ISOLATION AND PARTIAL CHARACTERIZATION OF VESICLES DERIVED FROM THE PLASMA MEMBRANE OF THE CHICKEN GIZZARD MUSCLE

by

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ABSTRACT

Plasma membrane vesicles, isolated from the chicken gizzard using differential centrifugation and sucrose gradient centrifugation, were biochemically characterized. Two fractions obtained from the sucrose gradient, Fractions 4 and 5 (32% and 34% sucrose respectively), were judged to be the most pure in plasma membranes as based on 5' nucleotidase and iodination studies. Both fractions had the same coomassie blue and PAS staining profile when electrophoresed and under electron microscopy both fractions consisted of membrane vesicles of varying size.

A Mg^{2+} stimulated ATPase activity was found to be present and highest in Fraction 5 while Fraction 4 exhibited little activity. This enzyme was inhibited in the presence of high concentrations of ATP and Mg. A similar ecto Mg^{2+} stimulated ATPase was observed in isolated smooth muscle cells. Phosphorylation using [γ-^{32}P] ATP was observed at 205,000, 165,000 and 145,000 daltons in Fraction 5 only. Mg promoted dephosphorylation of the 205,000 dalton band while Ca promoted phosphorylation of the 165,000 dalton band. All phosphorylated peaks were sensitive to hydroxylamine treatment. These results would seem to indicate that there is a difference in membrane orientation between Fraction 4 and Fraction 5.

The membrane orientation in Fractions 4 and 5 was then examined using acetylcholinesterase and sialic acid was external plasma membrane markers. Fraction 4 was found to contain mainly inside-out vesicles in contrast to Fraction 5, which was thought

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to consist mainly of right-side-out plasma membrane vesicles. The orientation differences were further examined using lactoperoxidase catalyzed iodination using ^{125}I.

Iodination of Fraction 4 resulted in the appearance of ^{125}I in a band migrating in SDS electrophoretigrams with an apparent molecular weight of 100,000 daltons, and minor labelling was seen at 205,000 and 55,000 daltons. 0.05% Triton X-100 significantly enhanced labelling of all three bands. Iodination of Fraction 5 resulted in labelling of all three bands, but treatment of the membranes with Triton X-100 enhanced labelling only at 100,000 daltons. Iodination of intact single cells resulted in an iodination pattern similar to that of Fraction 5 in the absence of Triton X-100.

Attempts were made to further purify the membranes using concanavalin A - Sepharose affinity chromatography. After Fraction 4 was applied to the column, four peaks of protein could be eluted. The first two peaks, eluted in the absence of α methyl-D-mannoside were thought to consist of inside-out vesicles as judged by iodination and acetylcholinesterase sidedness studies. The other two peaks, eluted in the presence of α methyl-D-mannoside were thought to contain unsealed plasma membrane vesicles. Over 90% of the originally applied protein was eluted, 20% being contained in the two peaks eluted in the presence of α methyl-D-mannoside.

Fraction 5 behaved quite differently on the affinity columns. Approximately 90% of the originally applied protein could not be eluted even in the presence of α methyl-D-mannoside.

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Of the two peaks eluted, one peak obtained in the absence of α methyl-D-mannoside, was thought to consist of inside-out plasma membrane vesicles. The second peak, eluted in the presence of α methyl-D-mannoside was thought to contain unsealed membrane vesicles as indicated by sidedness studies.

It was concluded that Fractions 4 and 5 represent plasma membrane preparations of differing orientation, Fraction 5 being predominantly right-side-out and Fraction 4 after affinity chromatography mainly insight-out. These two fractions may have some applicability in investigating the asymmetry of various membrane transport systems.

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INTRODUCTION

Although the studies with isolated preparations rich in smooth muscle have provided valuable information on many features of membrane phenomena in this type of cells, perusal of the literature reveals that some basic questions have not been answered satisfactorily as yet. For example, it is recognized that the mechanism of Ca extrusion from the cell plays a key role in the control of tension, but the present knowledge of the mechanism is rather incomplete. Indeed, it has not even been established whether the energy required for extrusion of Ca against a steep gradient of the negative chemical potential of this ion is provided by hydrolysis of ATP, or spontaneous influx of Na (BLAUSTEIN, 1977).

It must be appreciated, however, that study of these and other problems is complicated by factors largely unique to smooth muscle preparations. The dimensions of smooth muscle cells make it rather unlikely that a number of the methods already applied successfully to studies on other types of muscle cells, such as the monitoring of the intracellular concentration of Ca^{2+} by measurement of the luminiscence of injected aequorin (ALLEN & BLINKS, 1978) can be used for smooth muscle studies. Secondly, the large extracellular space with its high concentration of fixed charged groups makes it difficult, if not impossible, to obtain reliable information on the transmembrane ion fluxes from evaluation of isotope flux data. Last, but not least, cells other than smooth muscle are frequently present in isolated preparations, and unless this fact is taken into account, some of the observations may be interpreted incorrectly.

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To eliminate the last two factors, isolated individual cells have been introduced by the group of FAY (FAY, 1973; FAY, 1977; SCHEID et al., 1979), but the studies have been restricted almost exclusively to cells isolated from the stomach muscularis of Bufo marinus by enzymic digestion. It remains to be shown that this approach would apply equally well to smooth muscle cells from mammaliam tissues.

An attractive alternative is to study membrane phenomena using the isolated plasma membrane. The advantages that this approach offers are obvious. Unfortunately, relatively little attention has been paid to the fact that, if meaningful results are to be obtained, the preparation must meet certain criteria.

If the preparation were to be used, e.g., for a study of Ca transport, it should be at least essentially free of contamination by membranes of sarcoplasmic reticulum. In other types of muscle this cellular component is known to exhibit a Ca^{2+} - stimulated ATPase activity, which, in contrast to the mitochondrial Ca^{2+} - stimulated ATPase, cannot be inhibited selectively (CARAFOLI & CROMPTON, 1978). Clearly, an ideal preparation should be free of contamination by membranes derived from intracellular organelles.

There are, however, other criteria which an ideal preparation should meet. In order to remove the contaminating membranes and components of the cytoplasm, a multi-step fractionation procedure is usually required. The problem here

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is that, in the course of fractionation, some important, but loosely bound components of the plasma membrane may become lost, or, conversely, some components that are not associated with the membrane in situ may become firmly attached to the final preparation. Ideally the process of fractionation should produce a preparation whose compoisition, conformation and other features are identical to those exhibited by the plasma membrane in situ.

This problem is much more complicated than it may seem to be at first sight, because the membrane in situ is typically under the influence of an electrical field of appreciable strength and the latter may have a pronounced effect on the conformation and disposition in the membrane of any component possessing at least a dipole or a charged group. The significance of the fact that the two surfaces of the membrane are typically exposed to solutions of markedly different composition should not be underestimated.

Finally, if the membrane isolation procedure is to be of any value, its yield should be reasonably high, particularly because the quantity of tissues rich in smooth muscle that can be obtained from typical laboratory animals is usually quite limited.

A. Isolation of Plasma Membranes

In general, the initial step in the preparation of isolated plasma membranes is homogenization, which is usually achieved by application of shear forces (BIRNIE, 1972). This is followed by differential centrifugation to separate the plasma

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membranes from the cell debris, nuclei and mitochondria (GRAHAM, 1972; GRAHAM, 1975; NEVILLE, 1975; SCHAPIRA, 1975; SCHIMMEL & KENT, 1977; WALLACH & SCHMIDT-ULLRICH, 1977). The resulting crude "microsomal" preparation can be further fractionated by density gradient centrifugation. Typically, plasma membranes and endoplasmic reticulum are found at lower densities (1.14 - 1.15 g x cm^{-3}) while mitochondria are at higher densities (1.16 - 1.18 g x cm^{-3}) (PRICE, 1974; TOLBERT, 1974). If warranted, further purification of the preparation can be attempted using techniques such as affinity chromatography (CUATRECASES, 1973; PHARMACIA, 1974; SHARON & LIS, 1975; HYNES, 1976; WALSH et al., 1976; BRUNNER et. al., 1977).

The homogenization technique is of critical importance in that it also determines the final yield of plasma membranes. There are three basic types of homogenization mechanical, liquid and gaseous (BIRNIE, 1972; GRAHAM, 1975; WALLACH & SCHMIDT-ULLRICH, 1977). Mechanical preparation using mechanical shear employs two basic techniques. The first, the freeze thaw technique, used less frequently, involves using cycles of freezing and thawing which result in the disruption of cells by intracellular ice crystal formation. In the second type of mechanical shear, employed by commercial units like the Polytron and MSE homogenizers, the sample is drawn into a working head where it is mixed by rotating blades and sheared during expulsion from the working head. Membranes are usually obtained in the form of vesicles rather than cell sheets. Until recently, it was felt that the drawbacks of mechanical shear far outweighed the advantages.

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Drawbacks to this method include the possible damage to the plasma and intracellular membranes by the high local temperatures generated during homogenization and disruption of most organelles. On the other hand, mechanical shear of this type if quite effective even for homogenization of tissues rich in collagen and elastin.

In liquid shear homogenization, the disruptive forces are considerably weaker than those of mechanical shear. Tissues are disrupted by being forced through a narrow space between a moving pestle and the wall of the containing vessel. Using this method, soft tissues are readily homogenized without concomitant disruption of cellular organelles. In the homogenate, the plasma membranes are usually present in the form of sheets rather than vesicles, but spontaneous vesicle formation occurs with time. Commercially available homogenizers of this type include the Potter-Elvehjem and the Dounce homogenizers.

The third method of homogenization is gaseous shear. Nitrogen cavitation using a Parr bomb involves equilibrating a stirred cell suspension with oxygen-free nitrogen at pressures between 500 and 800 lb./in.^{2} for periods of 15-20 minutes (HUNTER & COMMERFORD, 1961). Cell disruption occurs upon sudden release of the pressure due to the gas expanding within the cell or by the shearing forces of the rapidly forming bubbles of gas in the liquid phase. Gaseous shear, however, requires that the tissue be in the form of isolated single cells. The preparation of single cells itself is associated with drawbacks including alteration of the plasma membranes by proteases found in commercial collagenase preparations (RODBELL, 1964; BAGBY et al., 1971; FAY & DELISE, 1973;

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RODBELL & KRISHNA, 1974; SMALL, 1977). The plasma membranes (PMs), endoplasmic reticulum and nuclear membranes form very small vesicles, which makes further density gradient separations necessary. Other cell organelles are generally maintained intact.

Osmotic lysis (BARBER & JAMIESON, 1973) used infrequently, has been applied to the isolation of membranes from skeletal muscle (McCOLLESTER, 1962). In this method, segments are incubated at high temperatures and then excess distilled water is added. This leads to an abrupt dissolution of intracellular components, leaving only muscle plasma membranes. At the same time, however, partial solubilization of peripheral PM proteins occurs accompanied by an increase in membrane permeability.

With few exceptions, none of the shear techniques provides perfect homogenization of the starting material. It is often necessary, therefore, to remove the unhomogenized fragments by filtration. The filtrate contains in addition to plasma membranes, intact and disrupted organelles as well as soluble and insoluble components of the cytoplasm. As mentioned earlier, the filtrate is then fractionated by differential centrifugation, a technique based on differences in buoyant densities of the various cell components. For example, nuclei sediment rapidly in a gravitational field of 2000 g. After the cell debris, nuclear membranes, intact mitochondria and other organelles have been removed by centrifugations at lower g forces (2000 g - 17,000 g), the microsomal fraction containing PM is usually pelleted by centrifugation at 100,000 g (GRAHAM, 1975;

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SCHIMMEL & KENT, 1977; WALLACH & SCHMIDT-ULLRICH, 1977).

Many investigators consider the 100,000 g pellet sufficiently enriched in plasma membranes to warrant its use in various studies. However, it has clearly been demonstrated that this enriched 100,000 g plasma membrane fraction still contains contamination from fragmented organelles. These fragments, oftenin the form of vesicles, may seriously bias any results observed in this plasma membrane fraction. The demonstrated presence of contamination warrants a third stage of membrane isolation such as the use of density gradient centrifugation. This involves centrifugation of the microsomal fraction on a sucrose density gradient at high g forces (120,000 g) for long periods of time (2-24 hours) which allows the individual components of the microsomal fraction to reach their own buoyant densities. The gradient used must span the full range of densities exhibited by the various membrane fragments present. The gradient material must be water soluble, which requirement is not met by either dextran or Ficoll. Separations that do use the latter rely on differences in quantitative rises in buoyant density when fixed charges are neutralized by Mg^{2+} (STECK, 1974a; GRAHAM, 1975) but in general, sucrose gradients are more commonly used. The plasma membranes fraction derived from the sucrose gradient is quite often substantially free from contamination by intracellular membranes, though not necessarily 100% pure. This raises a number of important questions. Can or should the membranes be further purified and how does one assess the increases in plasma membranes not only in a fourth stage of membrane isolation but in each stage?

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Further purification of the plasma membranes has involved one basic approach. That is, by selectively altering the density of the PM in stage 1 or stage 3, better separation can be effected based on larger differences in the buoyant densites of plasma membranes as compared to intracellular membranes. This has been achieved by attaching lectins to plasma membrane carbohydrate moieties (CUATRECASES, 1973; NICHOLSON, 1974; SHARON & LIS, 1975; HYNES, 1976; BARCHI et al., 1977; WALLACH & SCHMIDT-ULLRICH, 1977). The labelling of the PM with plastic microspheres affords the same advantage (LIM et al., 1975). Digitonin, incubated with the microsomal pellet prior to gradient centrifugation, has been found to increase the density of PM over other intracellular membranes (LEWIS et al., 1975; MAGARGAL et al., 1978). Not only is it possible to alter the density of the plasma membrane, but also that of the contaminating components. A good example of this is seen in the separation of plasma membranes from sarcoplasmic reticulum in heart muscle (LEVITSKY et al., 1976). The SR was allowed to accumulate Ca in the presence of ATP and oxalate and the SR then removed by gradient centrifugation.

The validity of the whole process of membrane isolation rests on the ability to assess the results of each step used. The degree of plasma membrane purification can be monitored in two ways. Removal of the contaminants can be assessed by selectively measuring a feature such as an enzymatic activity of the respective organelle in the supernatant and pellet. A few examples of enzymatic markers are: mitochondria (BONNER, 1955; DONALDSON et al., 1972; TOLBERT, 1974): succinate dehydrogenase, cytochrome c oxidase, fumarase and NADH cyt c reductase (rotenone sensitive);

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GOLGI APPARATUS (FLEISCHER & KERVINA, 1974): galactosyltransferase; LYSOZOMES (HUBSCHER & NEST, 1965; HODGES & LEONARD, 1974): acid phosphatase; ENDOPLASMIC RETICULUM (NORDLIE & ARION, 1966; TOLBERT, 1974): glucose-6-phosphatase, NADPH cyt c reductase and protein synthesis. Some of the above markers are found with a non-specific distribution in other organelles, and therefore have limited value as markers. The above assessment may be supplemented by semi-quantitative examination of the fraction under the electron microscope.

The gradual enrichment of plasma membranes can be followed by markers specific for the plasma membrane (WALLACH & WINZLER, 1974). Such markers include endogenous chemical markers (cholesterol/phospholipid ratios), enzyme markers (5' nucleotidase, adenylate cyclase, ATPases) (RODBELL & KRISHNA, 1974; WIDNELL, 1974), virus receptors, covalent labels and immunological markers. Closely related to the last marker are lectin binding sites. Lectins are thought to bind to cell plasma membrane surfaces reacting with terminal non reducing sugars in glycoproteins and/or lipids. Inherent in this work is the assumption that the label does not permeate the plasma membrane (SCHIMMEL & KENT, 1977; WALLACH & SCHMIDT-ULLRICH, 1977).

Once it has been established that the plasma membrane preparation is of acceptable purity, one must consider the state of the plasma membranes themselves. It is known that the plasma membranes can be in the form of membrane sheets or vesicles. Also, the vesicles, may be right-side-out (RO) or

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inside-out (IO). Therefore, before using the preparation to investigate plasma membrane properties we must know the orientation of the membrane. As well, it may be possible to invoke a fifth stage of membrane isolation to separate out the unsealed vesicles, RO vesicles and IO vesicles. This would allow one to examine specific phenomena associated with the cytoplasmic membrane surface and/or the external membrane surface.

B. The Orientation Problem

Each biological membrane operates differently on the two compartments it separates. Being anisotrophic in its function, the plasma membranes has been shown to be assymetric with respect to the composition of the two surfaces (STECK, 1974b). Identification of the components at each surface would do much to define the structure.

Investigators have approached the problem of orientation from two directions (STECK, 1974a; STECK & KANT, 1974). The initial one involves the assessment of membrane sidedness using marker enzymes specific for either the external or cytoplasmic surface. Detergents can be used to make both membrane surfaces equally accessible to the enzyme substrates used. Selective protein extraction also occurs during detergent treatment (HELENIUS & SIMONS, 1975; TANFORD & REYNOLDS, 1976). This must be appreciated. Results observed using preparations containing a mixture of right-side-out, inside-out and unsealed plasma membrane vesicles are thought to reflect the general orientation of the membranes.

The second approach to elucidation of orientation involves assessment as above, combined with a fifth stage of membrane

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fractionation in which attempts are made to separate the purified plasma membrane preparation further on the basis of membrane orientation. In general there are three techniques available for a fifth stage of isolation. These are the use high polymer gradients (STECK, 1974a) combined with aqueous partition/counter current distribution (AP/CCD) (DODGE et al., 1963; ALBERTSSON, 1970; WALTER & KROB, 1976; WALTER, 1978), free flow electrophoresis (FFE) (HANNIG & HEIDRICH, 1974; HANNIG, 1975a; HANNIG, 1975b) and affinity chromatography (MURTHY & HERCZ, 1973; PHARMACIA, 1974; HYNES, 1976; WALSH et al., 1976; BRUNNER et al., 1977). Each has been applied with varying degrees of success.

High density Ficoll polymer gradients combined with AP/CCD have proven ideal for separating membranes of differing orientation from certain tissues (STECK, 1974a). Sealed vesicles are first separated from unsealed ones using Ficoll density gradients. The separation depends on the fact that sealed vesicles are not collapsed as on sucrose gradients, but instead they expand. They therefore have buoyant densities different from unsealed vesicles. It is the low solubility and permeability of Ficoll which permits this. Glycerol, for example, is not acceptable since it readily permeates sealed vesicles. Once the sealed membranes have been separated from the sealed vesicles, AP is used to separate the two populations of vesicles remaining. Aqueous partition separations rely on the exploitation of subtle physicochemical differences between the two different membrane surfaces. These surface properties are highly dependent on ionic conditions and pH. One can further effect separations by modifying the polymers used in the partition. Utilization of

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polymer ligands, such as derivatives of dextrans and lectins, specific for membrane receptors shows great promise.

The most well known application of the above technique is that of Steck (STECK, 1974a) using red blood cell ghosts (rbcg). In the initial investigation he localized marker enzymes on both membrane surfaces. Localized on the cytoplasmic surfaces of rbc were Na+/K+ ATPase, gylceraldehyde-3-phosphate dehydrogenase, adenylate cyclase, protein kinase and NADH cyt c reductase. Externally localized were acetylcholinesterase, sialic acid residues and the ouabain binding site of Na+/K+ ATPase. These markers were used to check on the isolation of vesicles with different orientations. Unsealed ghosts were removed from RO and IO vesicles by Ficoll gradients. A dextran T110 and glycol 6000 partition was then used to separate the RO vesicles from the IO vesicles; with separation occurring only under certain ionic conditions and temperatures. Interestingly it was observed that high ionic strength lead to accumulation of all the vesicles at the interface. Halide ion drove the vesicles into the lower phase while phosphate reversed this trend.

Under investigation as a technique for the separation of RO vesicles is free flow electrophoresis (FFE) (HANNIG & HEIDRICH, 1974; HANNIG, 1975a; HANNIG, 1975b). This method exploits differences in surface charge, density and size between RO and IO vesicles. The mixture of plasma membrane vesicles is injected into a continuously flowing buffer with an applied electric field at right angle to the flow direction.

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The vesicles separate according to electrophoretic mobility during flow. Problems arise since thermally undisturbed flow of the liquid curtain can only be obtained at high flow velocities which may result in turbulence. Sufficient deflection only occurs with longer and smaller chambers and at high field strengths. The latter is ruled out as the heat generated increases with the field strength squared but by employing very low ionic strength media for the separation buffer the heat problem can be avoided. As well, it should be noted that higher liquid curtain velocities further reduce the heat problem, the probability still exists that turbulence may occur.

The limiting factor at low ionic strengths appears to be the instability of the vesicles (STECK, 1974a). Further effects can occur with contamination from DNA, RNA and cell nuclei which bind to the membrane surfaces screening charge. To date, RO and IO rbc vesicles, viruses, bacteria, proteins and nuclei acids have been separated.

The last major method of current practical use in the separation of preferentially oriented vesicles is affinity chromatography and related techniques. Lectins, such as, WGA, Con A, RGA, are covalently linked to solid supports like dextran, agarose, sepharose or nylon fibres. RO vesicles and unsealed vesicles are thought to bind to the covalently linked lectin by specific sugar moities located on the external plasma membrane surface while IO vesicles are not absorbed and pass through the column. The bound membranes are then eluted by adding a sugar which competes with the membranes for the lectin binding sites.

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As mentioned earlier a variation on this theme includes adding lectin to a mixture of RO and IO vesicles followed by density gradient centrifugation to separate the higher density RO vesicles from the IO ones (CUATRESAS, 1973).

There have been two major studies using Con A affinity chromatography to separate populations of IO and RO vesicles, but with conflicting results. Using porcine lymphocyte homogenates Walsh (WALSH et al., 1976) isolated what appeared to be IO lymphocyte plasma membranes as judged by marker studies, immunoprecipitation, and ferritin linked Con A experiments. 40% of the protein applied to the column was recovered. However, 50% could not be eluted under any conditions and appeared to be due to high affinity non-specific multivalent binding. The study also revealed a number of drawbacks to this type of membrane purification. Lectins inhibit certain enzymes and also cause capping of surface markers. The low osmolarity of the buffers may lead to an increase in membrane permeability.

In the second study (BRUNNER et al., 1977), anywhere from 60 to 70% of the applied membranes remained bound to the lectin linked sepharose beads. The membranes, however, could be eluted in the presence of α methyl-D-mannoside accompanied by mechanical stirring of the Con A linked sepharose beads. Whether the eluted protein was Con A or membranes is subject to conjecture as no electrophoretic gels were run and no enzyme marker assays were done. As well, mechanical stirring of the beads may have been associated with fragmentation.

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The above technique has the advantages of being relatively inexpensive and easy to carry out. However, the fact that 50% of the protein is often not recoverable does not auger well for the isolation of a RO set of membranes.

All the previous methods outlined involve the use of a membrane preparation that has a variable ratio of RO:IO:unsealed membrane vesicles which may be dependent upon the homogenzation method used. The separation procedures could be eliminated if it became possible to control the membrane orientation during preparation and predict membrane orientation based on the method of preparation. To date both questions remain basically unanswered. Only Steck (STECK, 1974a; STECK & KANT, 1974) has successfully prepared IO and RO vesicles from rbc ghosts, by varying the ionic milieu. While it is still not clear how the ionic milieu determines vesicle sidedness, one might speculate that the labelling of plasma membranes with lectins prior to homogenization may alter the RO:IO membrane vesicle ratio.

Despite the many methods available and the many theories on how to increase yields of preferentially oriented vesicles, the attainment of this goal is still quite far away. The IO and RO plasma membranes isolated from the rbc membrane were the result of fortunate observation and rigorous characterization. It is only through the latter that we can begin to understand which factors are responsible for the orientations seen in plasma membrane preparations.

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There are many plasma membrane preparations from various tissues which suffer from problems of contamination and indefinite membrane orientation. These preparations often have failed to use more than 2 stages in the membrane isolation while others have failed to carry out proper characterization of the membrane preparation. These deficiencies are no more apparent than in the case of smooth muscle though more recently some good studies have appeared.

