimulus of secretion at the Sites 1, 2 and 4 (Figure 28).

The same stimulus of secretion may also modulate the activity of ATPase

system (Reaction 12) of the plasma membranes (Site 5) and subsequently alter the flux of cations (Figure 28) across the cell membranes. They might in part be responsible for the coupling between a stimulus and the secretory mechanism (10 - 14). However, the present findings along with those of Pritchard (15) and of Ridderstrap and Bonting (211) indicate that this enzyme system (Site 5) is not directly involved in the process of exocrine secretion of proteins.

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Abstract:


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