C. Smooth Muscle Preparations

As shown in Table I, plasma membrane fractions derived from smooth muscle have been prepared by liquid and mechanical shear techniques. Many of the investigations lack proper evaluation of the purity of their plasma membrane preparations. Most studies in the past simply used differential centrifugation to obtain a 100,000 g microsomal plasma membrane preparation (PREISS & BANASCHAK, 1975; ZELCH et al., 1975; GODFRAIND et al., 1976; RANGACHARI et al., 1976; WEBB & BHALLA, 1976; NISHIKORI et al., 1977; BHALLA et al., 1978a, 1978b). Some have replaced the 100,000 g pellet by a 40,000 g pellet (SHIBATA & HOLLANDER, 1974; FITZPATRICK & SVENTIVANYI, 1977). More recent studies combine differential centrifugation with simple four step gradients (WEI et al., 1976a, 1976b, 1976c; JANIS et al., 1977; AKERMAN & WIKSTROM, 1978; VALLIERES et al., 1978). The smooth muscle used was obtained from arteries, the uterus and the ileum.

The simplest of all plasma membrane preparations would be to use the crude homogenate. This kind of approach serves no real

purpose under these conditions because of the contamination by organelles and problems with vesicle orientation. Measurement of membrane ATPase activities is contradicted due to the presence of actinomyosin ATPase. Furthermore the lysozomal enzymes will eventually degrade all membrane systems.

Microsomal preparations of smooth muscle have involved the use of a 4,000 g or 100,000 g pellet. Many of these studies were done using vascular smooth muscle. The main emphasis of these studies was Ca^{2+} transport and measurement of ATPase activities associated with the membranes. The characterization of the membranes varied from the combination of electron microscopy with one marker such as succinate dehydrogenase (ZELCK et al., 1975; CYLMAN et al., 1976; NISHIKORI et al., 1977; KRALL et al., 1978) to more exhaustive marker studies (CHATURVEDI et al., 1978; KUTSKY & GOODMAN, 1978; THORENS & HAUESLER, 1978; MATLIB et al., 1979; THORENS, 1979) - See Table 1. The limited use of markers in a microsomal preparation is unacceptable. To measure Ca^{2+} uptake or release in a membrane preparation without knowing the various possible types of membranes present is unadvisable. A good example of this is in 100,000 g microsomal preparations considered by many to be sarcoplasmic reticulum (SHIBATA & HOLLANDER, 1974; WEBB & BHALLA, 1976; FITZPATRICK & SZENTIVANYI, 1977; BHALLA et al., 1978a, 1978b). This hypothesis has been advanced on the basis of the marker enzyme NADH cyt c reductase. While mitochondria are excluded by electron microscopy studies the probability that plasma membranes are present is acknowledged but rarely checked by using standard marker enzymes and no attempts have been made to determine the

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orientation and/or permeability of the membrane, this latter point being crucial in the observation of efflux or influx of various ions. The use of NADH cyt c reductase as a sarcoplasmic reticulum marker is now suspect as this activity has also been shown to be present in the plasma membranes and other cell organelles (SOTTOCASA, 1967; SOTTOCASA, 1971; KELBERG & CHRISTENSEN, 1979). Electron microscopy is at best only semi-quantitative. Also, the presence of plasma membranes in the 100,000 g sarcoplasmic reticulum pellet has been unequivocally demonstrated (HURWITZ et al., 1973; MOORE et al., 1975; VALLIERES et al., 1978; WUYTACK et al., 1978; MATLIB et al., 1979; THORENS, 1979). It has been shown that this pellet contains sufficient mitochondrial and plasma membranes to effect Ca^{2+} accumulation observed in the 100,000 g pellets.

Another drawback to studies using 100,000 g pellets has sarcoplasmic reticulum or plasma membranes preparations is seen in studies comparing Ca^{2+} uptake in microsomes prepared from the aortas of hypertensive and normotensive rates (BHALLA et al., 1978b). It is thought that certain ultrastructural changes occur in smooth muscle cells of aortas in hypertensive animals. To treat both aortas under identical preparation conditions is questionable because it is by no means certain that both tissue types will behave in the same manner. Furthermore, it is difficult to assign much significance to studies of Ca^{2+} uptake by vesicles whose origin, orientation and contamination by other membranes is not known.

Many investigators realizing the drawbacks of the microsomal preparations have further purified the plasma membranes gradients (KIDWAI, 1974; MAGARGAL et al., 1978; WUYTACK et al., 1978; MATLIB et al., 1979; THORENS, 1979). Most studies have

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utilized visceral smooth muscle. Several investigators have carried out extensive characterization of the final preparations. A typical example is seen in the preparations of myometrial plasma membranes. The sarcoplasmic reticulum, prior to homogenization, was loaded with ^{3}H-leucine and this was used to check the viability of NADH cyt c reductase as a sarcoplasmic reticulum marker (MATLIB et al., 1979). Electron microscopy can be made semi-quantitative by attaching WGA Lectin to the plasma membranes prior to homogenization (VALLIERES et al., 1978). It was fully appreciated that this may change membrane densities.

Invesitgations by various authors to find suitable markers for smooth muscle cell components have met with some success (MAGARGAL et al., 1978; VALLIERES et al., 1978; MATLIB et al., 1979). By changing the density of plasma membranes on the gradient using digitonin, it has been shown that oleyl CoA: lysolecithin acetyltransferase is a specific marker for the SR (MAGARGAL et al., 1978).

Interesting results have been obtained using gradient centrifugation. The first of these is a Mg^{2+} - stimulated ATPase thought to be located in the plasma membrane fractions which may be the Mg^{2+} - stimulated ATPase seen in the so called 100,000 g sarcoplasmic reticulum preparations (MOORE et al., 1975; WEI et al., 1976; JANIS et al., 1977; VALLEIRES et al., 1978; MATLIB et al., 1979). This Mg^{2+} - stimulated ATPase obscures any Na+/K+ ATPase activity that might be present in the membrane preparation.

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The second interesting point is that the location of the plasma membranes on the gradients appears to be variable. In some preparations using the rat aorta, the plasma membranes are found at higher densities in the gradient than are the sarcoplasmic reticulum membranes. This is in contrast to the results of other studies in which the plasma membranes are found at lower densities relative to the sarcoplasmic reticulum (HURWITZ et al., 1973; MOORE et al., 1975; WEI et al., 1976a, 1976b; THORENS & HAEUSLER, 1978). It may be that the plasma membranes are binding to denser fragments of other membranes.

Studies using gradient centrifugations to compare plasma membranes from arteris of hypertensive animals to those of non-hypertensive animals raise similar questions to those mentioned earlier in the discussion of microsomal preparations (MOORE et al., 1975; WEI et al., 1976c). What is necessary in these studies are marker and sidedness assays of each fraction obtained after each step in the procedure. Fortunately, this is now being done (VALLIERES et al., 1978; MATLIB et al., 1979).

It would be very improper to assume that the drawbacks mentioned above apply universally to all plasma membrane preparations. This is obviously incorrect, since there have been some nice studies on membrane characterization of skeletal, cardiac and even smooth muscle. It is apparent, however, that, until recently, the majority of smooth muscle preparations have been inadequately characterized.

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D. Rationale

The basic aim of the present study was to prepare and characterize a well defined preparation of plasma membranes from smooth muscle. Such a preparation requires a tissue rich in smooth muscle cells and relatively free from fat as well as connective tissue. The smooth muscle should be readily available in large quantities as well. Ideally suited for these purposes is the chicken stomach muscularis. This muscle, based on the results of various studies (CALHOUN, 1954; McCLEOD et al., 1964; KING & McCLELLAND, 1975; SOBIESZEK & SMALL, 1976) is thought to consist principally of visceral smooth muscle. A certain degree of caution is required as skeletal muscle is present in neighbouring regions of the digestive tract. As much as 10 to 15 grams of smooth muscle can be obtained from one domestic chicken.

The next stage in the preparation of the plasma membranes is homogenization. Liquid shear would require that the tissue be dispersed into single cells prior to homogenization, a process that may effect the plasma membranes of the cells. The best approach at first is to use mechanical shear of muscle cubes by a Polytron homogenizer. It was borne in mind that the conditions used could be optimized to give maximal plasma membrane yields at the various stages of the procedure.

The crude homogenate contains a mix of cell components, many of which are fragmented. Differential centrifugation combined with marker studies allows removal of much of the contamination. However, differential centrifugation in itself

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would not yield a sufficiently purified plasma membrane preparation requiring a fourth stage of isolation. Sucrose gradients are the easiest to use and allow for fine separations of contaminating organelles. The gradients used would have to be sufficiently long and large to allow for this separation. The final plasma membrane preparation obtained from the gradient, once characterized for purity and sidedness, was expected to be basically free of contaminants. It is unlikely, however, that the vesicles would have uniform orientation of the membrane. If the membranes are ill defined with regards to their orientation it would become necessary to further purify the preparation. There wre three choices. Free flow electrophoresis is very expensive, the resolution is poor and the experimental conditions are quite varialbe. AP/CCD is dependent on many variables and there are difficulties encountered in removing partitioning compounds from the membranes. The easiest method to use seemed to be affinity chromatography, and if a suitable ligand can be found, the most efficient. Its only drawback is the danger of high affinity non specific binding of the plasma membranes. As a possible method of first choice it should be used. Failure of this technique would mean resorting to FFE or AP/CCD. What is extremely important is that the membranes be characterized at each step in the procedure with respect to purity and if at all possible with respect to sidedness, the latter being very critical in the latter stages of the isolation procedure. It was hoped that this approach would make it possible to obtain a suitable plasma membrane preparation.

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Table I

Summary of smooth muscle preparations.

1. Source of smooth muscle - aorta

AUTHORS	METHODS USED	PREPARATION	CHARACTERIZATION
PETERS et al., (1972)	Dounce homogenization Differential centrifugation Sucrose gradients	Single cells Lysozomes	Cytochrome oxidase 5' Nucleotidase DNA Phosphatases Glycosidases N - acetyl - glucosaminidase Napthylamidase Cathepsin A-D Monamine oxidase
HURWITZ et al., (1973)	Homogenization not indicated Differential centrifugation Sucrose gradients	Plasma membranes	Na^+/K^+ ATPase Ca^{2+} uptake 5' Nucleotidase NADH oxidase
HESS & FORD (1974)	Homogenization not indicated Differential centrifugation	Microsomal	Ca^{2+} uptake Cytochrome oxidase $Na^+/K+$ ATPase 5' Nucleotidase Mg^{2+} ATPase
KIDWAI (1974)	Polytron homogenization Differential centrifugation Sucrose gradients	Plasma membranes Mitochondria	Succinate dehydrogenase 5' Nucleotidase NADH oxidase
BHALLA et al., (1978a, 1978b) FITZPATRICK & SZENTTIVANYI (1977) MOORE et al., (1975) SHIBATA & HOLLANDER (1974) WEBB & BHALLA (1976)	Polytron homogenization Differential centrifugation	Microsomes Plasma membranes Sarcoplasmic reticulum	Ca^{2+} ATPase (N_3^- insensitive) cAMP Cytochrome oxidase Succinate dehydrogenase Phosphodiesterase I

Table I (cont'd)

1. aorta (cont'd)...

AUTHORS	METHODS USED	PREPARATION	CHARACTERIZATION
WEI et al., (1976a)	Polytron homogenization Differential centrifugation Sucrose gradients	Plasma membranes Mitochondria Endoplasmic reticulum	Phosphodiesterase I 5' Nucleotidase Cytochrome c reductase K^+ phosphatase (ouabain sensitive) ATPases Ca^{2+} accumulation Electron microscopy
CHATURVEDI et al., (1978) THORENS & HAEUSLER(1978)	Potter Elvehjem homogenization Differential centrifugation Sucrose gradients	Microsomes Plasma membranes	Na^+/K^+ ATPase 5' Nucleotidase Glucose-6-phosphatase NADH oxidase Cytochrome oxidase Ca^{2+} uptake
KUTSKY & GOODMAN (1978)	Polytron homogenization Differential centrifugation	Microsomes	5'Nucleotidase Mg^{2+} ATPase Succinate-cytochrome c reductase NADPH cyt c reductase Ca^{2+} uptake
MAGARGAL et al., (1978)	Vortex, Dounce homogenization Differential centrifugation Sucrose gradients Digitonin treatment	Plasma membranes	NADH ferricyanide reductase NADH cyt c reductase (rotenone insensitive) NADPH cyt c reductase Cytochrome oxidase 5' Nucleotidase Monamine oxidase Alkaline Phosphodiesterase I Acyl transferase
WEI et al., (1976b,1976c) THORENS (1979) Mesenteric artery used.	Polytron, Potter Elvehjem homogenization Differential centrifugation Sucrose gradients	Plasma membranes Mitochondria Endoplasmic reticulum	5' Nucleotidase Na^+/ K^+ ATPase Alkaline phosphatase Phosphodiesterase I Cytochrome c oxidase Ca^{2+} uptake
CLYMAN et al., (1976) Umbilical artery used.	Potter Elvehjem homogenization Differential centrifugation	Microsomes	None

Table I (cont'd)

1. aorta (cont'd)...

AUTHORS	METHODS USED	PREPARATION	CHARACTERIZATION
PREISS & BANASHEK(1975) Carotid artery used.	Homogenizer not indicated Sucrose gradient	Microsomal	Adenylate cyclase 5' Nucleotidase
WUYTACK et al., (1978) Coronary artery used.	Potter Elvehjem homogenization Differential centrifugation Sucrose gradients	Plasma membranes	Ca^{2+} uptake NADH cytochrome c reductase (rotenone insensitive) Choline phosphotransferase Cytochrome oxidase

2. Smooth muscle source - ileum

AUTHORS	METHODS USED	PREPARATION	CHARACTERIZATION
GODFRAIND et al., (1973,1976,1977)	Potter Elvehjem homogenization Differential centrifugation	Microsomes Mitochondria	Ca^{2+} uptake ATPases 5' Nucleotidase Cytochrome oxidase
ZELCK et al., (1975)	Homogenizer not indicated Differential centrifugation	Nuclei Mitochondria	Electron microscopy Succinate dehydrogenase
NILSSON et al., (1977)	Homogenizer not indicated Differential centrifugation Sucrose gradients	Plasma membranes Mitochondria Nuclei	5' Nucleotidase Phosphodiesterase I ^{3}H-leucine uptake Cholesterol/phospholipid Cytochrome c oxidase ^{3}H-ouabain binding
RAEYMAEKERS et al.,(1977)	Potter Elvehjem homogenization Differential centrifugation	Mitochondria	O_2 consumption Cytochrome c oxidase Ca^{2+} uptake

Table I (cont'd)

3. Source of smooth muscle - myometrium

AUTHORS	METHODS USED	PREPARATION	CHARACTERIZATION
JANIS et al., (1976)	Polytron homogenization Differential centrifugation Sucrose gradients	Plasma membranes Mitochondria Smooth endoplasmic reticulum Rough endoplasmic reticulum	5' Nucleotidase glucose-6-phosphatase Mg^{2+} ATPase Ca^{2+} uptake Ouabain sensitive phosphatase Cytochrome c oxidase
RANGACHARI et al., (1976)	Potter Elvehjem homogenization Sucrose gradients	Microsomes	Adenyl cyclase 5' Nucleotidase
MATLIB et al., (1979)	Polytron homogenization Differential centrifugation Sucrose gradients	Plasma membranes	Succinate cytochrome c reductase NADH cyt c reductase (rotenone sensitive and insensitive) Monamine oxidase ^{3}H-WGA labelling of PM ^{3}H-oxytocin binding sites Mg^{2+} ATPase 5' Nucleotidase NADPH reductase
NISHIKORI et al., (1977)	Polytron homogenization Differential centrifugation	Microsomal	Succinate dehydrogenase
KRALL et al., (1978)	Polytron homogenization Differential centrifugation	Microsomes	Protein Kinase
VALLIERES et al., (1978)	Parr bomb Differential centrifugation Sucrose gradients	Plasma membranes	Adenyl cyclase 5' Nucleotidase Mg^{2+} ATPase Phosphodiesterase I Cytochrome c oxidase ^{125}I-WGA labelling of plasma membranes (PM) Electron microscopy

MATERIALS

Concanavalin A - linked to Agarose (Con A-Agarose), α methyl-D-mannoside, Triton X-100, lactoperoxidase, acrylamide, ammonium persulphate, Tris (hydroxymethyl) aminomethane (TRIS), 2 (N-Morpholino) ethane sulphonic acid (MES) and all enzyme substrates were obtained from Sigma Chemical Company, vanadium-free ATP from Boehringer Manheim, concanavalin A-linked to Sepharose (Con A-Sepharose) and Sepharose 4B from Pharmacia Fine Chemicals, collagenase (Grade A) from Cal Biochem, Coomassie Blue R-250, sodium dodecylsulphase (SDS), N,N,N',N' - Tetramethyl ethylenediamine (TEMED) from Biorad Laboratories.

Radioactive carrier free [^{125}I] NaI (100 mCi/ml) was obtained from Amersham and New England Nuclear. Adenosine 5'-triphosphate, tetra (triethylammonium) salt, [γ-^{32}p] (20-40 Ci/mmol) was purchased from New England Nuclear as well as Omnifluor. Unless otherwise stated, scintillation grade toluene, MIRACLOTH and all common chemicals were received from North American Chemical Supply Limited.

Domestic chickens (Rhode Island Reds, 18-24 months old) were obtained from the U.B.C. Poultry Farm Unit. Animals were killed by breaking their necks. Gizzards were immediately excised and used.

METHODS

A. Enzyme Assays

5'-nucleotidase

The method used was that of WIDNELL (1974) and BURGER & LOWENSTEIN (1975). The release of inorganic phsophate from 5'-AMP is measured. To 0.9 ml of substrate solution (11.11 mM 5'-AMP, sodium salt; 1.11 mM magnesium chloride; 0.1 M TRIS-HCl, pH 8.5 at 22°C) at 37°C, 50 µl of membrane suspension (10-20 µg protein) is added. After incubation for 20 minutes at 37°C, 50 µl of 50%

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trichloroacetic acid (TCA) and 0.5 ml of Ames reagent (1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate in 0.5 M sulphuric acid) (AMES, 1966) are added with stirring. The samples are then incubated at 37°C for a further one hour. Colour development is then stopped by addition of 0.5 ml ice cold distilled water. The samples are then centrifuged at 2000 g, 0°C, 3 minutes in a Sorvall RC-2B centrifuge and the absorbance of the supernatant read at 660 nm. Blanks without either 5'-AMP or membranes are run with each sample. Nanomoles of phosphate released are calculated based on a standard curve obtained using inorganic phosphate and the procedure above leaving out membranes. Results are expressed as mole phosphate released per hour per mg protein.

NADPH/NADH antimycin A-insensitive cytochrome c reductase

The methods used were those of TOLBERT (1974), HATEFI & RIESKE (1967) and HODGES & LEONARD (1974). To 2.80 ml of substrate solution (0.375 mg/ml cytochrome c; 45 mM sodium phosphate, pH 7.0 at 20°C; 1.8 mM sodium cyanide) is added 20 µl of antimycin A solution in ethanol (2 mg/ml). This is followed by the addition of 100 to 200 µl of membrane preparation (40-80 µg protein) after which the rate of change in absorbance at 550 nm is recorded for 5 minutes. Once the initial rate has been established, 100 µl of NADH or NADPH (3 mg/ml) is added. The new rate of change in absorbance at 550 nm is recorded and substracted from the initial rate to yield the actual rate. Micromoles of cyt c consumed per unit time are calculated assuming the extinction coefficient of cyt c at 550 nm to be

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18.5 mM^{-1} cm^{-1}. Specific activities are expressed in moles per hour per mg protein.

NADH antimycin A-sensitive cytochrome c reductase.

This assay is carried out as the assay for NADPH/NADH antimycin A-insensitive cytochrome c reductase except that 45 mM glycine-sodium hydroxide buffer, pH 9.0 is substituted for sodium phsophate buffer and antimycin A omitted.

acid phosphatase

The method used was a modification of the methods of HODGES & LEONARD (1974) and HUBSCHER & WEST (1965). To 0.9 ml substrate solution (3.33 mM p-nitrophenol phosphate; 1.67 mM magnesium sulphate; 36.7 mM TRIS-MES, pH 5.5 at 25°C) is added 100 l of membrane suspension (20-30 µg protein). For determination of K+ stimulated acid phosphatase the substrate solution is made to contain 55.5 mM potassium chloride. This solution is then incubated at 37°C for 20 minutes after which 50 µl of 50% TCA and 0.5 ml of Ames reagent (1 part 10% ascorbic and 6 parts 0.42% ammonium molybdate in 0.5 M sulphuric acid) (AMES, 1966). The samples are then incubated at 37°C for one hour after which 0.5 ml of ice cold distilled water is added. Samples are next centrifuged at 2000 g, 3 min., 0°C in a Sorvall RC-2B centrifuge. The supernatants are withdrawn and the absorbance of the supernatant at 660 nm determined. Nanomoles of phosphate released are read from a 0-10 nanomole calibration curve using the above procedure. Blanks without either p-nitrophenol phsophate or membranes are run for each sample. Specific activities are expressed as moles phosphate released per hour per mg protein.

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succinate dehydrogenase

The method used was based on those of BONNER (1955) and TOLBERT (1974). To a 4.0 ml quartz cuvette are added 0.3 ml of 0.1 M potassium cyanide; 0.3 ml of 0.01 M potassium ferricyanide; 0.2 ml of 0.2 M sodium succinate and 2.0 ml of 55 mM sodium phosphate buffer, pH 7.2. At time zero, 0.20 ml of membrane suspension (40-80 µg protein) is added and the change in absorbance at 400 nm recorded. Control samples without either the membrane preparation or succinate were processed in the same manner. Specific activities are calculated using an extinction coefficient at 400 nm, at 20°C, of $0.892 \times 10^3 M^{-1} cm^{-1}$ for potassium ferricyanide. Results are expressed in moles succinate utilized per hour per mg protein.

glucose-6-phosphatase

The method used was that of NORDLIE & ARION (1966). To 0.90 ml of substrate solution (3.33 mM glucose-6-phsophate; 111 mM sodium cacodylate buffer, pH6.5) at 37°C is added 50 µl of membrane suspension (10-20 g protein). This mixture is then incubated at 37°C for 20 minutes after which 50 µl of 50% TCA and 0.5 ml of Ames Reagent (1 part 10% ascorbic acid to 6 parts 0.42% ammonium molybdate in 0.5 M sulphuric acid) (AMES, 1966) are added. The samples are then incubated for 1 hour at 37°C after which 0.5 ml of ice cold distilled water is added. Samples are then centrifuged at 2000 g, 3 min. 0°C in a Sorvall RC-2B centrifuge, following which the supernatants are withdrawn and their absorbance at 660 nm measured. Blanks without either glucose-6-phosphate or membrane suspension were

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also run with each sample. The absorbance data are converted to micromoles of phosphate by use of a standard curve obtained by the above procedure using inorganic phosphate in place of glucose-6-phosphate and membrane suspension. Results are expressed in moles phosphate released per hour per mg protein.

Lowry Protein Assay

The method is based on the original procedure of LOWRY et al. (1951). The following stock solutions are prepared fresh and stored at room temperature prior to use. Reagent A is prepared in the following order: 2% sodium carbonate in 0.1 M sodium hydroxide, 49 ml; 2% potassium tartrate, 0.5 ml; 1% copper sulphate, 0.5 ml. Reagent B is the Folin-Ciocalteau 2 N diluted 1:1 with distilled water.

The sample to be assayed is made up to 1.0 ml with distilled water. At time zero, 5.0 ml reagent A is added and the mixsture stirred. 0.5 ml of reagent B is added after 10 minutes with stirring and the absorbance at 750 nm read 20 minutes later. A standard curve is obtained in the same manner using BSA (0-250 µg/ml). If TRIS buffer or sucrose is present in the samples to be assayed appropriate control samples must be included (ROBSON et al., 1968; GERHARDT & BEEVERS, 1968).

Characterization of Mg^{2+} Stimulated ATPase

(a) Determination of Optimum Conditions

TRIS-ATP (vanadium free, see HUDGINS & BOND, 1977; BEAUGE & GLYNN, 1977; JOSEPHSON & CANTLY, 1977) concentrations of 0, 0.05, 0.10, 0.20, 0.40, 0.80, 1.20, 2.00, 3.00, 4.00 mM were used. The substrate solutions were buffered with 50 mM TRIS-HCl, pH7.2.

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To 0.9 ml of each substrate solution at 37°C, 50 µl of membrane suspension (10-20 µg protein) are added. The samples are then incubated for 10 minutes at 37°C following which the reactions are terminated using 50 µl of 50% TCA and 0.5 ml of Ames Reagent (1 part 10% ascorbic acid to 6 parts 0.42% ammonium molybdate in 0.5 M sulphuric acid) (AMES, 1966). Colour development is allowed to proceed for 20 minutes at 37°C after which 0.5 ml of ice cold water are added and the absorbance of each sample read at 660 nm. Blanks without enzyme were run with each sample, controls without ATP at frequent intervals. Micromoles phosphate released are calculated based on a phosphate standard curve using inorganic phosphate. Specific activities are expressed in umoles phosphate liberated per hour per mg protein. Optimum Mg^{2+} total and ATP total concentrations were then used for the next series of experiments.

(b) pH Optima and Na^+, Li^+, K^+ and Ouabain Sensitivity

For determination of the pH optimum, the following pH's at 37°C were used: 6.00, 6.50, 7.00, 7.20, 7.40, 7.60, 7.80, 8.00, 8.50 and 9.00. For each pH value, substrate solutions were prepared as in Table II to test for stimulation by ions and inhibition by ouabain.

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Table II

Substrate Solutions to Test Mg^{2+} - Stimulated ATPase Sensitivity to pH, Na^+, Li^+, K^+ and Ouabain

1	2	3	4	5	6
$[ATP]^a_{max}$	$[ATP]_{max}$	$[ATP]_{max}$	$[ATP]_{max}$	$[ATP]_{max}$	$[ATP]_{max}$
50 mM Buf[b]	50 mM Buf	50 mM Buf	50 mM Buf	50 mM Buf	50 mM Buf
	$[Mg^{2+}]^a_{max}$	$[Mg^{2+}]_{max}$	$[Mg^{2+}]_{max}$	$[Mg^{2+}]_{max}$	$[Mg^{2+}]_{max}$
		120 mM Na^+	120 mM Li^+	120 mM Na^+	120 mM Na^+
				20 mM K^+	20 mM K^+
					2 mM Ouabain

[a] optimal concentrations based on the results of part (a).

[b] pH at 37°C - 6.0, 6.5 Cacodylate Buffer, - 7.0-8.5 TRIS·HCl, - 9.0 Glycine-NaOH Buffer

To 0.9 ml of each substrate solution at 37°C are added 50 µl of membrane suspension (10-20 µg protein). The samples are then incubated at 37°C for 10 minutes after which 50 µl of 50% TCA solution and 0.50 ml of Ames reagent (1 part ascorbic acid to 6 parts 0.42% ammonium molybdate in 0.5 M sulphuric acid) (AMES, 1966) are added with stirring. Following further incubation at 37°C for 20 minutes, 0.50 ml of ice cold distilled water are added. The absorbance at 660 nm of each sample is read and recorded. Blanks were run for each sample deleting the 50 µl of membrane suspension. Controls without ATP were also run on a random spot

check basis. Micromoles phosphate released were calculated based on a standard curve of 0 to 50 µmoles inorganic phosphate. Specific activities were expressed in micromoles phosphate released per hour per mg protein.

(c) Velocity Profile

Twenty ml of substrate solution were prepared using the optimal Mg^{2+} total and ATP total concentrations derived from part (a), using 50 mM TRIS-HCl, pH7.2. For the time course 0.9 ml of substrate were used with 50 µl membrane suspension (10-20 µg protein) for each time. The reaction times used were 0, 3, 5, 10, 15 and 30 minutes at 37°C, after which the reactions were terminated by the addition of 50 µl of 50% TCA and 0.50 ml of Ames Reagent (see parts (a) and (b)). Phosphate released was calculated based on standard curves.

acetylcholinesterase

The assay was based on the methods of STECK & KANT (1974). The assay was done in duplicate with 100 µl of the membrane suspension per sample. The 100 µl aliquots of membrane suspension (50-60 µg protein) were pipetted into the bottom of a 1 cm semi-micro quartz cuvette and mixed with an equal volume of either 5 mM sodium phosphate, pH 8.0 or (0.40% Triton X-100 (v/v) in 5 mM sodium phosphate, pH 8.0). The concentration of detergent was chosen so that all latent enzymatic activity would be released with little or no activation. The volume was made to 0.70 ml with 100 mM sodium phosphate, pH 7.5, then 50 µl of 5, 5' - dithiobis (2-nitrobenzoic acid) (DTNB) stock

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solution (10 mM DTNB; 100 mM sodium phosphate, pH 7.5; sodium bicarbonate: DTNB, 3:8; stored at -5°C until used) is added. Finally, 50 µl of acetylcholine chloride or bromide (12.5 mM in H_2O), stored frozen) is added. The cuvette contents are then mixed by inversion and the rate of change in absorbance at 412 nm recorded. An increase in absorbance of 17.0 corresponds to 1 µm of product based on an extiction coefficient of 13.6×10^4 $M^{-1}cm^{-1}$ at 412 nm for DTNB. To establish the baseline, the absorbance was recorded also prior to addition of the substrate. Final results are expressed in nmoles per hour per mg protein.

measurement of sialic acid

The method used was a modification of the methods of WARREN (1955; 1959) and STECK & KANT (1974). A neuraminidase (sialidase) solution was prepared by dissolving 1 mg of Clostridium Perfringens neuraminidase (type G from Sigma Chemical Co.) in 1 ml of 0.03% aqueous solution of bovine serum albumin. The solution was stored at 4°C. For the assay proper, the stock soltuion is diluted 10 fold with 0.1 M TRIS-acetate buffer, pH 5.7 ± 0.40% Triton X-100. To ensure that the enzyme is active with respect to the sialoglycoproteins and sialoglycolipids present in the membrane preparation, the amount of N-acetyl neuramic acid (NANA) released by the enzyme was compared to that released by a 1 hour exposure of the preparation to 0.05 M sulphuric acid at 80°C.

Prior to use membrane preparation are dialyzed in an Amicon ML-2 diafiltration unit to remove interfering chromophores

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as 2-deoxyribose. Duplicate 100 µl aliquots of membrane suspension (60-80 µg protein) are mixed with or without the detergent. The sample is incubated for 30 minutes at room temperature in a test tube with a Teflon lined screw cap. Released NANA is then determined as follows.

After the 30 minute incubation 100 µl of sodium metaperiodate (0.2 M in 9 M phosphoric acid) was thoroughly mixed into each sample and allowed to react for at least 20 minutes. Following this, 1.5 ml of sodium arsenite solution (10% w/v in 0.5 M sodium phosphate) is mixed into each sample and allowed to react for at least 20 minutes. Following this, 1.5 ml of sodium arsenite solution (10% w/v in 0.5 M sodium phosphate) is mixed in vigorously with the samples. After 2 minutes the mixing is repeated, and is followed by the addition of 3.0 ml of thiobarbituaric acid solution (0.6% of w/v in 0.5 M sodium sulphate).

Next the tightly capped tubes are placed in a boiling water bath for exactly 15 minutes and thereafter colled under tap water to room temperature. Two ml from each sample is withdrawn and extracted with 2 ml cyclohexanone. This was done by vigorous shaking for 15 seconds. The two phases were separated by centrifugation of the samples at 2000 rpm/20°C/5 minutes in an International SBV type centrifuge. The rosy pink cyclohexanone (upper phase) layer is transferred to a 1 cm path length cuvette and the absorbances at 549, 562, and 532 nm read. Micromoles NANA released were calculated in two ways.

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The first: moles NANA = $\frac{\text{vol (ml) x A549 nm}}{57}$ doesn't correct for 2-deoxyribose contamination which interferes. If 2-deoxyribose contamination is suspected, the NANA absorbance maxima (A562 nm) and the 2-deoxyribose maxima (A532 nm) are used to calculate the micromoles of NANA released, which =

$$\text{vol (ml)} \left[\frac{133\ A562\ nm}{(32.6)\ (133)-(26)(8)} - \frac{8\ A532\ nm}{(32.6)\ (133)-(26)\ (8)} \right]$$

Other equations are available using the absorbance at 549 and 532 nm for other interfering chromophores. Results are expressed as nmoles sialic acid released per mg of protein.

B. Plasma Membrane Isolation

The method used for isolation of smooth muscle plasma membrane (PM) was modified from the procedure of KIDWAI (1974). The gizzards were excised from freshly sacrificed chicken, cleaned of fat and placed in ice cold buffer A (0.25 M sucrose; 3.0 mM Mg^{2+}; 1 mM TRIS-MES, pH 7.4, at 22°C). All subsequent operations were carried out at 4°C. The stomach muscularis was removed and 2 grams of visceral smooth muscle (visibly free of connective tissue) were minced with scissors and placed in 30 ml buffer A. The muscle (chunks 2 mm x 2 mm 3 mm) was then homogenized for 30 seconds at a setting of 6.0 using a Brinkmann Polytron PT-20. After one minute the muscle was rehomogenized for 40 seconds. Homogenization times were selected on optimal yields of 5' - nucleotidase specific activity in the first 5 gradient fractions (see discussion page 141).

The homogenate was then filtered through 2 crossed layers of Miracloth. The filtrate was made up to 38 ml and centrifuged at 2000 g, 10 minutes, $0^{o}C$ in a SS-34 angle rotor in a Sorvall RC-2B centrifuge. The supernatant was carefully removed and saved. The pellet was normally discarded, unless marker assays were to be done. The supernatant was then centrifuged as above except at 15,000 g_{av}, 15 minutes, $0^{o}C$. The pellet was discarded, and the supernatant centrifuged at 100,000 g_{av}, 75 minutes, $0^{o}C$, in a SW-27 rotor in a L5-65 Beckman Ultracentrifuge. The resulting pellet was saved and the supernatant discarded unless marker assays were to be done. The pellet was then resuspended in a 2.5 ml of 0.25 M sucrose and layered on a discontinuous sucrose gradient consisting of 2.5 ml each of 27.0, 30.0, 32.0, 34.0, 35.0, 36.5, 40.0, 43.0, 45.0 and 66.0% sucrose. The layered gradients were then centrifuged at 12,000 g_{av}, 150 minutes, $0^{o}C$ in a Beckman L5-65 ultracentrifuge using a SW-27 swinging bucket rotor. Following centrifugation each layer was carefully removed by a hypodermic syringe and either used immediately or stored frozen at $-20^{o}C$. Membrane enzymatic activity was stable for 2 weeks.

To remove the bulk of sucrose from the fractions, which was necessary in those situations when sucrose interferes with the enzyme assay, the fractions were diluted 1:1 with distilled water and centrifuged at 140,000 g_{av}, 150 minutes, $0^{o}C$ using a Type 65 Beckman angle rotor in a L5-65 Beckman ultracentrifuge. Recovery of membranes was greater than 85% using this procedure. Addition of 5.0 mM $CaCl_2$ to the diluting solution prior to centrifugation increased yields only 3%.

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Membranes can be stored for 3-4 weeks in the pellet form.

It should be added that an Amicon ultrafiltration M1-2 unit was also used to remove sucrose. Using this procedure 40% of the membranes were lost by absorption to the filter. Sucrose was most effectively removed by using dialysis tubing and dialyzing against 20 volumes of 20 mM TRIS-HCl, pH 7.0, 1 mM magnesium chloride, 1 mM EDTA and 1 mM $CaCl_2$ for 2 days with regular changes of dialyzing buffer.

C. Cell Sheet and Single Cell Preparation

Cell sheets (LEWIS et al., 1975) 0.25 mm thick were obtained from chicken gizzard smooth muscle using a Sorvall Tissue Slicer. The cell sheets were immediately washed with TRIS-MES buffer (1 mM TRIS-MES, pH 7.2; 0.25 M sucrose; 1 mM magnesium chloride) twice. If the cell sheets were to be used for isolation of plasma membranes, they were suspended in 35 ml of Buffer A (see earlier) and treated as described in the plasma membrane isolation procedure. The only difference was that the Polytron homogenization was done in two - 10 second bursts; one minute apart (again optimized on the basis of 5' nucleotidase activity of the final product).

If the cell sheets were to be used to prepare individual cells (BAGBY et al., 1971; LEWIS et al., 1975; RODBELL et al., 1964; FAY & DELISE, 1973; FAY & SINGER, 1977; SMALL, 1977) they were suspended in 9.0 ml of Buffer B- (100 mM sodium phosphate, pH 7.4; 0.25 M sucrose) containing 0.03% grade

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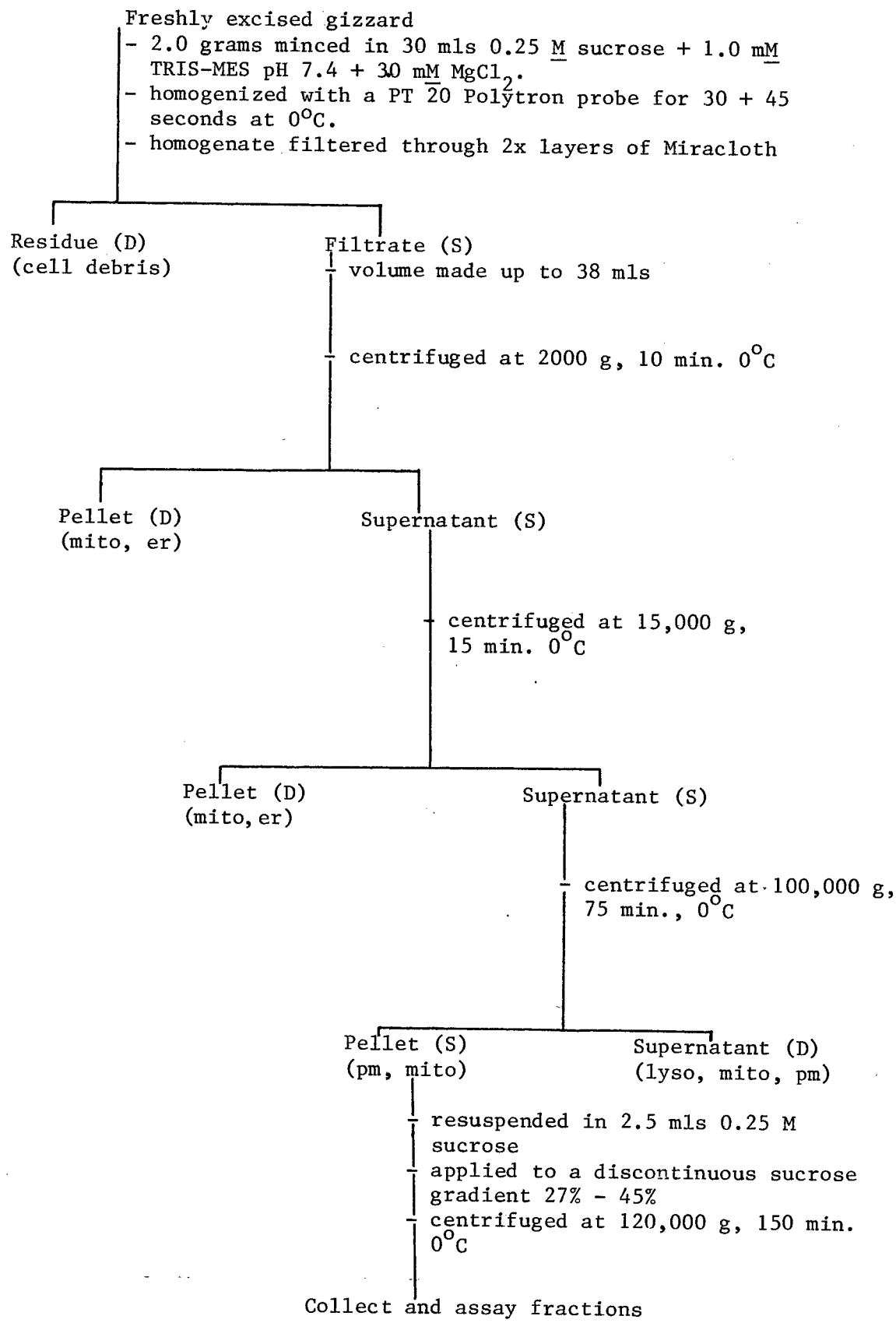

Figure 1 Scheme for isolation of smooth muscle plasma membranes from the chicken gizzard. Abbreviations are as follows: pm=plasma membranes; lyso=lysozomes; mito=mitochondria; er=endoplasmic reticulum; S=saved; D=discarded fraction.

A collagenase (CALBIOCHEM) for 30 minutes at 37°C with aeration by 0_2:CO_2 (95:5). The resulting cell sheets and cells were then pelleted by centrifugation at 1000 g_{av}, 5 minutes, 0°C in a Sorvall RC-2B centrifuge. The supernatant was carefully removed and discarded. The pellet was gently resuspended in 9.0 ml Buffer B, with 0.15% grade A collagenase and aerated at 37°C by 0_2:CO_2 (95:5) for 20 minutes. The cell suspension was then recentrifuged as above and the formed pellet resuspended in Buffer A only. The single cells were immediately centrifuged as above. This procedure was repeated five times taking great care to not discard the pellet each time. The final pellet was resuspended in Buffer A and used. Cells were examined by phase contrast microscopy (See Figures 9 and 10) and were treated with trypan blue to test cell viability.

D. Gel Electrophoresis

The overall approach used for SDS (sodium dodecylsuphate) gel electrophoresis was that of BARTON (1978). The modifications used by LAMELLI et al. (1973) and FAIRBANKS et al. (1971a) were also incorporated. Gel formulations are given below in Table III. Reservoir buffers were 0.1% SDS in 0.1 M sodium phosphate.

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Table III

Gel Formulations Used for Electrophoresis

COMPONENT	5% separating gel STOCK	Ml Used	3% stacking gel STOCK	Ml Used
Acrylamide	22.2%	9.0 ml	30.0%	2.5 ml
Methylene-bis-acrylamide	0.60%		1.50%	
Ammonium persulphate	15mg/ml	2.0 ml	10 mg/ml	0.75 ml
TEMED	-	30 µl	-	10 µl
H_2O	distilled	9.0 ml	distilled	19.25 ml
Buffer	0.2 M sodium phosphate, pH 7.2; + 0.2% SDS	20.0 ml	1.25 M TRIS-HCl, pH6.8; + 1.0% SDS	2.50 ml

After preparation of the separating gel solution, 9 cm long gels were formed in 0.4 cm x 12 cm acid washed flint glass tubes. Each column was overlayered with 1.0 cm of water or isobutanol. Following polymerization, the overlay was removed and 1 cm stacking gel solution added atop the separating gel and overlayered with water or isobutanol until polymerization. The overlay was removed after polymerization and replaced by reservoir buffer. The gels were then placed in a Pharmacia Ge-4 electrophoresis apparatus and pre-run at 8 ma/gel and 8-13 volts/cm for 4 hours.

As the gels were pre-running, membrane suspension in 15.0% sucrose was prepared for electrophoresis. To 150 µl of membrane suspension were added 125 µl of sample buffer

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(0.01 M sodium phosphate, pH 7.2; 1% SDS; 0.14 M mercaptoethanol, 10% v/v glycerol; 0.002 M bromophenol blue). The samples were then capped and placed in a boiling water bath for 5-10 minutes to reduce disulphide bonds and to promote solubilization by SDS. Molecular weight markers were incubated as above. Those used were BSA (MW=70,000), Aldolase (MW=161,000) egg albumin (MW=43,000), cyt c (MW=12,398), Elastase (MW=25,900), actin (MW=43,000) and myosin (MW=210,000).

After sample preparation was complete, each sample was taken up in a disposable micropipette and discharged gently onto the top of the stacking gel. Electrophoresis was performed with the voltage at 4-5v/cm and current at 4-5mA/gel. The running time was about 10 hours. Variations in absolute migration distances were minimized by removing tubes individually from the electrophoresis apparatus one by one as the bromphenol blue tracking dye migrated 9.0 cm from the origin. Tracking dye positions were marked by slots in the gels.

The gels were normally stained for protein with Coomassie Blue R-250 (Biorad). Gels were stained as follows. After rinsing each gel twice with distilled water they were placed in 16 x 150 mm capped culture tubes. To each tube 30 mls of fixing, staining and distaining solutions were added as given below. The tubes were gently agitated at room temperature in a Dubnoff shaker.

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(1) 25% isopropanol; 10% acetic acid; 0.05% Coomassie Blue - 12 hours.

(2) 10% isopropanol; 10% acetic acid; 0.002% Coomassie Blue - 10 hours.

(3) 10% acetic acid; 0.001% Coomassie Blue - 8 hours.

(4) 10% acetic acid - 3 x 10 hours each.

The third and fourth steps are absolutely necessary if the background staining is to be reduced to minimum. When methanol was substituted for isopropanol the gels did not destain as well. Gels stained with Coomassie Blue from Sigma could not be destained either by diffusion or electrophoretic methods.

The Periodic-Acid Schiff's (PAS) procedure was used to stain the gels for carbohydrate (NEVILLE & GLOSSMAN, 1974). Because high concentrations of SDS produce an intense background, it was necessary to remove the detergent by carrying out the steps 1 - 4 described above, but without the presence of the protein stain. The fixed gels were then placed individually into slotted plexiglass tubes and suspended for the specified period to the following sequence of solutions. Each gel required 100 ml.

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(1) 0.5% periodic acid - 2 hours

(2) 0.5% sodium arsenite; 5% acetic acid - 1 hour

(3) 0.1% sodium arsenite; 5% acetic acid - 2 x 20 minutes

(4) 5% acetic acid - 10-20 minutes

Each solution was stirred vigorously at room temperature. The gels were then transferred to tubes containing 10 ml of Schiff's reagent (ACHARIUS & ZELL, 1969; NEVILLE & GLOSSMAN, 1974) and left overnight. Finally, they were returned to the slotted tubes and incubated in 0.1% sodium bisulphite in 0.01 M HCl for several hours (solution changed once every hour) until the rinse solution failed to turn pink upon addition of formaldehyde. Rose pink bands appeared after 2 hours in the Schiff's reagent. No variable background absorption was seen.

The gels were scanned using a Gilford spectrophotometer equipped with a Model 2420 Linear Transport Accessory. The Coomassie Blue stained gels were scanned at 530 nm, those stained by the PAS procedure at 560 nm. The slit width was 0.1 mm in either case. When samples containing radioactively labelled components were run, gels were not stained but rinsed in water twice, fixed with 8% TCA (15 minutes) and then sliced into 2 mm sections for counting (see [γ^{32}P] ATP labelling experiments). Duplicate gels for staining only were also run.

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E. Iodination Experiments

(a) Iodination of Muscle Chunks, Cell Sheets and Free Cells with ^{125}I.

Approximately 2 grams of cell sheets (or 2 x 2 x 2 mm muscle chunks) were washed with 200 ml of ice cold 0.25 M sucrose in 50 mM sodium phosphate, pH 7.2 (Buffer C). The sheets were then placed into a polypropylene centrifuge tube containing 0.43 ml of 10 µM lactoperoxidase, 9.025 ml of Buffer C and 12 µl of carrier free [^{125}I]NaI (1.2 m Ci) (ROMBAUTS et al., 1967; Morrison, 1970, 1974; MORRISON & BAYSE, 1970; MORRISON & SCHONBAUM, 1976; Hynes, 1976). A total of 40 µl of 1.6 mM hydrogen perioxide was added in 1 µl portions every 30 seconds for 20 minutes. Following the last addition, the cell sheets were washed on a Buchner funnel filtration unit with 1.5 litres of Buffer C containing 5 mM sodium iodide. The cell sheets were then homogenized as described under the membrane isolation procedures. Following membrane isolation, Fractions 4 and 5 of the sucrose gradient were immediately collected and samples withdrawn for SDS gel electrophoresis. Two gels were run for each sample, one for Coomassie Blue staining and the other for ^{125}I counting. The remaining F4 and F5 were then subjected to the iodination procedure to be described in Section B. Controls without lactoperoxidase and/or hydrogen perioxide were also run. Samples of homogenized cell sheets or muscle chunks were removed for gel electrophoresis. ^{125}I was detected using a Nuclear Chicago gamma counter (see Section c) (FAIRBANKS et al., 1967; BOGDANOVE & STRASH, 1975; LEINEN & WITCLIFFE, 1978).

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When free cells isolated from 1.0 grams of muscle were to be labelled with ^{125}I, the procedure was similar except that the volume was reduced to 3.0 ml because of the smaller quantity of the cells but the final concentrations of lactoperoxidase, ^{125}I and hydrogen peroxide remained unaltered. The reaction was stopped by the addition of 0.75 ml of 0.1% sodium azide in a 25 mM sodium iodide solution. The cells were then immediately pelleted by centrifugation at 1000g, 5 minutes, 0°C in a Sorvall RC-2B centrifuge. The pellet was suspended in 3.0 ml of Buffer C and recentrifuged as above. This procedure was repeated 5x until the supernatant was virtually free of ^{125}I. The pellet was then resuspended in 3.0 ml Buffer C and used immediately for electrophoresis. Controls were run as described previously and a control using homogenized ^{125}I labelled cells was also run. ^{125}I was determined as in part c and as described under gel electrophoresis.

(b) Iodination of Sucrose Gradient Fractions

For reasons to be discussed later three versions of the iodination procedure were employed to label isolated membrane fractions. (1) Membranes were iodinated in 400 µl of 32% sucrose (34% for Fraction 5) made 50 mM in sodium phosphate, pH 7.2, 0.8 µM in lactoperoxidase and 0.15 mCi/ml in [^{125}I] NaI (carrier free). At room temperature, 1 µl aliquots of 1.6 mM hydrogen peroxide were added (total volume added 30 µl) at 30 second intervals for 15 minutes. Reactions were terminated with the addition of 35 µl of 0.1% sodium azide in 28 mM sodium iodide or by adding 100 µl of electrophoresis sample buffer. The membranes in both cases were used

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immediately for SDS gel electrophoresis. In the experiment, lactoperoxide and/or hydrogen peroxide wre omitted as controls.

(2) 400 µl of membranes in sucrose (32-34) were distilled 1:1 with distilled water. The iodination was carried out using the final concentrations of lactoperoxidase, [^{125}I] NaI, hydrogen peroxide and soium phosphate as in (1). Following the last addition to hydrogen peroxide, 100 µl of 0.1% sodium azide in 25 mM sodium iodide solution was added and the sample centrifuged at 140,000g_{av}, 150 minutes, 0°C in a L5-65 Beckmann Ultracentrifuge using a Type 65 angle rotor. The resulting pellet was immediately resuspended in 0.25 M sucrose-50 mM sodium phosphate buffer, pH 7.2 and used for electrophoresis. Controls were run as in (1).

(3) Membranes in 0.8 ml were pelleted or dialyzed as described under membrane preparation to remove sucrose. The pellet was resuspended in 400 µl of 100 mM sodium phosphate, pH 7.2 using a syringe. The final concentrations of lactoperoxidase, ^{125}I and hydrogen peroxide were as in (1). Reactions were terminated and samples prepared as in (1). For iodination of sucrose fractions in detergent the only changes from (1) are that the membranes were made 0.05% and 0.40% in Triton X-100 20 minutes prior to the addition of hydrogen peroxide aliquots to start the reaction.

(c) Iodination of lactoperoxidase - "iodolactoperoxidase"

Five µl aliquots of a 64 µM lactoperoxidase stock solution (freshly prepared since self iodination increases with aging of the enzyme) were added to 10 different 400 µl portions of sodium iodide solutions, containg 0.00, 0.10, 0.20, 0.30, 0.40, 0.50, 1.00, 1.50, 2.00, and 5.00 mM sodium iodide respectively. Each solution was aldo made to be

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0.15 mCi/ml in [^{125}I] NaI and 50 mM in sodium phosphate, pH 7.2. To start the reaction 1 µl aliquots of a 1.6 mM hydrogen peroxide solution were added every 30 seconds for 15 minutes, after which 600 µl of 12.5% TCA was added. The suspension were then centrifuged at 5000g, 10 minutes, 0°C in a Sorvall RC-2B centrifuge using a SS-34 rotor lead. The supernatants were poured off and saved. The pellets were then counted in a Nuclear Chicago Model 1020 Gamma counter. Supernatants were counted as a check to ensure all samples contained the same initial radioactive carrier free [^{125}I] NaI. Controls were run omitting lactoperoxidase and/or hydrogen peroxide. The supernatants and pellets were also subjected to electrophoresis, stained and counted for ^{125}I to ensure that only lactoperoxidase was labelled and that equal amounts of the enzyme were used.

After determination of the optimal sodium iodide concentration required for optimal I^- incorporation by the lactoperoxidase, the above experiment was repeated using a constant iodide concentration of 0.3 mM but varying the hydrogen peroxide concentration used. After determination of the optimum hydrogen peroxide concentration (1 µl/30 seconds for 15 minutes), the following cold labelling of lactoperoxidase was done. Five ~5 µl of lactoperoxidase were labelled with 0.5 mM sodium iodide (cold) and the optimum hydrogen peroxide concentration above. Following the last hydrogen peroxide addition, the lots were pooled, diafiltered at 0°C to 250 µl and the concentrate applied to a 1 x 40 cm Sephadex G-100 column at 0°C. The lactoperoxidase came off the column shortly

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after the void volume as indicated by UV absorption at 280 nm and previous runs using ^{125}I labelled lactoperoxidase on the column. The fractions with the lactoperoxidase were pooled and the non-radioactive iodine iodinated lactoperoxidase concentrated by diafiltration to 200 µl. This was immediately assayed for specific activity protein and electrophoresed. The specific activity found was identical to the unlabelled enzyme. Self-iodinating ability of the cold labelled lactoperoxidase was assessed by comparison with non-iodinated lactoperoxidase. The conditions used were those above. It was found that the non radioactively labelled iodolactoperoxidase self labelling with I^- was less than 10% of that observed with the non-iodinated variety.

(d) Effect of Triton X-100 on Lactoperoxidase Self Labelling

Six 400 µl aliquots of a 0.25 M sucrose -50 mM sodium phosphate, pH 7.2. buffer solution containing 0.00, 0.05, 0.10, 0.20 and 0.40% of TX-100 (v/v) respectively were all made 0.8 M in lactoperoxidase and 0.15 mCi/ml in [^{125}I] NaI. To each tube for 15 minutes at 30 second intervals, 1 µl aliquots of 1.6 mM hydrogen peroxide were added subsequently. Reactions were terminated by the addition of 50 µl of 0.1% sodium azide in 25 mM sodium iodide solution or by the addition of 100 µl gel electrophoresis sample buffer proceeded by heating at 100°C for 10 minutes and all the samples subjected to gel electrophoresis. Gels were prepared as described for either staining with Coomassie Blue or counting.

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(e) Studies of Membrane Iodination Times and Effect of [^{125}I] NaI on Specific Activity

Nine sets of membranes were iodinated and the samples prepared as described in section b(1) except for the following changes: a) For the first 6 sets of membranes, the 1.6 mM hydrogen peroxide additions were 1 µl aliquots/30 seconds for 0, 5, 10, 15, 25 and 30 minutes respectively; b) For the remaining 3 sets, hydrogen peroxide was added as in b(1) but using three different specific activities of [^{125}I] NaI of 0.075 mCi/ml, 0.15 mCi/ml, and 0.30 mCi/ml. Controls were run as described in b(1).

F. Membrane Extraction Procedure

To make the extraction experiments feasible, (FAIRBANKS et al., 1971; STECK & YU, 1973; STECK, 1974b; COLEMAN et al., 1976; KAHLENBURG, 1976) the membranes had to be available in sufficiently large quantity so that the proteins extracted during the extr-ction procedures could be detected on SDS gel electrophoresis. To accomplish this, sixteen 0.8 ml fractions of membranes obtained directly from sucrose gradients F4 and F5 were diluted 1:1 with distilled water and centrifuged as described in the Membrane Preparation section. The pellets were pooled and suspended in 15 mM sodium phosphate pH 7.5. The suspension contained 1.0 mg of protein/ml. The supernatants and pellet suspension were subjected to gel electrophoresis as described earlier to see if changes in the protein composition of the membranes occurred due to the water dilution prior to sedimentation.

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For the extraction experiments, 50 µl of membrane suspension was used and extracted with 250 µl extracting media listed in Table IV. Following extraction, samples were centrifuged at 150,000g_{av}, 120 minutes, 0°C in a Type 65 rotor using a Beckman L5-65 ultracentrifuge.

Table IV

Extraction Media Used in Membrane Extraction

Media	Concentrations	Conditions
H_2O	-	20 min., 0°C
sodium phosphate, pH 7.5	5 mM	20 min. 0°C
pCMBS[a] in 5 mM sodium phosphate, pH 7.5	0.01, 0.20, 2.00 mM	
TX-100 in 5 mM sodium phosphate pH 7.5	0.01, 0.05, 0.50% (v/v)	25 min., 25°C
DMMA[b]	0.05 mg/ml, 2.0 mg/ml 5.0 mg/ml neutralized to pH 7.5 with sodium hydroxide	
EDTA[c] in 5 mM sodium phosphate, pH 7.5	0.50 mM	25 min., 0°C
Digitonin	0.36 mg/ml in 5 mM sodium phosphate, pH 7.5	25 min., 0°C

[a] pCMBS refers to p-Chloromercuribenzene sulphonic acid

[b] DMMA refers to Dimethyl maleic anhhydride

[c] EDTA refers to Ethylenediamine tetraacetate

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Following centrifugation the pellets were suspended in 150 µl buffer (sodium phosphate, pH 8.0 and supernatant 300 µl) were analyzed for protein and then immediately electrophoresed as described under the gel electrophoresis section (using 150 µl supernatant and 75 µl pellet suspension). Coomassie Blue staining was done and all gels scanned. Peaks were assigned R_f values and compared.

G. Column Chromatography on Con A-Agarose or Con A-Sepharose

(a) One ml of membrane suspension (1-2 mg protein) in 50 mM sodium phosphate, pH 6.8, was loaded at room temperature onto a Con A-Agarose column (1 x 5 cm) (CUATRECASES, 1973; MURTHY & HENEZ, 1973; SHARON & LIS, 1975; WALSH et al., 1976; BRUNNER et al., 1977). The column had previously been prepared by washing with (1) 20 ml of acetate buffer (0.2 M sodium chloride; 6.0 mM magnesium chloride; 5 mM sodium acetate, pH 6.5), (2) 60 ml of 0.20 M sodium chloride in 5 mM sodium acetate, pH 6.5, and finally, (3) 20 ml of 50 mM sodium phosphate, pH 6.5 The applied membranes were eluted initially with 50 mM sodium phosphate, pH 6.5, at a flow rate of 12 ml/hr. for 4 hours, after which the eluting buffer was supplemented with αmethyl-D-mannoside in the final concentration of 100 mM. The column was allowed to run for an additional 4 hours at the above rate, after which the eluting buffer, was replaced by (50 mM sodium borate, pH 7.5; 150 mM α-methyl-D-mannoside). The elution rate increased to 24 ml/hr. and the column ran for 2 hours.

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The 1 ml fractions collected were immediately assayed for protein using the Lowry assay and the Biuret method. Samples of peak fractions were then subjected to gel electrophoresis as described earlier. Peak fractions were also used for enzyme assays and iodination.

(b) Using Con A-Sepharose the above mentioned procedure was repeated yielding similar results. Controls were run using Sepharose 4B and Agarose columns.

(c) Radioactively labelled membranes from F4 or F5 were prepared as described earlier and also applied to the affinity column. Samples were treated as in (a) except that no iodination of the effluent was carried out, only Coomassie Blue staining and radioactive counting of the gels.

H. Phosphorylation Studies

(a) General

Membrane Fractions 4 and 5 (25 - 50 µg of protein in the respective sucrose solution) were made up to the final volume of 1.05 ml by addition of components listed in Table V and mixed. This and all the subsequent steps were carried out at $0^{\circ}C$ (NAGANO et al., 1965; AVRUCH & FAIRBANKS, 1972; KNAUF et al., 1975; CHA & SOOLEE, 1976; LANE, 1976).

Table V

Components Used in Phosphorylation Studies

Sample 1	2	3	4	
0.1 mM $MgCl_2$	0.1 mM $MgCl_2$ 0.5 mM $CaCl_2$	0.1 mM $MgCl_2$ 100 mM $NaCl^2$	0.1 mM $MgCl_2$ 100 mM $LiCl^2$	
5	6	7	8	9
0.1 mM $MgCl_2$ 100 mM Choline	0.1 mM $MgCl_2$ 100 mM $NaCl^2$ 25 mM KCl	0.1 mM $MgCl_2$ 100 mM $NaCl^2$ 0.5 mM $CaCl_2$	Control	Hydroxylamine[a]

a - Used 0.4 M hydroxylamine hydrochloride freshly prepared. Extraction done as by Knauf et al. (1974) except that the sample was centrifuged at $150,000g_{av}$; 47,000 rpm, 2.5 hr., in a Beckman L5-65 ultracentrifuge using a Type 65 rotor.

[γ-^{32}P] - ATP was added to give the final ATP concentration of 3.74 M and the final specific activity of 28 Ci/ mol. After 30 seconds, 100 µl of 50% TCA was added and the samples spun down at $150,000g_{av}$, 60 minutes, 0^{o}C in a Beckman 25-65 ultracentrifuge using a Type 65 rotor. The TCA precipitated pellets were then washed once with and resuspended in 200 µl 10% sucrose - 50 mM TRIS-HCl, pH 7.2. Each sample was solubilized in 3% SDS sample buffer at 37^{0}C for 25 minutes. The total volume of the solubilized samples, 350 µl now, was divided into two 175 µl aliquots that were electrophoresed. Following electrophoresis, one sample was used for counting and the other

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for Coomassie Blue staining. The counting of ^{32}P radiation was done by slicing the gel into 2 mm slices and placing them into scintillation vials with 1 ml of 0.05% SDS. The vials were then incubated for 24 hours at 37°C. Next, 20 ml of Triton-toluene scintillation fluid (1 litre TX-100; 2 litres toluene; 16.9 grams of OMNIFLUOR) were added to each vial. Samples were cooled to 0°C and then counted using a Mark II Nuclear Chicago Scintillation counter. ^{32}P counting efficiency was approximately 85% as determined by adding a known ^{32}P dpm to the various vials.

Various controls were run throughout the experiments. To see whether phosphorylation was affected by trace impurities on the labelled [γ-^{32}P] ATP, phosphorylation was measured using [γ-^{32}P] ATP at 25 fold lower specific activities. It was found that the results wre unaffected by variations in the specific activity. Reactions were terminated using 10% TCA with 0.2 mM ATP and 1.0 mM orthophosphate or 10% TCA with 0.2 mM ATP or 10% TCA with 1.0 mM orthophosphate rather than TCA alone.

(b) Time Course

In order to determine optimum phosphorylation, membranes (100 µl protein) in 1000 µl of 30% sucrose with 20 mM TRIS HCl, pH 7.8 at 0°C, were phosphorylated for 0, 15, 30, and 45 seconds using 3.7 µM ATP made 28 mCi/mmol with [γ-^{32}P] ATP. The reactions were terminated as described earlier, and the samples processed in a similar manner.

(c) Time Course

In order to determine more precisely the nature of teh Mg^{2+} dephosphorylation and Ca^{2+} phosphorylation the membranes were phosphorylated as above for 15 seconds after

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which the sample was made 0.1 mM in Mg^{2+} or 0.5 mM in Ca^{2+} for a further 15 seconds. The reactions were then terminated as described in part (a). Controls were run checking phosphorylation with Mg^{2+} and ATP added at the same time for 15 - 30 seconds. The same applies to Ca^{2+}. Samples were processed as usual.

I. Electron Microscopy

F4 and F5 were obtained directly from the sucrose gradient and deposited on a Millipore Filter (0.22 μ pore) by means of a Sweeney Syringe. The deposited membranes or 0.5 mm cubes of muscle were post fixed for 1 hour with Karnovsky's fixative (KARNOVSKY, 1969) in 1% osmium tetroxide, stained for 1 hour in saturated aqueous uranyl acetate, dehydrated through a graded alchol series and embedded using standard procedure in Epon Araldite resin. Gold sections cut on a Reichart OMU 3 ultramicrotome, were stained with Reynolds lead citrate, and examined under Phillips 300 transmission electron microscope.

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RESULTS

A. General

The muscle in the chicken stomach muscularis has been shown to consist principally of visceral smooth muscle (Figures 2 and 3) (CALHOUN, 1954). As seen in the electron micrographs the cells are of varying size and each cell contains a central nucleus. Also, present are mitochondria, however, there was little evidence of sarcoplasmic reticulum being present. This latter point being especially interesting in view of the enzyme marker assays done for the sarcoplasmic reticulum (SR). Using Polytron homogenization of the smooth muscle, differential centrifugation, and gradient centrifugation we obtained membrane fractions enriched in plasma membranes (Figure 1, see page 40). The homogenization times and centrifugation rates were optimized to yield maximal specific and total activities of 5' nucleotidase in the first 5 layers of the sucrose gradients. (45 seconds: F4 - 0.50 µm/mg/hr, 0.005 µm/mg/hr; F5 - 0.61 µm/mg/hr, 0.006 µm/hr: 60 seconds: F4 - 0.61 µm/mg/hr, 0.007 µm/mg/hr; F5 - 0.90 µm/mg/hr, 0.009 µm/hr: 75 seconds: F4 - 0.58 µm/mg/hr, 0.022 µm/hr; F5 - 0.99 µm/mg/hr, 0.025 µm/hr: 90 seconds: F4 - 0.47 µm/mg/hr, 0.007 µm/hr; F5 - 0.73 µm/mg/hr, 0.006 µm/hr).

B. Membrane Marker Studies

The isolation of the plasma membranes (PMs) was monitored by measuring the specific activities of the enzymes listed in Table VI. Based on 5' nucleotidase activities, the PMs were

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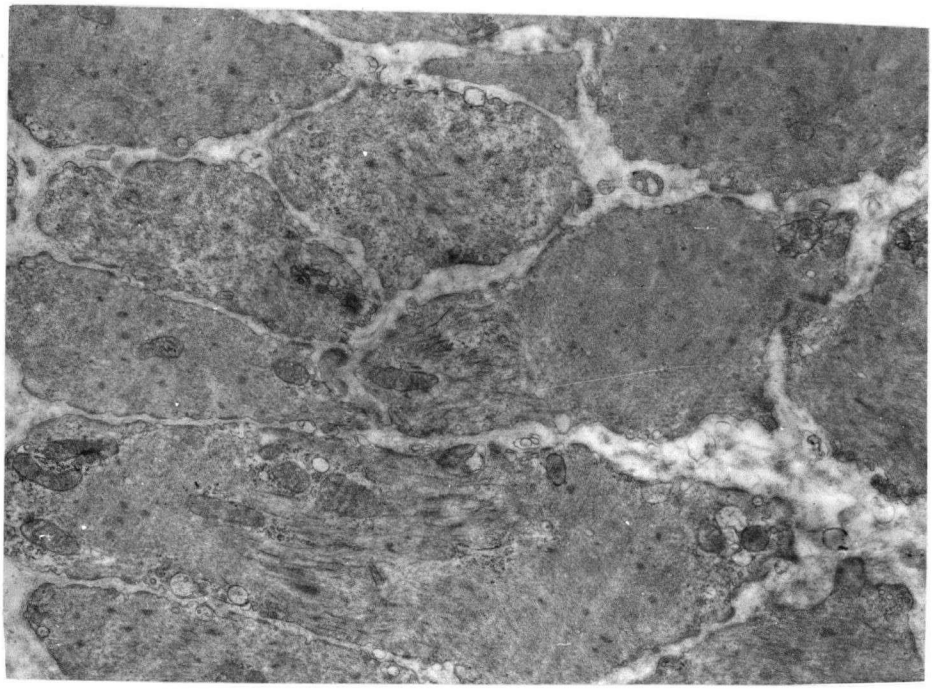

Figure 2. Electron micrograph of chicken gizzard smooth muscle. The field shows a number of cells. Magnification at 12,000x.

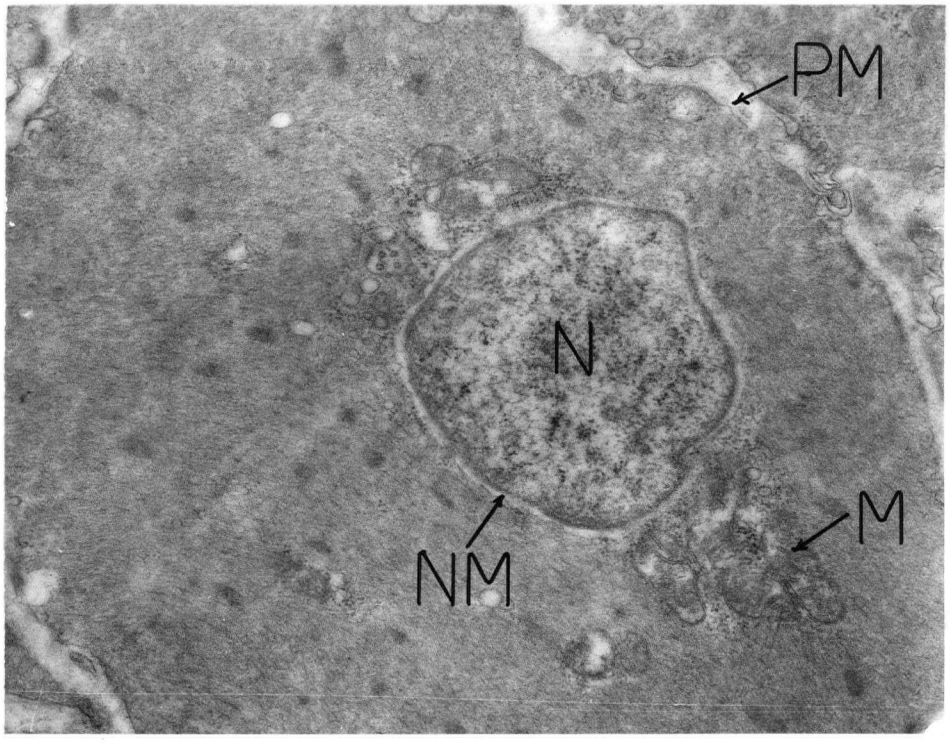

Figure 3. Electron micrograph of a chicken gizzard smooth muscle cell. The field shows various organelles (M - mitochondria, N - nucleus, NM - nuclear membrane, PM - plasma membrane). Magnification at 21,000x.

Table V I

Total and specific activities of selected marker enzymes at various stages of the fractionation procedure. The specific activities are in µmole / mg protein / hour, the total activities are in µmole / hour.

Marker Enzyme	Residue	Filtrate	2,000 x g pellet	2,000 x g supernatant	15,000 x g pellet	15,000 x g supernatant	100,000 x g pellet	100,000 x g supernatant
acid phosphatase								
specific activity	0.033	0.044	0.026	0.056	0.036	0.056	0.057	0.032
total activity	0.65	5.01	0.24	4.36	0.15	4.62	0.29	3.99
% yield	11.4	89.0	4.0	77.0	2.6	81.0	5.0	70.0
K^+ - stimulated acid phosphatase								
specific activity	0.046	0.026	0.10	0.068	0.051	0.017	0.063	0.026
total activity	0.91	2.96	0.94	5.30	0.18	1.40	0.33	3.24
succinate dehydrogenase								
specific activity	0.75	1.45	8.59	1.24	2.88	0.96	2.87	0.53
total activity	14.80	165.00	80.70	97.00	14.40	79.04	14.80	66.50
% yield	8.0	92.0	45.0	54.0	8.0	41.6	8.2	37.0
NADPH cyt c reductase								
specific activity	0.21	0.23	0.37	0.16	0.72	0.07	0.17	0.05
total activity	4.17	26.11	3.43	12.50	3.60	10.20	0.86	6.25
% yield	14.0	86.0	11.0	41.5	12.0	34.0	2.8	21.0
5 ' nucleotidase								
specific activity	0.031	0.030	0.072	0.040	0.077	0.028	0.310	0.005
total activity	0.59	3.42	0.68	3.12	0.27	2.26	1.60	0.62
% yield	15.0	85.0	17.0	78.0	6.7	56.0	40.0	16.0

Table VIIa

Specific activities of marker enzymes. Fractions obtained from sucrose gradients. Specific activities in μmole/mg protein/hour. See Table VIIbalso.

Fraction Number	5' nucleotidase	Mg^{2+} ATPase[a]	^{125}I c.p.m./μg protein	succinate dehydrogenase	NADH cyt c[b] reductase	Protein (μg)	% sucrose
1	0.05	0.33	420	1.20	1.09	563	8.0
2	0.86	8.06	703	0.34	0.63	775	27.0
3	0.93	17.25	714	n.d.	n.d.	500	30.0
4	0.57	8.97	743	2.29	0.11	387	32.0
5	1.11	23.30	760	0.37	0.01	263	34.0
6	1.10	22.72	690	0.37	0.01	225	35.0
7	0.27	19.86	420	10.39	0.62	263	36.5
8	0.17	14.57	310	9.90	1.70	200	40.0
9	0.12	14.43	200	0.11	0.65	113	42.0
10	0.05	4.20	n.d.	4.58	1.13	250	43.0
11	0.07	n.d.	n.d.	0.09	0.28	463	44.0
12	0.04	n.d.	n.d.	n.d.	n.d.	375	45.0
13	0.01	n.d.	n.d.	n.d.	n.d.	100	66.0

[a] Specific activities in μmole/mg protein/minute.

[b] Refers to Antimycin sensitive NADH cyt c reductase.

Table VIIb

Specific activities of marker enzymes. Fractions obtained from sucrose gradients. NADH cyt c reductase activity measured in the presence of Antimycin A. Specific activities in µmol/mg protein/hour. The results of this table can be compared directly to those of Table VIIa.

Fraction Number	NADH cyt c reductase	NADPH cyt c reductase	glucose-6-phosphatase	acid phosphatase $-K^+$	acid phosphatase $+K^+$	Protein (µg)	% sucrose
1	3.24	0.23	0.11	0.19	0.25	563	8.0
2	7.08	0.63	0.25	0.21	0.31	775	27.0
3	7.50	0.34	0.11	0.28	0.39	500	30.0
4	4.70	0.44	0.09	0.10	0.11	387	32.0
5	4.60	0.28	0.09	0.05	0.05	263	34.0
6	4.80	0.23	0.04	n.d.	n.d.	225	35.0
7	6.67	0.37	0.07	n.d.	n.d.	263	36.5
8	4.38	0.32	0.03	0.09	0.10	200	40.0
9	0.24	0.25	0.03	0.11	0.13	113	42.0
10	0.81	0.09	0.10	n.d.	n.d.	250	43.0
11	2.82	0.18	0.12	n.d.	n.d.	463	44.0
12	n.d.	n.d.	0.01	n.d.	n.d.	375	45.0
13	n.d.	n.d.	n.d.	n.d.	n.d.	100	66.0

purified ten fold during the first five steps of the isolation procedure (Figure 1). In the process of the gradient centrifugation, the PMs were purified another 2 to 4 fold based on 5' nucleotidase values (Tables VIIa and VIIb), the specific activity of 5' nucleotidase being highest in Fraction 5. The validity of using 5' nucleotidase as a PM marker was checked by using lactoperoxidase catalyzed iodination of the PM prior to homogenization. Table VIIa shows that Fraction 4 (F4) and Fraction 5 (F5) contained the highest specific activity of ^{125}I. The difference between the ^{125}I labelling results and the 5' nucleotidase results gave us the first indications that there may be a difference between the orientation of the membranes in F4 compared to F5.

The contamination of the various PM gradient fractions was followed by standard markers for the SR, mitochondria, and lysozomes. Mitochondria, assessed using succinate dehydrogenase and NADH antimycin A sensitive cyt c reductase activities, were removed mainly in the 2,000g centrifugation step. This result was quite surprising since higher g forces are usually required to sediment out mitochondria. Despite this result, there was still a 2 to 4 fold increase in the specific activity of succinate dehydrogenase in the microsomal pellet, followed by a further 4 to 5 fold increase after gradient centrigugation. This increase in specific activity was found in Fractions 7 and 8 of the sucrose gradient. K^+ stimulated acid phosphatase, thought to be found mainly in lysozomes, showed little increase in specific activity at any stage of the PM isolation procedure. Maximal specific activities were found in the first 3 layers of the gradient.

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While dependable markers for the SR are rather controversial, it has been felt that NADH antimycin A insensitive cyt c reductase was specific for the SR. Recently, some doubt has been cast on this hypothesis as PMs and other organelles are thought to contain the above mentioned enzyme as well (SOTTOCASA et al., 1967; SOTTOCASA, 1971; KELBERG & CHRISTENSEN, 1979). Similarily glucose-6-phosphatase is no longer regarded as being specific for the SR. However, NADPH cyt c reductase, one of the more commonly used SR markers, is thought to be specific for these membranes. Based on studies with this enzyme, the SR was localized to fractions 1 to 3, 7, 10 and 11. There was definite contamination of Fractions 4 and 5 but this represented less than a 2 fold increase in specific activity over the filtrate. The distributions of glucose-6-phosphatase and NADH antimycin A insensitive cyt c reductase were not exactly the same as that of NADPH cyt c reductase. The exact meaning of this is treated in the Discussion section (see also Tables VIIa and VIIb).

Based on the above results, F4 and F5 were judged to be the purest PM fractions obtained from the gradient. They were shown to consist of vesicles under the electron microscope (Figures 4 and 5) but no quantitative results were derived from the electron micrographs other than vesicle size (which appeared quite variable). Further characterization of the membranes using gel electrophoresis, showed the PAS and coomassie blue staining profiles of F4 and F5 to be the same (Figures 15 and 16, Table IX). These results would indicate that any differences observed in membrane properties do not appear to arise from structural heterogeneity.

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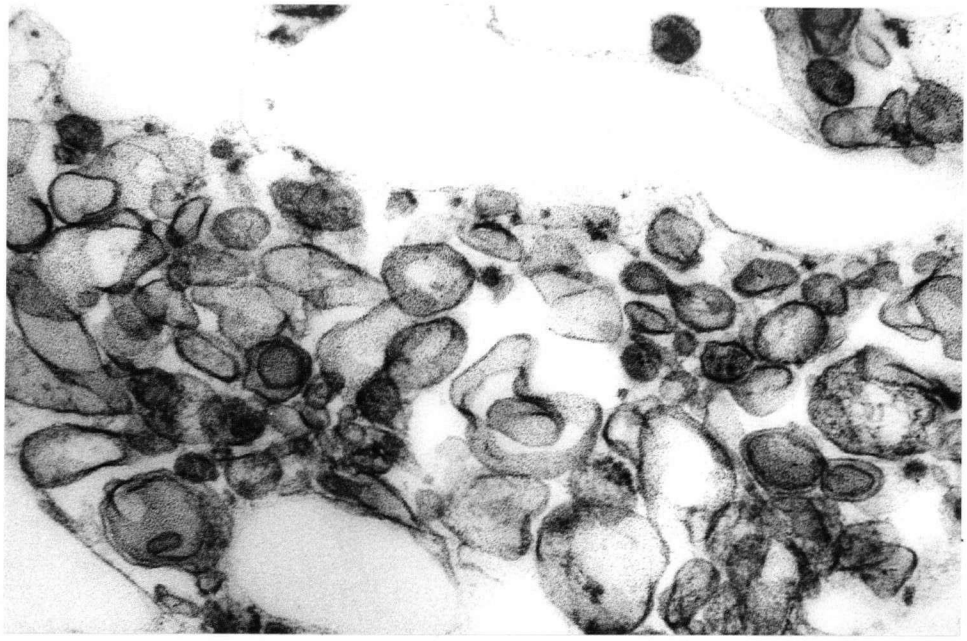

Figure 4. Electron micrograph of Fraction 4 plasma membranes isolated from the chicken gizzard smooth muscle. Magnification at 47,000x.

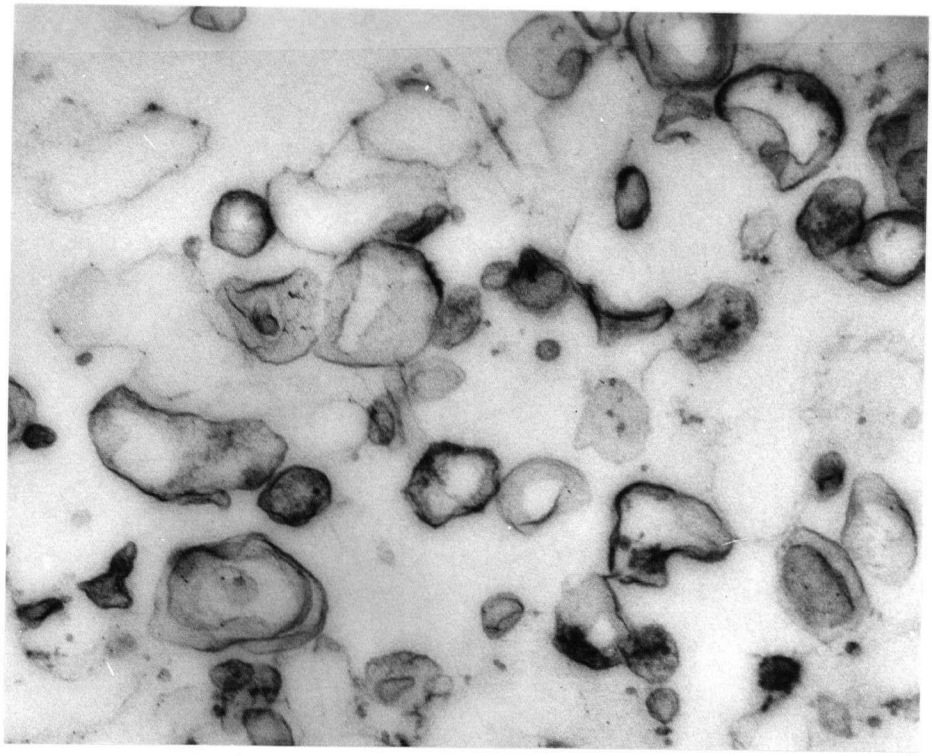

Figure 5. Electron micrograph of Fraction 5 plasma membranes isolated from the chicken gizzard smooth muscle. Magnification at 60,000x.

C. Plasma Membrane Mg^{2+} Stimulated ATPase Activities

(a) Optimization of Mg^{2+} stimulated ATPase activity - When the gradient fractions were being characterized with respect to contamination, attempts were made to observe some Na^+/K^+ ATPase activity. As shown in Table VIIa, only a very active Mg^{2+} stimulated ATPase was observed. Maximal specific activity was observed in F5, this in contrast, to the rather low specific activity observed in F4. These results when combined with the aforementioned PM marker results, provided further evidence for F4 having the opposite orientation to F5. It was felt atthis time that a thorough analysis should be done of the Mg^{2+} stimulated ATPase activity observed in F5 since there have been many reports of similar Mg^{2+} stimulated ATPases in other smooth muscle preparations. Some investigators now consider Mg^{2+} stimulated ATPase as a specific PM marker (VALLIERES et al., 1978).

Using F5 , the specific activity of the Mg^{2+} stimulated ATPase was optimized for Mg^{2+} and ATP (Figure 6) and under conditions of optimal activity the enzyme was tested for Na^+, Li^+ and K^+ sensitivity, ouabain inhibition and pH effects (Figure 7). K^+ stimulation was only observed at very high pH values, while ouabain and the various other cations tested had little effect. The pH optima of the enzyme was 7.6 at 37^oC. When Ca^{2+} stimulation was tried no effect was noted. The enzyme appeared to be a Mg^{2+} stimulated ATPase.

Assumingthat $[MgATP]^{2-}$ was the enzyme substrate, a plot of velocity versus substrate concentration was done (Figure 8).

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Figure 6. Optimization of Mg^{2+} ATPase activity in Fraction 5. Plot of specific activity of Mg^{2+} ATPase versus $[Mg^{2+}]_{total}$ at various [ATP]. ATPase assay was as described under " Materials and Methods ". △, [ATP] = 0.00 mM; ■, [ATP] = 0.05 mM; □, [ATP] = 0.10 mM; ◇, [ATP] = 0.20 mM; ●, [ATP] = 0.40 mM; ○ , [ATP] = 0.80 mM; ◆, [ATP] = 1.20 mM; ▼ , [ATP] = 2.00 mM; ▲ , [ATP] = 4.00 mM. Specific activities represent the mean of 5 values.

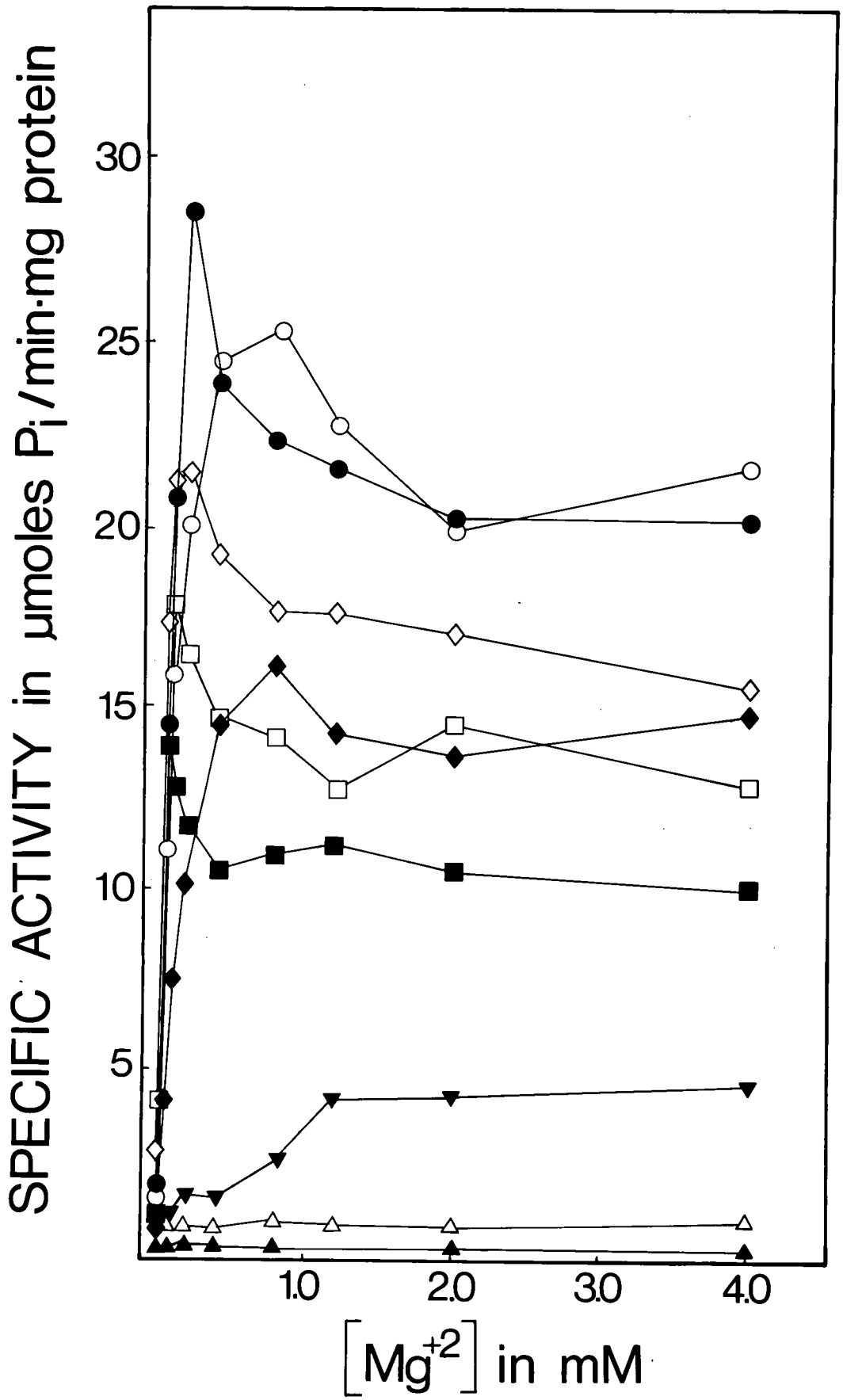
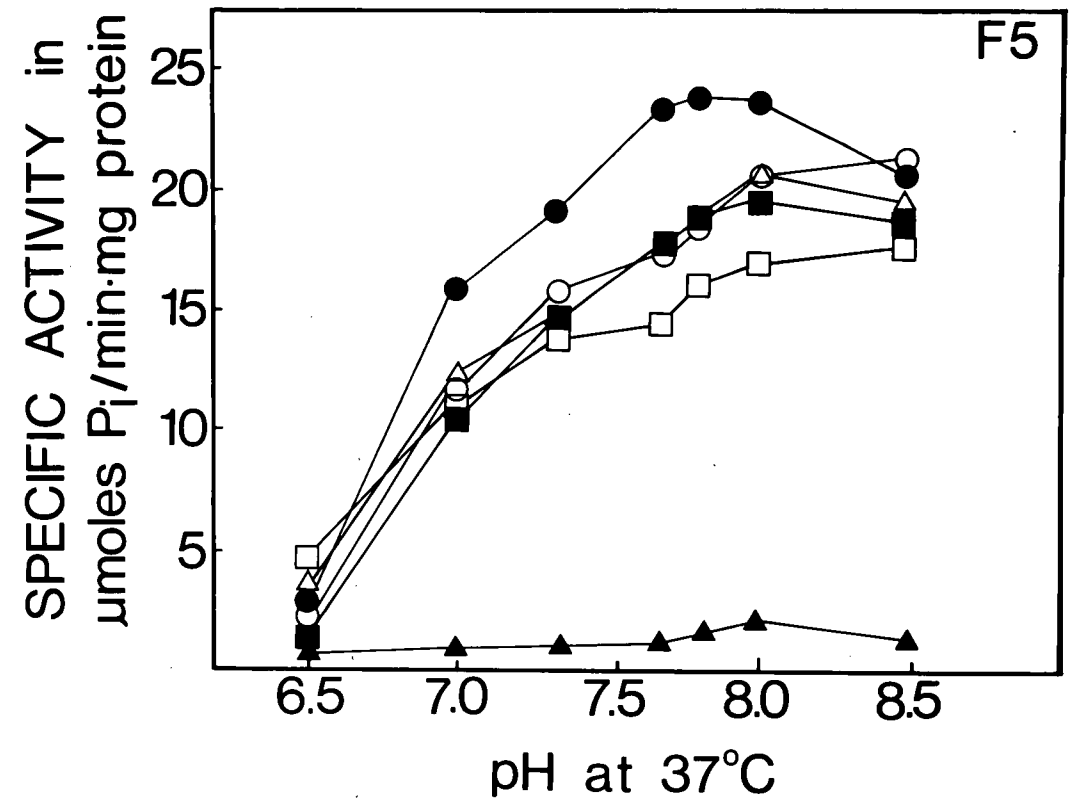
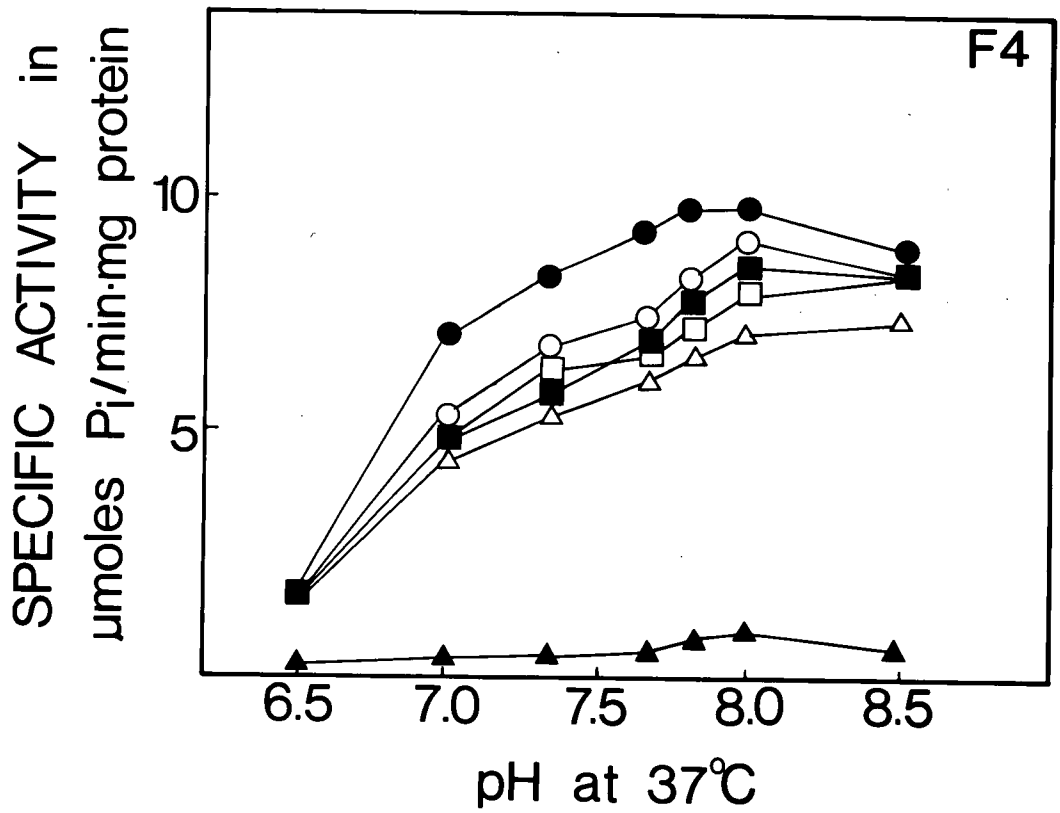

SPECIFIC ACTIVITY in µmoles P_i/min·mg protein
30
25
20
15
10
5
1.0
2.0
3.0
4.0
$[Mg^{+2}]$ in mM

Figure 7. Effects of pH, Na, K, Li and ouabain on the Mg^{2+} ATPase activity of Fraction 4 (bottom) and Fraction 5 (top). Fraction 4: ▲, 0.40 mM ATP; ●, 0.40 mM ATP + 0.20 mM Mg^{2+}; ○, 0.40 mM ATP + 0.20 mM Mg^{2+} + 120 mM Na^{+}; △, 0.40 mM ATP + 0.20 mM Mg^{2+} + 120 mM Na^{+} + 20 mM K^{+}; □, 0.40 mM ATP + 0.20 mM Mg^{2+} + 120 mM Li^{+}; ■, 0.40 mM ATP + 0.20 mM Mg^{2+} + 120 mM Na^{+} + 20 mM K^{+} + 1 mM ouabain. Fraction 5 (all concentrations as for Fraction 4): ▲, ATP; ●, ATP + Mg^{2+}; ○, ATP + Mg^{2+} + Na^{+}; △, ATP + Mg^{2+} + Na^{+} + K^{+}; □, ATP + Mg^{2+} + Li^{+}; ■, ATP + Mg^{2+} + Na^{+} + K^{+} + ouabain. Specific activities are the mean of 5 values.


F5
SPECIFIC ACTIVITY in µmoles Pi/min·mg protein
25
20
15
10
5
6.5
7.0
7.5
8.0
8.5
pH at 37°C


F4
SPECIFIC ACTIVITY in µmoles Pi/min·mg protein
10
5
6.5
7.0
7.5
8.0
8.5
pH at 37°C

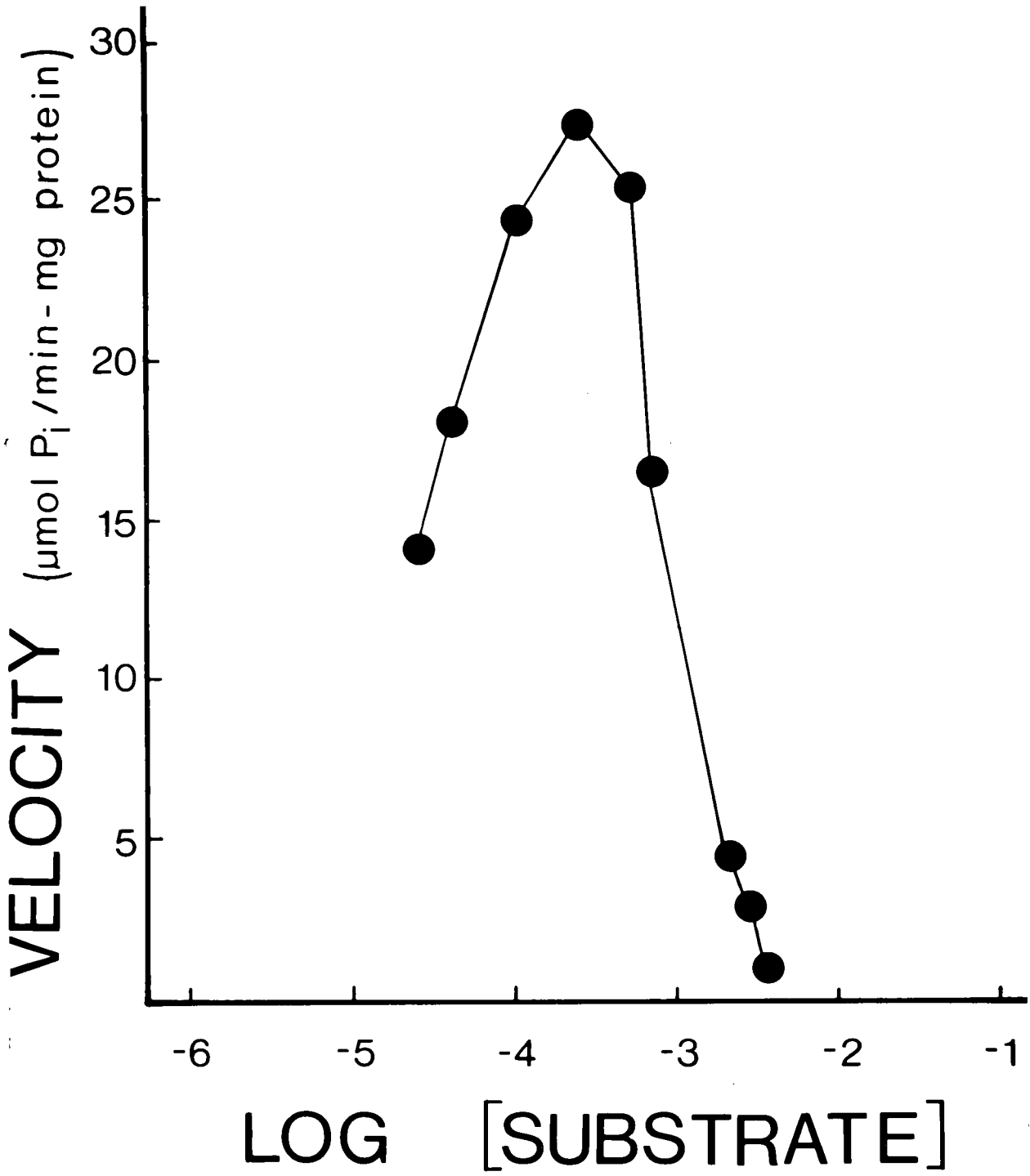


Figure 8. Plot of rate versus logarithm of the substrate [MgATP]$^{-2}$ for the Mg^{+2}ATPase observed in Fraction 5. The enzyme appears to be inhibited by high substrate concentrations.

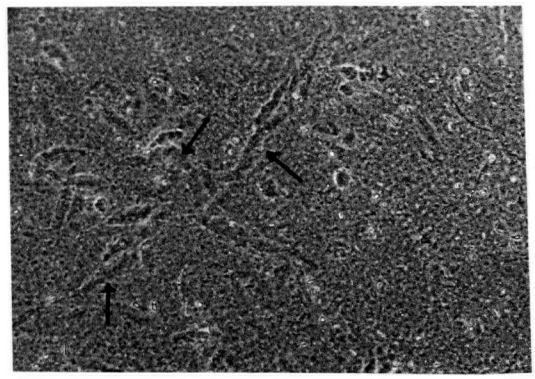


Figure 9. Phase contrast micrograph of a suspension of single smooth muscle cells (see arrows). Magnification 80x.

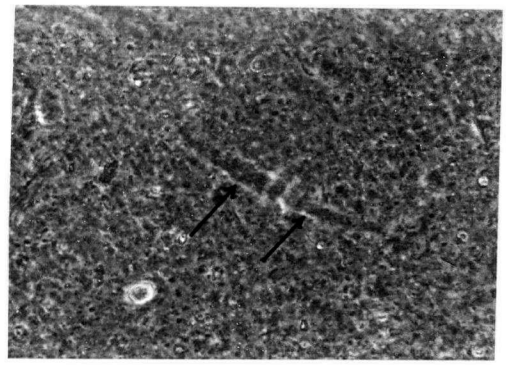


Figure 10. Phase contrast micrograph of an isolated smooth muscle cell (see arrows). Magnification 160x.

The results are representative of an enzyme that is inhibited by high subsrtate concentrations. Based on the maxima, we might calculate a Km of 5×10^{-5} M, Ks' of 2×10^{-3} M and a Vmax of 2.5×10^{7} M/sec. As we shall see later the assumption that $[MgATP]^{2-}$ is the substrate, has to be somewhat modified. The presence of the Mg^{2+} stimulated ATPase on the external surface of F5's PM correlated well with the ecto Mg^{2+} stimulated ATPase activity noted in suspensions of single smooth muscle cells derived from the chicken gizzard (Figures 9 and 10). The ecto Mg^{2+} stimulated ATPase on the cells had similar characteristics to the Mg^{2+} stimulated ATPase found in F5.

(b) Phosphorylation of the plasma membranes in F4 and F5 - All the results in this section are directly comparable. The amounts of protein, as judged by gel electrophoresis, were identical in all runs. The ATPase was further characterized by phosphorylation of F4 and F5 PMs with $[\gamma\text{-}^{32}P]$ ATP. For F5 in the presence of $[^{32}P]$ ATP only, the ^{32}P incorporated was maximized with respect to incubation times used (Figure 11). In the presence of $[^{32}P]$ ATP, F5 exhibited three major peaks; Peak A - 205,000 daltons; Peak B - 165,000 daltons; Peak C - 145,000 daltons. When F4 was labelled with $[^{32}P]$ ATP, only a small amount of ^{32}P was incorporated when compared with F5 (Figures 12a, 12b and 12c). These results point toward an ATP binding site being accessible from the external PM surface only, while the fact that some F4 was labelled may have been indicative of some of the vesicles in this fraction being either leaky, unsealed or of mixed RO/IO orientation. $[^{32}P]$ ATP added in the presence of Mg^{2+} caused a decrease in the ^{32}P

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incorporation of Peak A in F5 (Figure 12b). A decrease of 35% was seen in Peak B of F5, this being half of that noted for Peak A (F5), whereas in F4 no detectable changes were noted. When Ca^{2+} was substituted for Mg^{2+} in F5 a slight decrease was noted in the phosphorylation of Peak A, this compared to a 50% increase in the ^{32}P incorporation of Peak B (Figure 12c). Again, no real effect was noted in F4.

To show that Mg^{2+} was promoting dephosphorylation of the bound ^{32}P in F5, a time delay study was done in which Mg^{2+} was added 15 seconds after the addition of [^{32}P] ATP. The reaction was terminated after an additional 15 seconds. The results (Figures 14a and 14b) show that Mg^{2+} promotes dephosphorylation of Peak A. When Ca^{2+} replaced Mg^{2+} in the time delay experiment (Figure 14a), a 3 fold increase was noted in the ^{32}P incorporation of Peak B. Peak A showed little or no increase. As illustrated in Figure 14a, addition of hydroxlamine reduced the ^{32}P activity incorporated into F5 membranes (labelled with [^{32}P] ATP) which indicates that the labelled phosphoryl groups in Peaks A, B and C were bound to acyl moieties. Both the enzymes involved in Peak A and B appear to have sites localized externally on the F5 membranes but these sites are inaccessible in F4 due to an apparent difference in orientation. All phosphorylated enzymes (Peaks A, B and C) are binding ^{32}P by acyl moieties. The correlation of the Mg^{2+} stimulated ATPase activity at 37°C with the labelling by ^{32}P at 205,000 daltons is extremely tempting. Based on these results this may be partially justifiable, but it is by no means certain.

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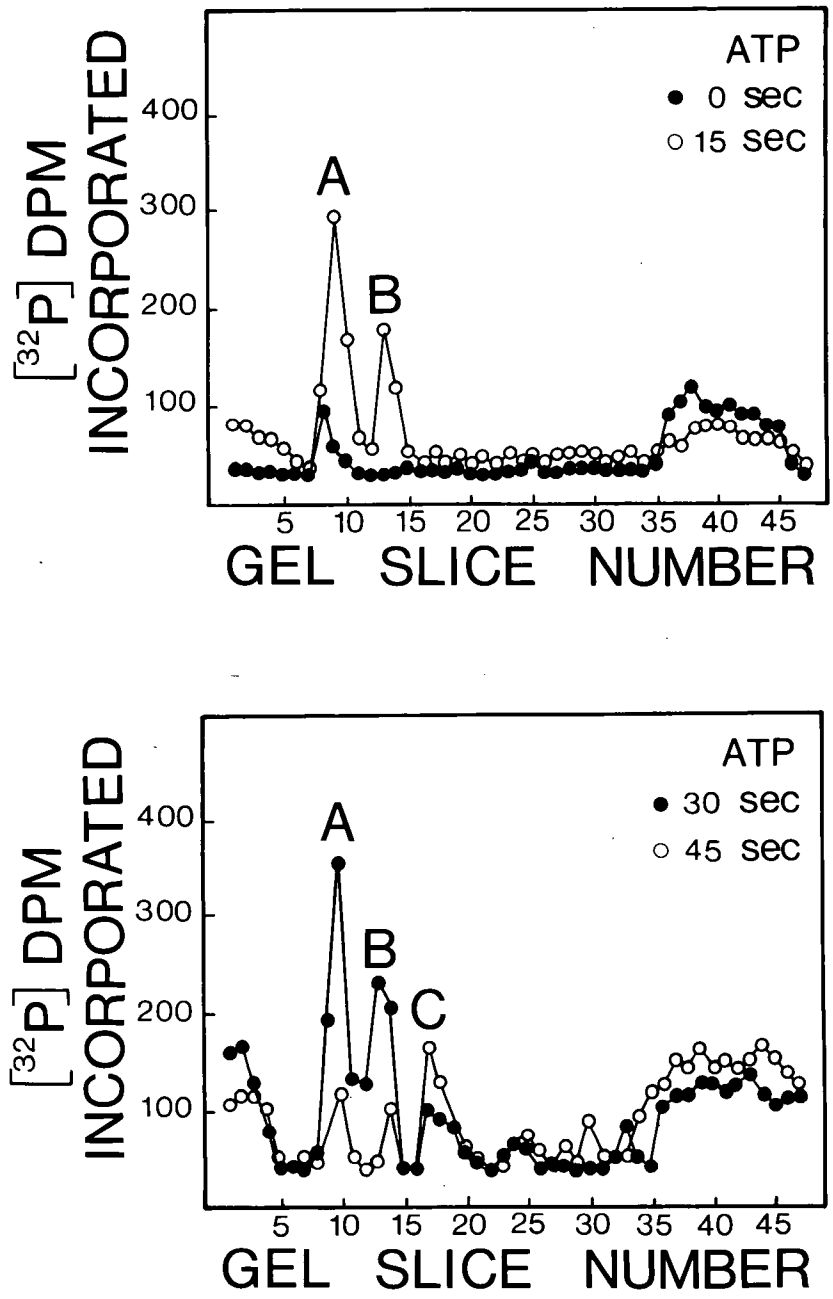
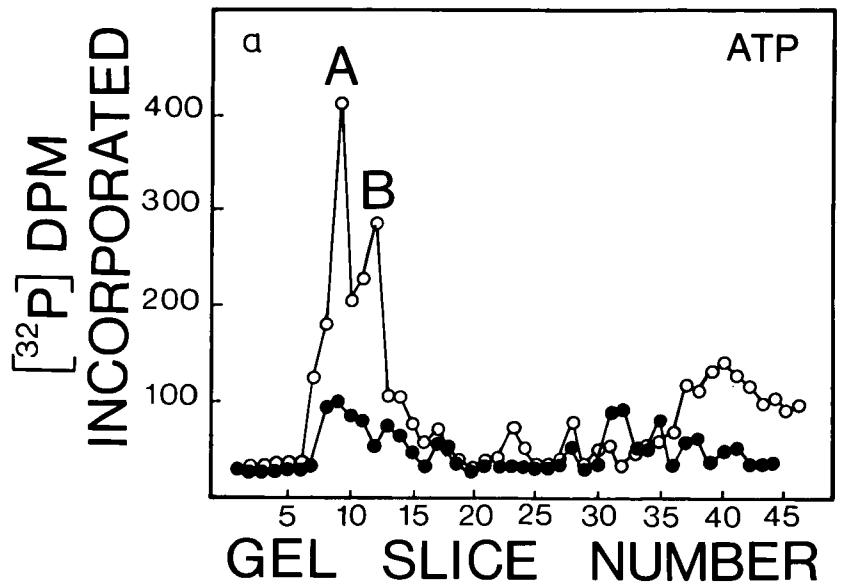
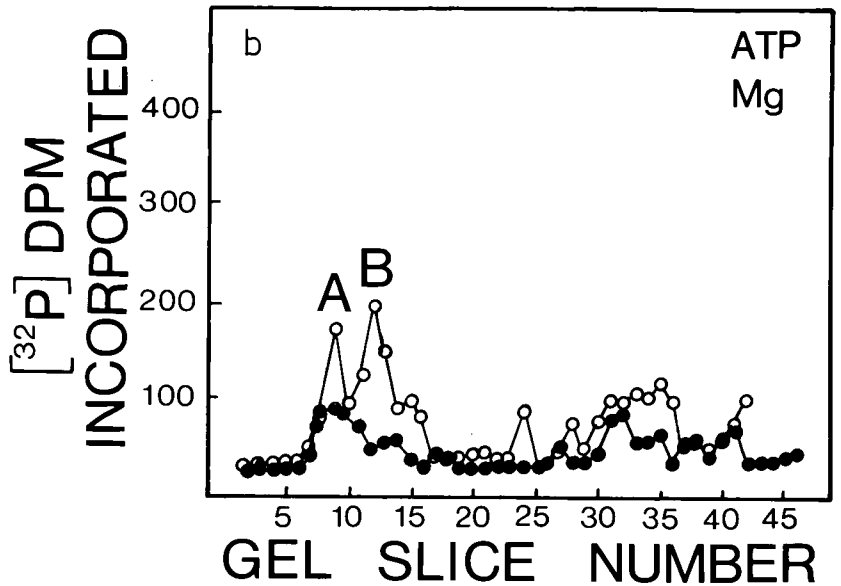


Figure 11. Phosphorylation patterns of Fraction 5 at various incubation times using [γ-^{32}P] ATP. Procedure is as described under " Materials and Methods ". Peak assignments are as follows:A - 205,000 MW; B - 165,000 MW; C - 145,000 MW. Peaks in Figures 12 to 14 are assigned similarly.

Figure 12. Phosphorylation patterns of Fraction 4 (●—●) and Fraction 5 (○—○). Conditions are as described under " Materials and Methods ". (a): 3.74 μM ATP ([γ-^{32}P] ATP, 40 Ci/nmol); (b) : 3.74 μM ATP ([γ-^{32}P] ATP, 40 Ci/nmol) + 0.10 mM Mg^{2+}; (c) 3.74 μM ATP ([γ-^{32}P] ATP, 40 Ci/nmol) + 0.50 mM Ca^{2+}.


a
ATP
A
B
[32P] DPM INCORPORATED
400
300
200
100
5 10 15 20 25 30 35 40 45
GEL SLICE NUMBER


b
ATP
Mg
A
B
[32P] DPM INCORPORATED
400
300
200
100
5 10 15 20 25 30 35 40 45
GEL SLICE NUMBER

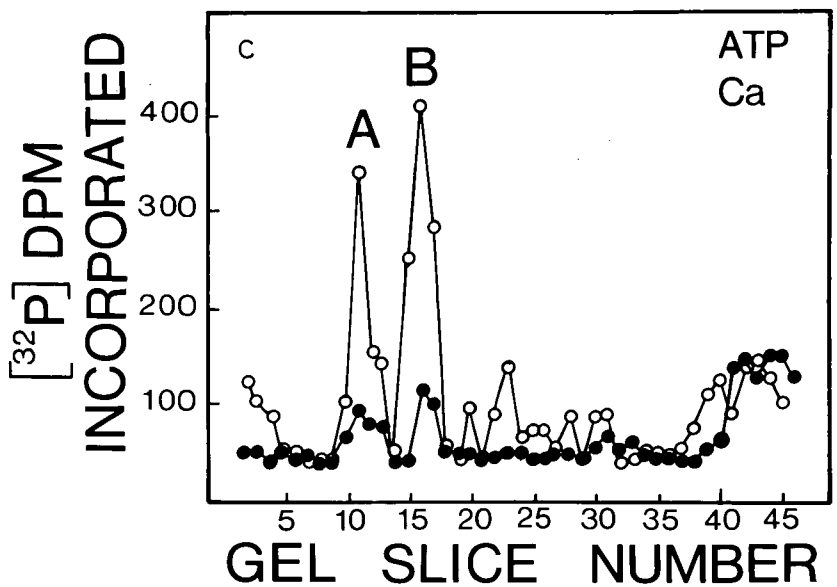
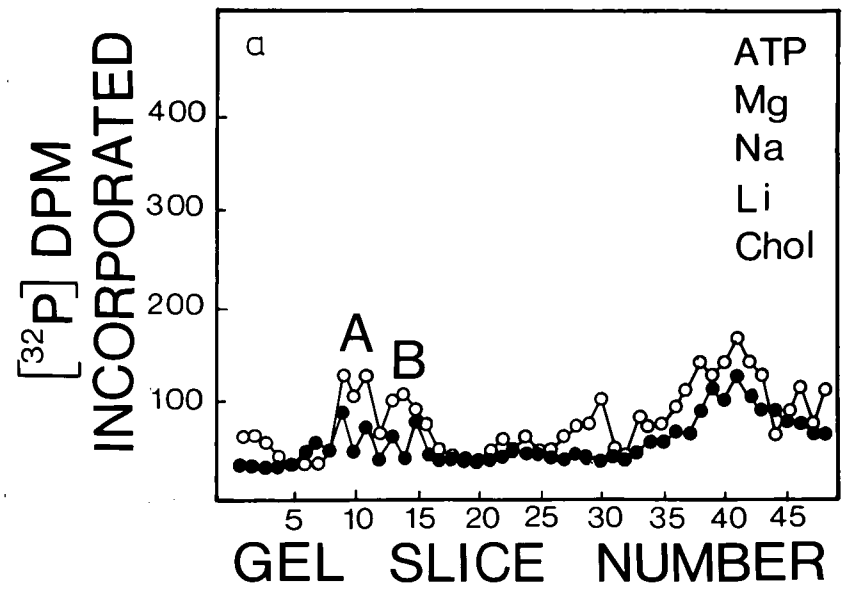
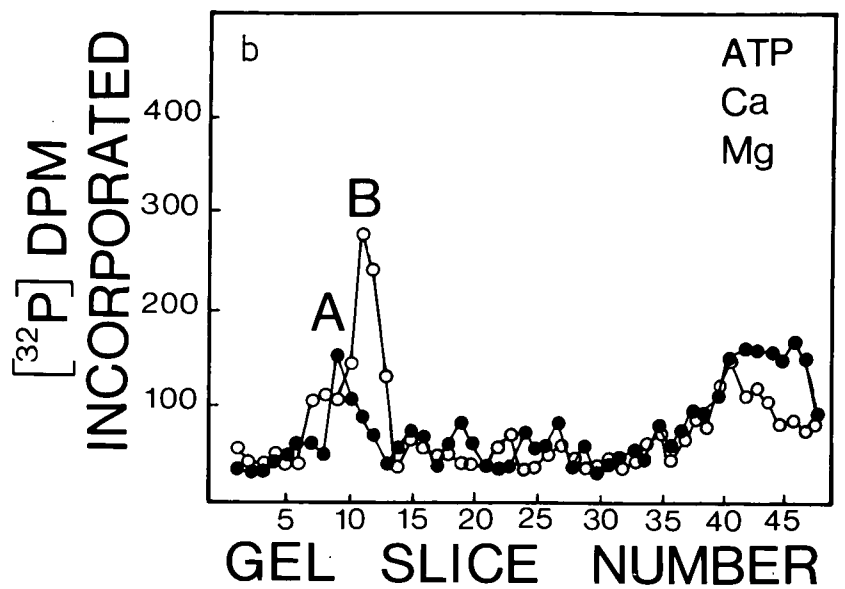

c
ATP
Ca
A
B
[32P] DPM INCORPORATED
400
300
200
100
5 10 15 20 25 30 35 40 45
GEL SLICE NUMBER

Figure 13. Phosphorylation patterns of Fraction 4 (●—●) and Fraction 5 (○—○). All media contained 3.74 μM ATP ([γ-^{32}P] ATP, 40 Ci/nmol). (a): ATP + 0.10 mM Mg^{2+} + 120 mM Na^{+} or Li^{+} or choline; (b): ATP + 0.10 mM Mg^{2+} + 0.50 mM Ca^{2+}; (c): ATP + 0.10 mM Mg^{2+} + 0.50 mM Ca^{2+} + 120 mM Na^{+} or Li^{+}. These results are directly comparable to Figures 11 to 14.


a
ATP
Mg
Na
Li
Chol
A
B
[32P] DPM INCORPORATED
100
200
300
400
5 10 15 20 25 30 35 40 45
GEL SLICE NUMBER


b
ATP
Ca
Mg
A
B
[32P] DPM INCORPORATED
100
200
300
400
5 10 15 20 25 30 35 40 45
GEL SLICE NUMBER

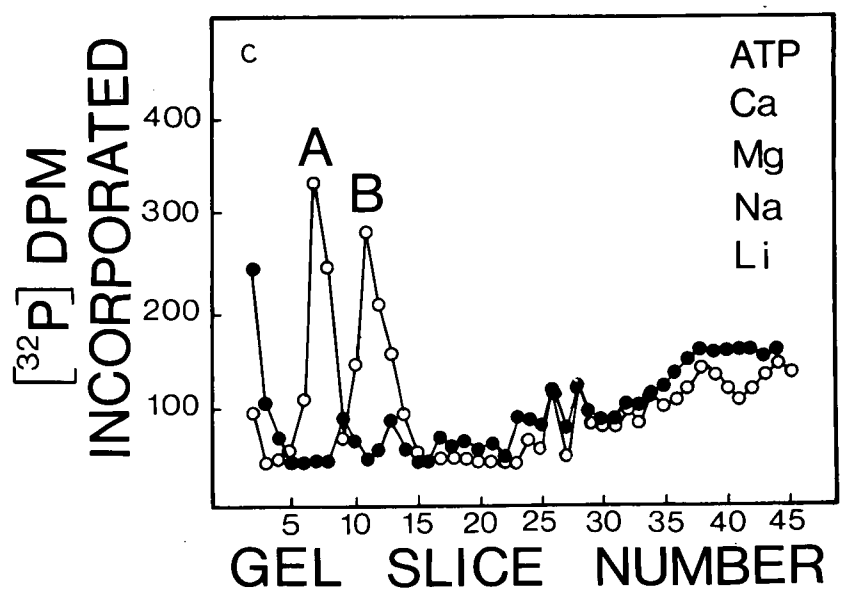
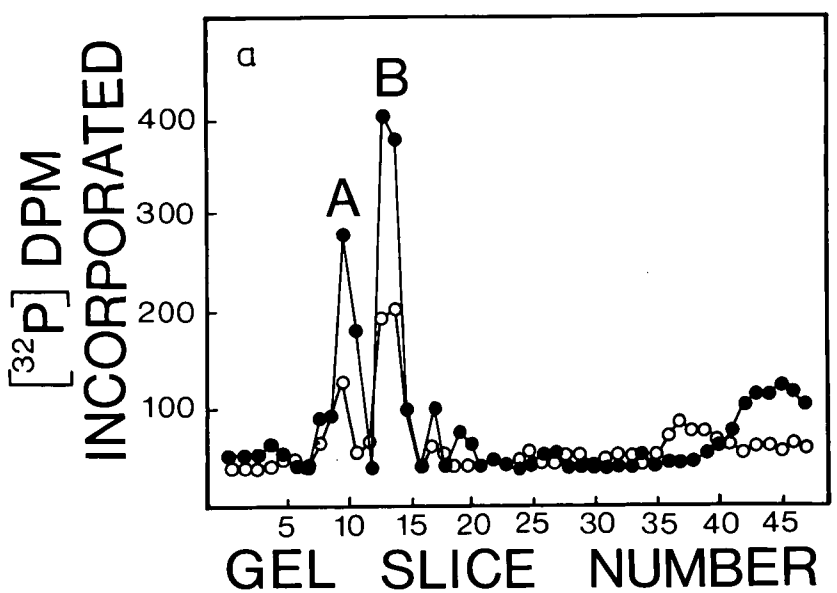
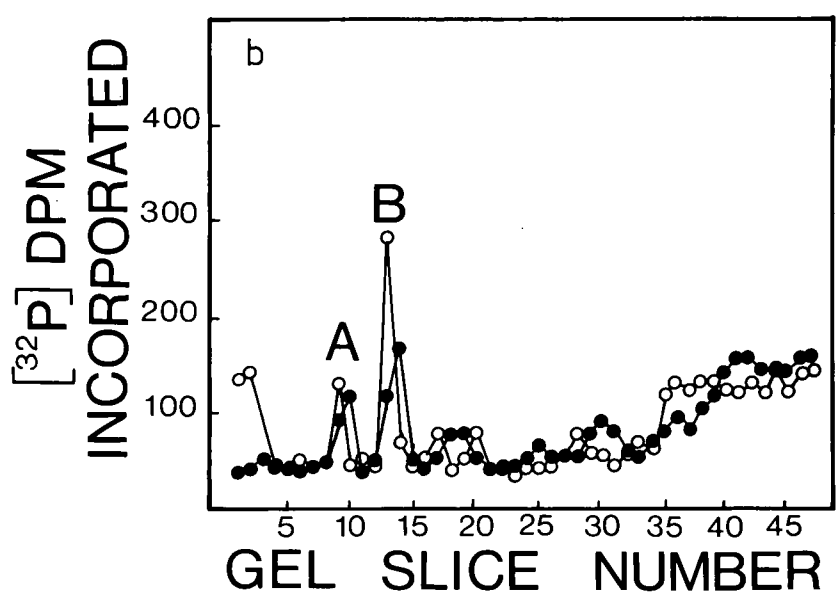

c
ATP
Ca
Mg
Na
Li
A
B
[32P] DPM INCORPORATED
100
200
300
400
5 10 15 20 25 30 35 40 45
GEL SLICE NUMBER

Figure 14. Phosphorylation patterns of Fraction 5. All media contained 3.74 μM ATP ([γ- ^{32}P] ATP, 40 Ci/nmol). (a): (O—O), ATP + 0.10 mM Mg^{2+}; (●—●), ATP + 0.50 mM Ca^{2+} (the latter being added 15 seconds after the addition of the ATP). (b): (O—O), ATP + 0.10 mM Mg^{2+}(the latter being added 15 seconds after the addition of the ATP); (●—●), hydroxylamine treatment of membranes phosphorylated with ATP for 15 seconds.


a
A
B
[32P] DPM INCORPORATED
400
300
200
100
5 10 15 20 25 30 35 40 45
GEL SLICE NUMBER


b
A
B
[32P] DPM INCORPORATED
400
300
200
100
5 10 15 20 25 30 35 40 45
GEL SLICE NUMBER

Finally F5, in the presence of Mg^{2+}, Ca^{2+} and [^{32}P] ATP (Figure 13b), showed a major decrease in Peak A phosphorylation. Peak B showed little or no change. F4 as before showed no real effect. These results for F5 may be due to competition of the two cations for a single binding site in Peak B or they are due to the effects of more than one binding site i.e. independent sites for Mg^{2+} and Ca^{2+}. Mg^{2+} is promoting dephosphorylation of this Peak B, while Ca^{2+} is promoting phosphorylation. When the membranes were phosphorylated in the presence of [^{32}P] ATP, Mg^{2+} and 120 mM Na^{+} or Li^{+} or choline, no increase in phosphorylation of any of the three peaks was noted in F4 or F5 (Figure 13a). However when Ca^{2+} was added to the above incubating media, non - specific increases in Peaks A and B phosphorylation were seen (Figure 13c). We can explain the increase in Peak B since we know Ca^{2+} increases its phosphorylation. However, the increase in Peak A is much more puzzling. Whether the Ca^{2+} is causing large conformational changes in the membrane enzymes is uncertain.

For each of the above experiments exhaustive control studies were done. To ensure that the ^{32}P bound was not adsorbed [^{32}P] ATP or $^{32}PO_4^{-3}$, reactions were stopped by means other than the addition of 5% TCA (see Methods section). To see whether phosphorylation was effected by trace impurities in the [γ - ^{32}P] ATP, phosphorylation was measured using 20-30 fold lower specific activities of [γ - ^{32}P] ATP. The same results were obtained regardless of the specific activity of the [γ - ^{32}P] ATP used.

Our original hypothesis that [MgATP] $^{2-}$ is the substrate for the Mg^{2+} stimulated activity observed has to be modified somewhat. It appears that ATP is binding prior to the binding of Mg^{2+}. The intermediate may still be the [MgATP] $^{2-}$ but this is purely conjecture.

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D. Orientation Studies Using Acetylcholinesterase and Sialic Acid

It was felt at this stage that F4 and F5 were certainly of differing orientation. F4 appeared to be enriched in inside-out (IO) vesicles while F5 was thought to contain predominantly right-side-out (RO) vesicles. This hypothesis was still far from conclusive at this stage so it was decided to characterize F4 and F5 with respect to their orientation using acetylcholinesterase (AchE) and sialic acid sidedness assays, both being externally localized on the cell PM. A 1.5 fold increase in AchE specific activity was observed in F4 when both membrane surfaces were made equally accessible to the substrate by the addition of 0.05% TX-100 prior to the assay (Table VIII). Similarily, after cleavage of sialic acid by neuraminadase there was a 3 fold increase in accessible sialic acid content of F4 with the addition of TX-100. F5 membranes showed little or no increase in AchE specific activity and sialic acid content in the presence of 0.05% TX-100. If one examines the actual increases in the specific activity of AchE and sialic acid content for F4 with the addition of TX-100, we find that the relative increases of the two markers differs. This appears to indicate that there is not a homogeneous distribution of these markers on the membrane surface. It should also be pointed out that the AchE specific activity was highest in Fractions 4 to 7. Sialic acid was found to be highest in Fraction 8, the mitochondrial enriched fraction.

E. Iodination Studies

Iodination was the next step in the investigation of membrane orientation. The investigation began with the labelling

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Table VIII

Accessibility of markers in sucrose gradient fractions. Sulphuric acid heading refers to total nanomoles of N - acetylneuramic acid / mg protein present in each assayed fraction. The concentration of detergent used was 0.05% Triton X-100 v/v.

Sucrose gradient fraction number	acetylcholinesterase[a]		sialic acid[b]		
	- Triton X-100	+ Triton X-100	- Triton X-100	+ Triton X-100	+ Sulphuric Acid
1	1.21	3.36	3.82	3.80	3.90
2	11.22	9.13	3.22	2.90	3.15
3	9.05	10.80	9.99	10.16	10.01
4	8.00	13.60	3.01	10.38	10.50
5	9.17	9.69	10.19	8.90	9.86
6	10.08	11.24	9.01	9.32	9.30
7	8.05	12.06	5.51	7.50	8.00
8	0.84	3.68	10.19	26.16	28.67
9	3.70	5.51	2.43	3.76	3.63
10	4.12	6.28	10.50	16.34	17.17
11	1.06	1.07	n.d.	n.d.	1.03
12	n.d.	n.d.	n.d.	n.d.	n.d.
13	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Expressed as nanomoles of product per milligram protein per minute.

[b] Expressed as nanomoles of N - acetylneuramic acid per milligram protein.

Table IX

Molecular weight assignments of bands and peaks depicted in Figures 15 to 41. Assignments are based on standard molecular weight markers as described under " Materials and Methods ".

Band	Peak	Molecular Weight
1	A, r	207,000
2	-	205,000 - 210,000
3		
4	-	191,000
5	B	165,000
6	C	136,000 - 145,000
7	-	130,000
8	s	100,000
9	-	82,000
10	t	55,000
11	-	45,000
12	-	Tracking Dye
13	-	-
14	-	31,000
15	-	93,000
14'	r'	207,000
15'	s'	100,000
16'	t'	55,000

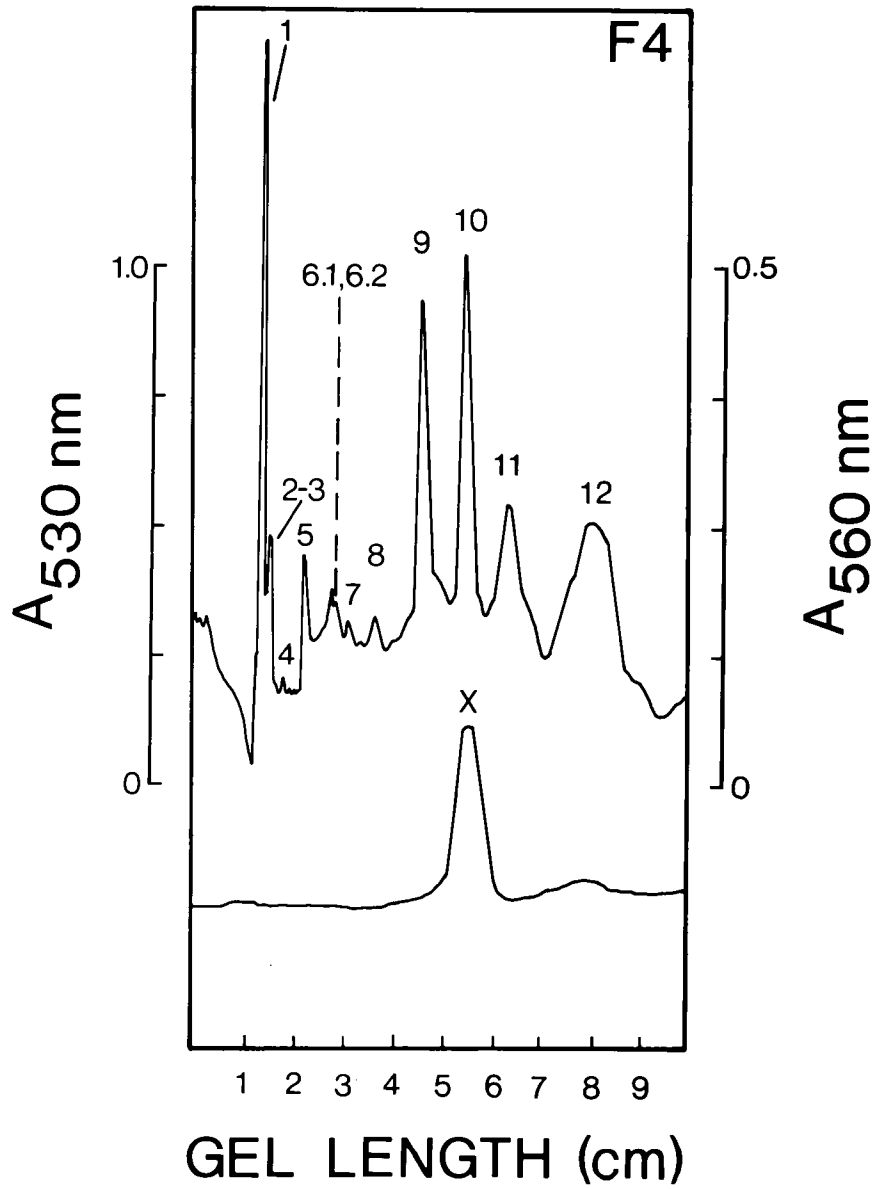

Figure 15. Coomassie blue staining pattern (top) and PAS profile (bottom) of Fraction 4. Protein used for coomassie blue staining (15 µg) was half that used for PAS staining (30 µg).

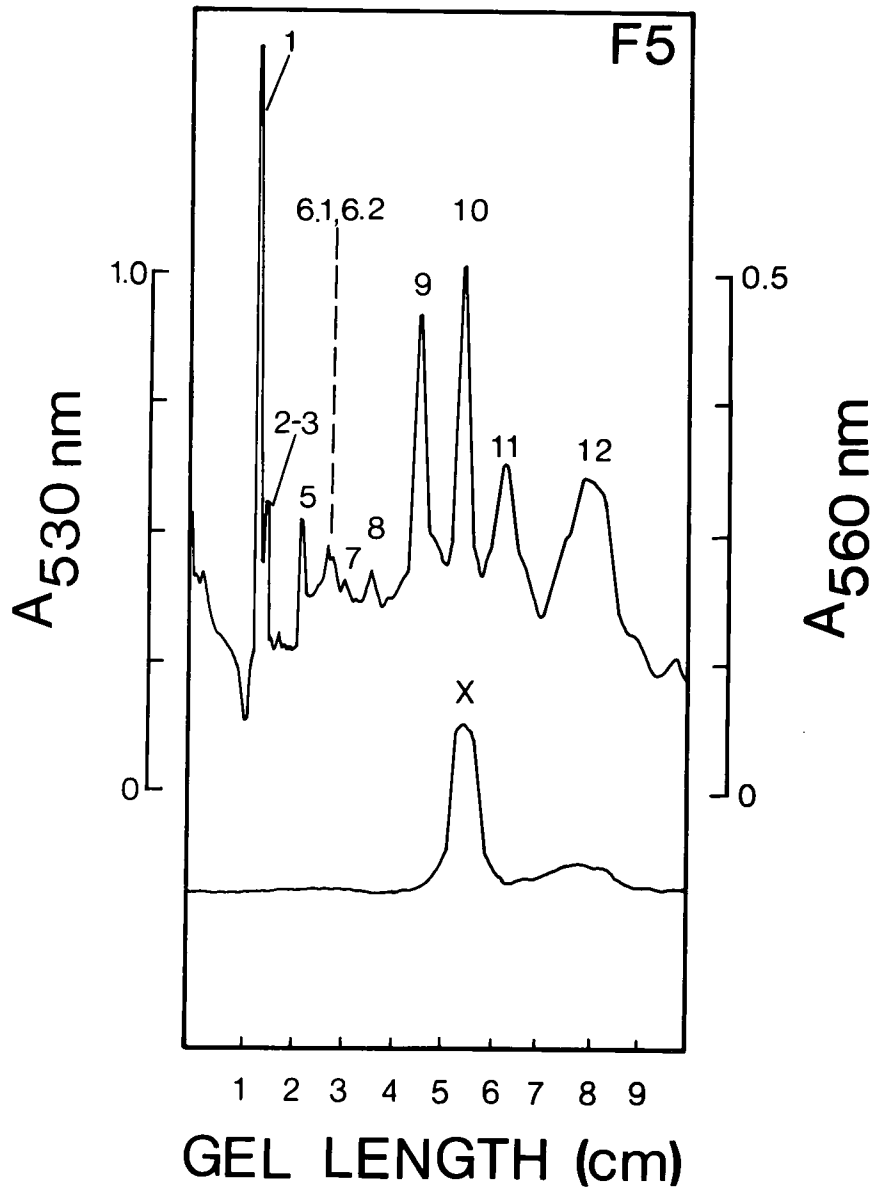

Figure 16. Coomassie blue staining pattern (top) and PAS profile (bottom) of Fraction 5. Protein used for coomassie blue staining (15 µg) was half that used for PAS staining (30 µg).

of the PM externally using 2 mm cubes of muscle, cell sheets, and single cell suspensions (Figures 17,18, and 19). When muscle cubes (Figure 17) were labelled with ^{125}I, labelling was seen at Band 16' (55,000 MW, peak t'). Cell sheets were labelled at Band 14' (205,000 MW, peak r') and Band 16'. There was no prominent labelling in the 100,000 MW region. The labelling of single cell suspensions (Figure 19) yielded similar results, however, there was a notable increase in the labelling of the 100,000 MW region (Band 15', peak s'). It should be pointed out that if certain regions of the protein profile have a low ^{125}I specific activity, it does not imply that the PM proteins have been labelled with a low specific activity. These gels (Figures 17 to 19) include the PM proteins and all other cellular proteins. Based on the above data we can conclude that at least two regions of the PM are accessible to the labelling species externally.

Next, the labelling of the muscle cubes and cell sheets was repeated. However, this time the muscle tissue was homogenized and processed to yield F4 and F5. The collected F4 and F5 were then examined for ^{125}I incorporation, F4 showing label incorporated at 205,000 MW (Band 1, peak r), 100,000 MW (Band 8, peak s) and 55,000 MW (Band 10, peak t) (Figures 20 and 22). Upon re-labelling of this fraction with ^{125}I, labelling was only noted at 100,000 MW. F5 showed a rather different behavior. Examination of F5 for initial incorporation showed the label at 205,000 MW, 100,000 MW and 55,000 MW (Figures 21 and 23). Re-labelling increased the loading in these regions. Not only do these preliminary results provide further evidence for the preferred orientations of F4 and F5 but they also provide us with information concerning the disposition of certain proteins in the membrane.

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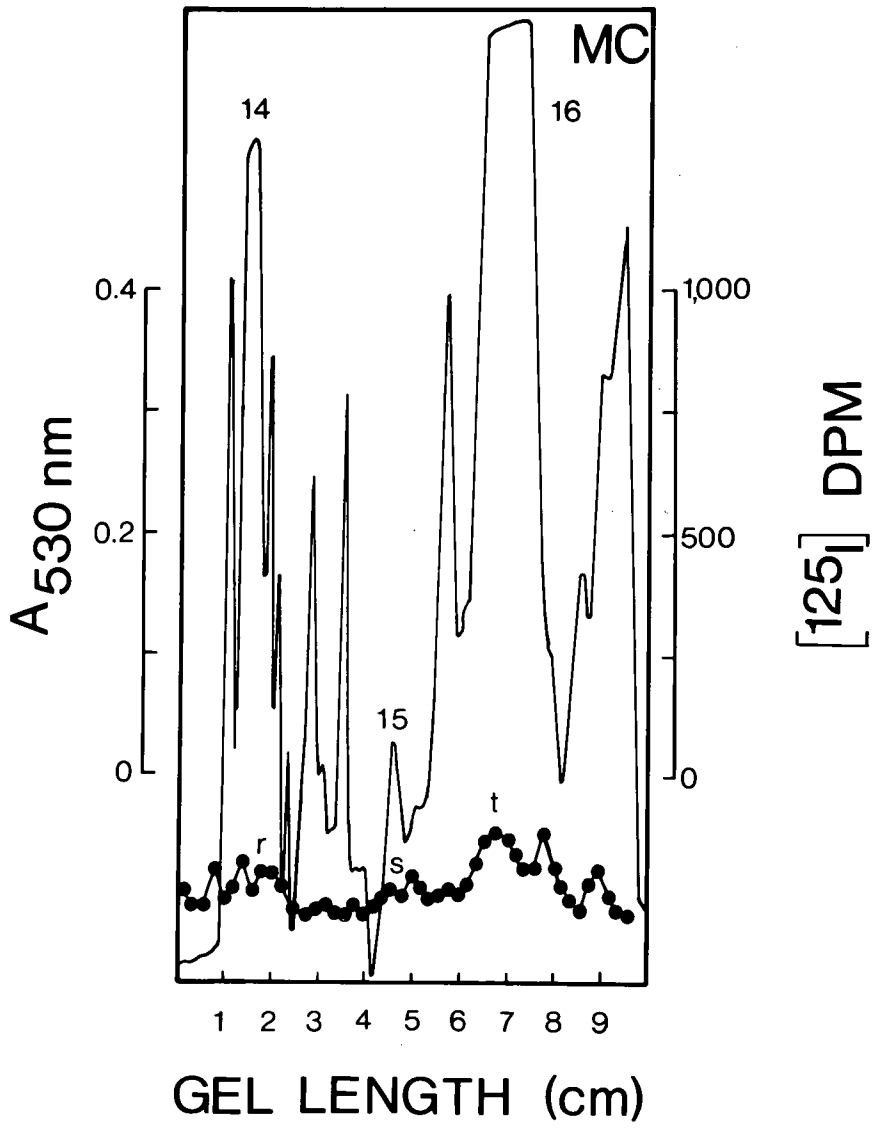

Figure 17. Labelling of muscle cubes with ^{125}I. The procedure used is described under " Materials and Methods ". (——), coomassie blue staining pattern of iodinated muscle; (●—●), iodination pattern of ^{125}I labelled muscle cubes.

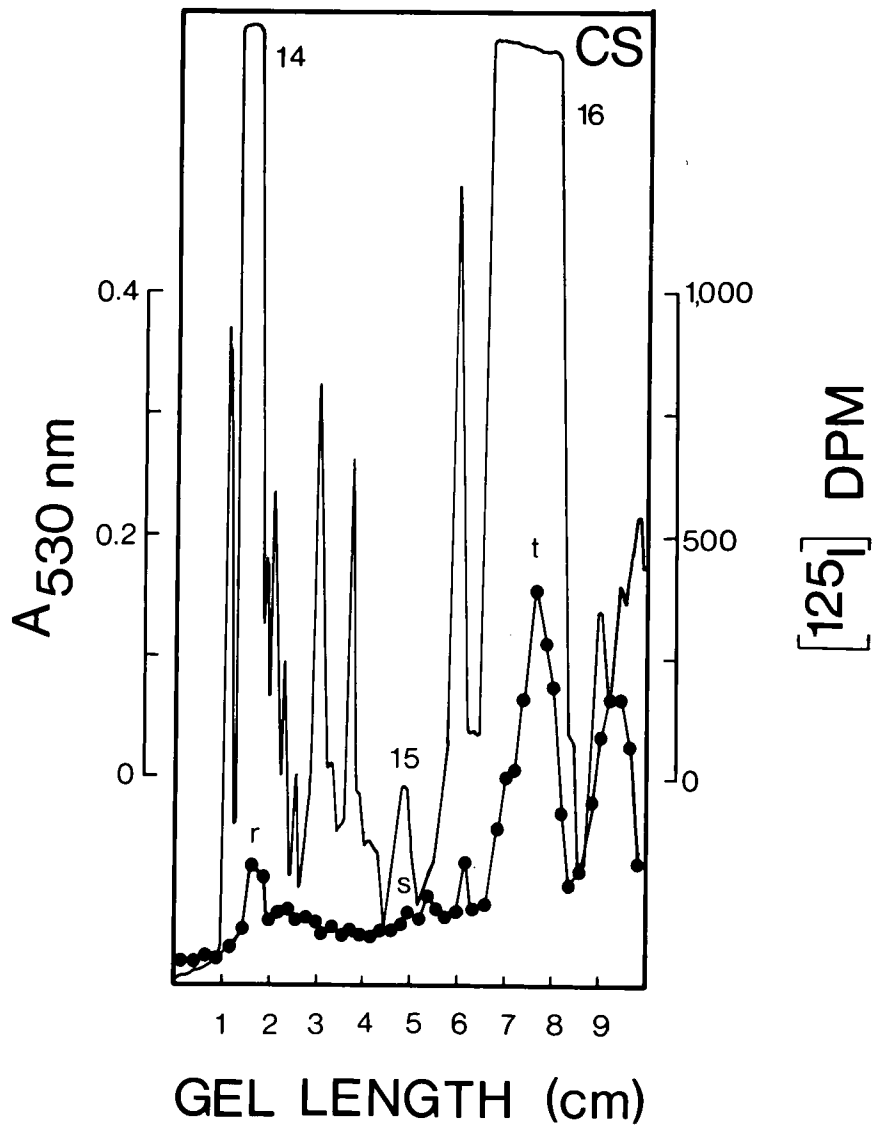

Figure 18. Labelling of cell sheets with ^{125}I. The procedure used is described under " Materials and Methods ". (——), coomassie blue staining pattern of iodinated cell sheets; (●—●), iodination pattern of ^{125}I labelled cell sheets.

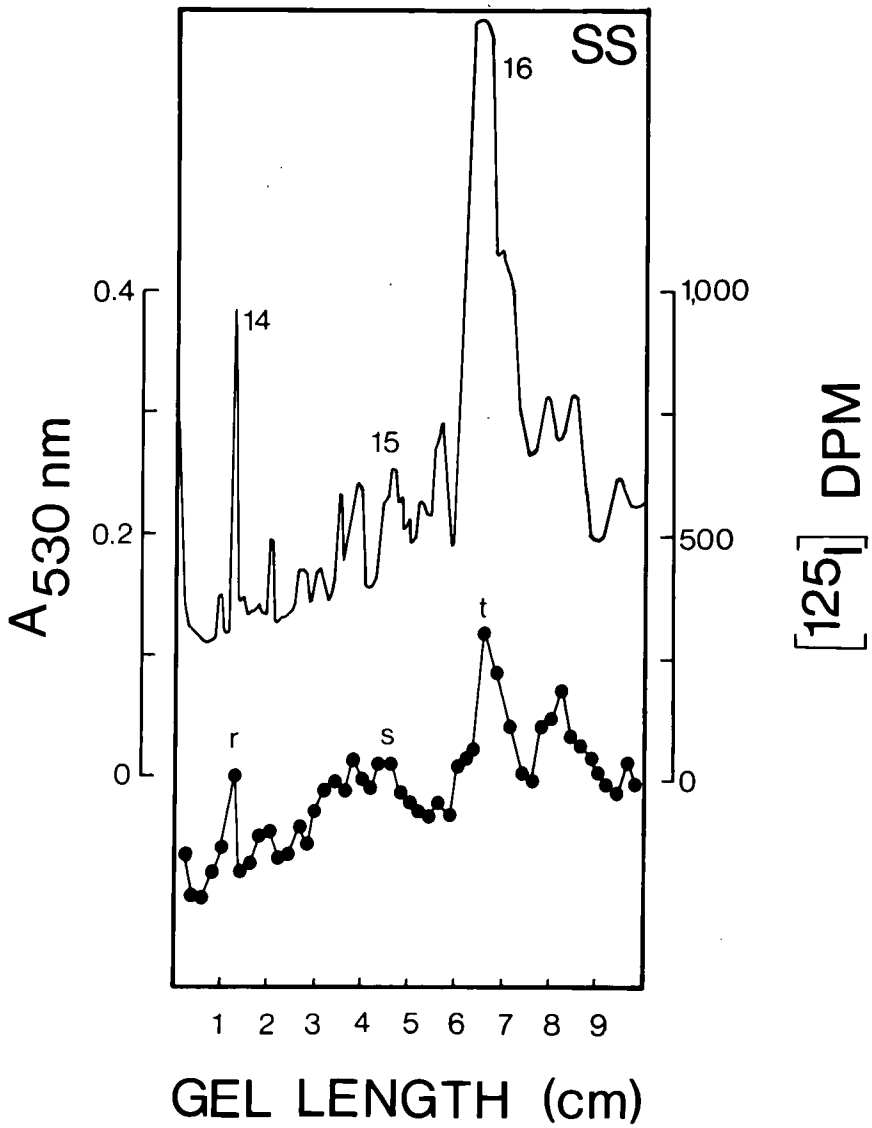

Figure 19. Labelling of a suspension of isolated single smooth muscle cells with ^{125}I. The procedure used is as described under " Materials and Methods ". (——), coomassie blue staining pattern of iodinated single cells; (●—●), iodination pattern of ^{125}I labelled single smooth muscle cells.

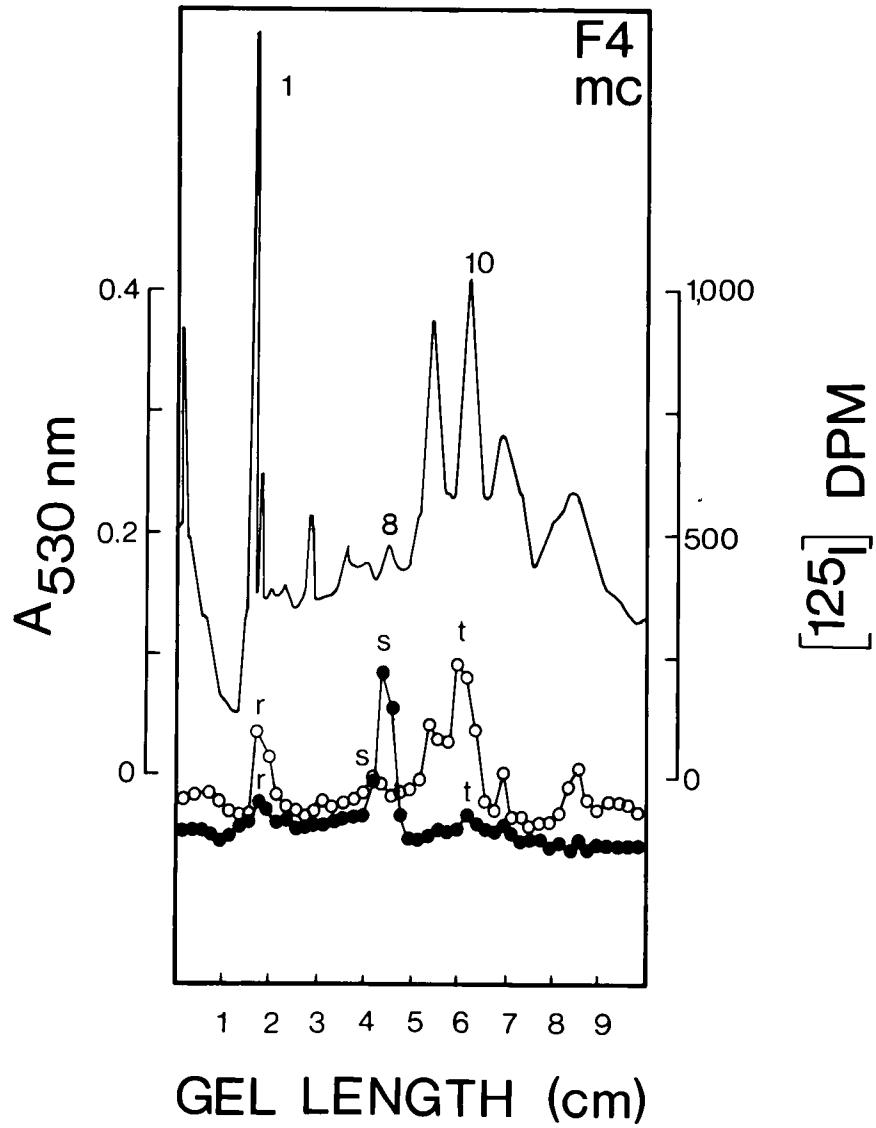

Figure 20. Iodination patterns of Fraction 4 using prelabelled muscle cubes. (——), coomassie blue staining pattern of Fraction 4; (O–O), iodination pattern of Fraction 4 obtained using muscle cubes labeled with ^{125}I prior to homogenization; (●–●), iodination pattern of Fraction 4 observed upon relabelling of Fraction 4 prepared from iodinated muscle cubes. For the exact procedure see under " Materials and Methods ".

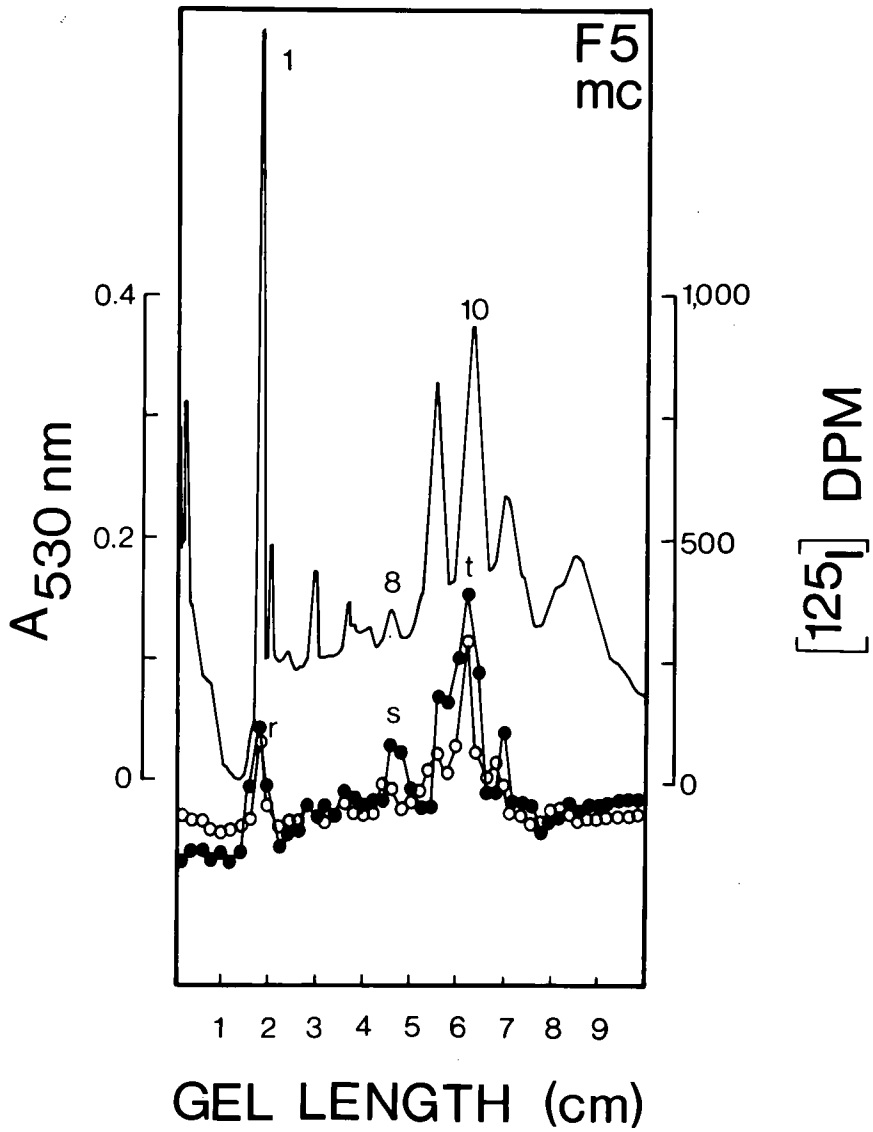

Figure 21. Iodination patterns of Fraction 5 using prelabelled muscle cubes. (——), coomassie blue staining pattern of Fraction 5; (O—O), iodination pattern of Fraction 5 obtained using muscle cubes labelled with ^{125}I prior to homogenization; (●—●), iodination pattern of Fraction 5 observed upon relabelling of Fraction 5 prepared from iodinated muscle cubes. For the exact procedure see under " Materials and Methods ".

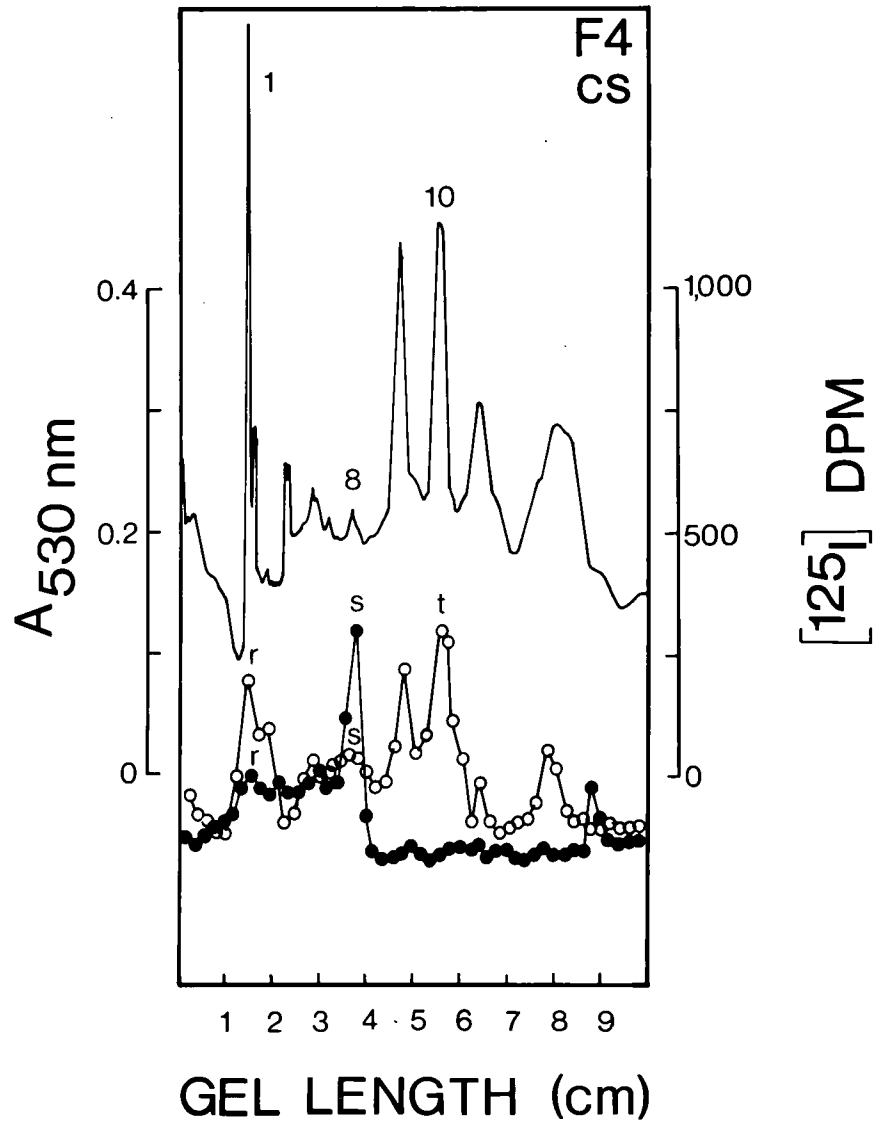

Figure 22. Iodination patterns of Fraction 4 using prelabelled cell sheets. (———), coomassie blue staining pattern of Fraction 4; (O—O), iodination pattern of Fraction 4 obtained using cell sheets labelled with ^{125}I prior to homogenization; (●—●), iodination pattern of Fraction 4 observed upon relabelling of Fraction 4 prepared from iodinated cell sheets. For the exact procedure see under " Materials and Methods ".

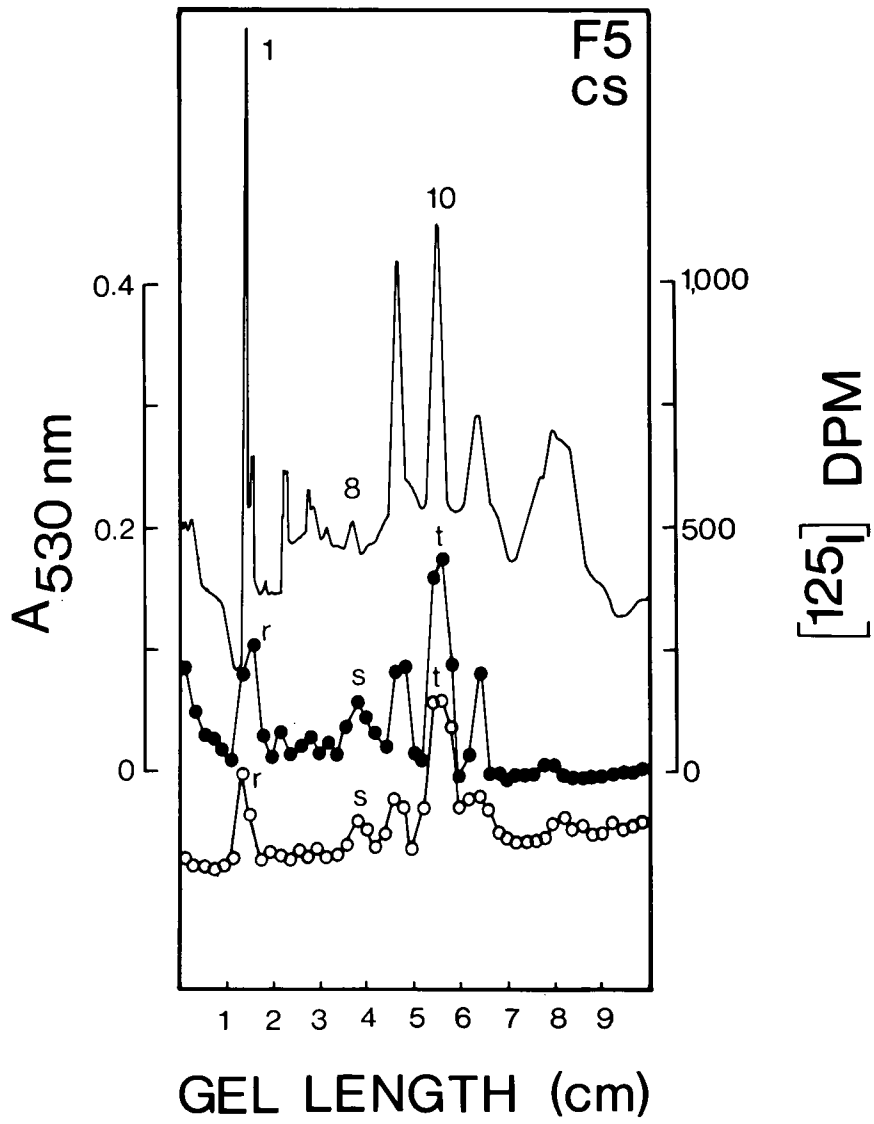

Figure 23. Iodination patterns of Fraction 5 using prelabelled cell sheets. (———), coomassie blue staining pattern of Fraction 5; (O—O), iodination pattern of Fraction 5 obtained using cell sheets labelled with ^{125}I prior to homogenization; (●—●), iodination pattern of Fraction 5 observed upon relabelling of Fraction 5 prepared from iodinated cell sheets. For the exact procedure see under " Materials and Methods ".

Similar results were obtained when F4 and F5 were labelled with ^{125}I using the three different methods outlined in the Methods section (the results of two of the methods are presented). The labelling of F4, directly from the gradient, shows that the label was incorporated at 100,000 MW with minor labelling of bands at 205,000 MW and 55,000 MW (Figures 24 and 26). As F4 was thought to consist predominantly of IO vesicles the labelling at 205,000 MW and 55,000 MW appears due to either leaky vesicles, unsealed vesicles or right-side-out vesicles. Incorporation of ^{125}I was noted at 205,000 MW, 100,000 MW and 55,000 MW for F5 (Figures 25 and 27). Some label was also seen to migrate with the tracking dye, representing possibly free $^{125}I^-$ or ^{125}I-labelled phospholipids. Based on the above we can conclude that F4 contains a 100,000 MW protein that is accessible to iodination from the external surface of F4. F5 contains 3 proteins that are accessible to the iodinating species externally. These are the 205,000 MW, 100,000 MW and 55,000 MW bands. If the 100,000 MW band is a single protein, it could be postulated that it spans the membrane. It cannot, however, be said that the 205,000 MW and 55,000 MW proteins do not span the membrane, because the cytoplasmic sites of labelling may not be accessible or may not contain any iodinatable residues. It is worth noting that the 205,000 MW band is thought to be the site of the ecto Mg^{2+} stimulated ATPase described earlier. Using the above information it should be possible to show in a controlled study that F4 is indeed IO and that F5 consists of mainly RO oriented membrane vesicles.

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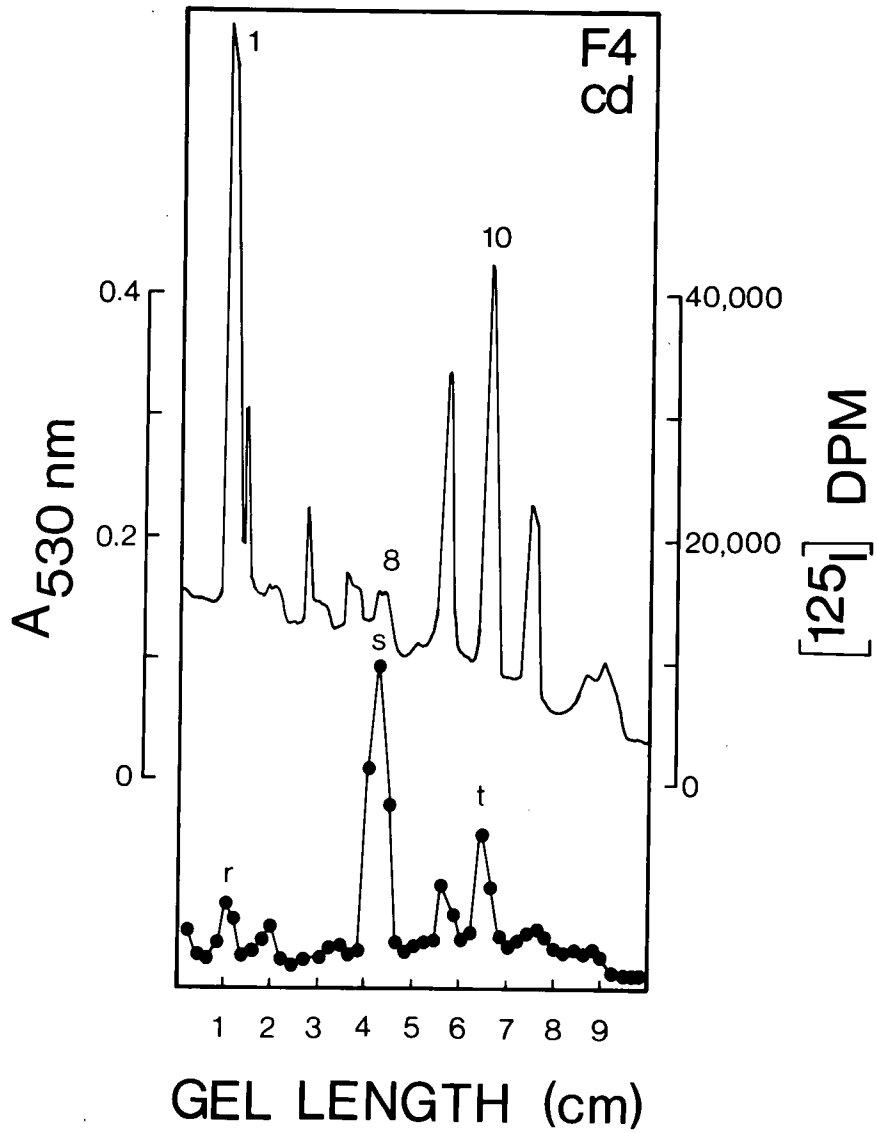


Figure 24. Iodination of Fraction 4. (——), coomassie blue staining profile of iodinated Fraction 4; (●—●), iodination profile of Fraction 4. Fraction 4 was prepared for iodination as described under " Materials and Methods, iodination studies, section B2 ".

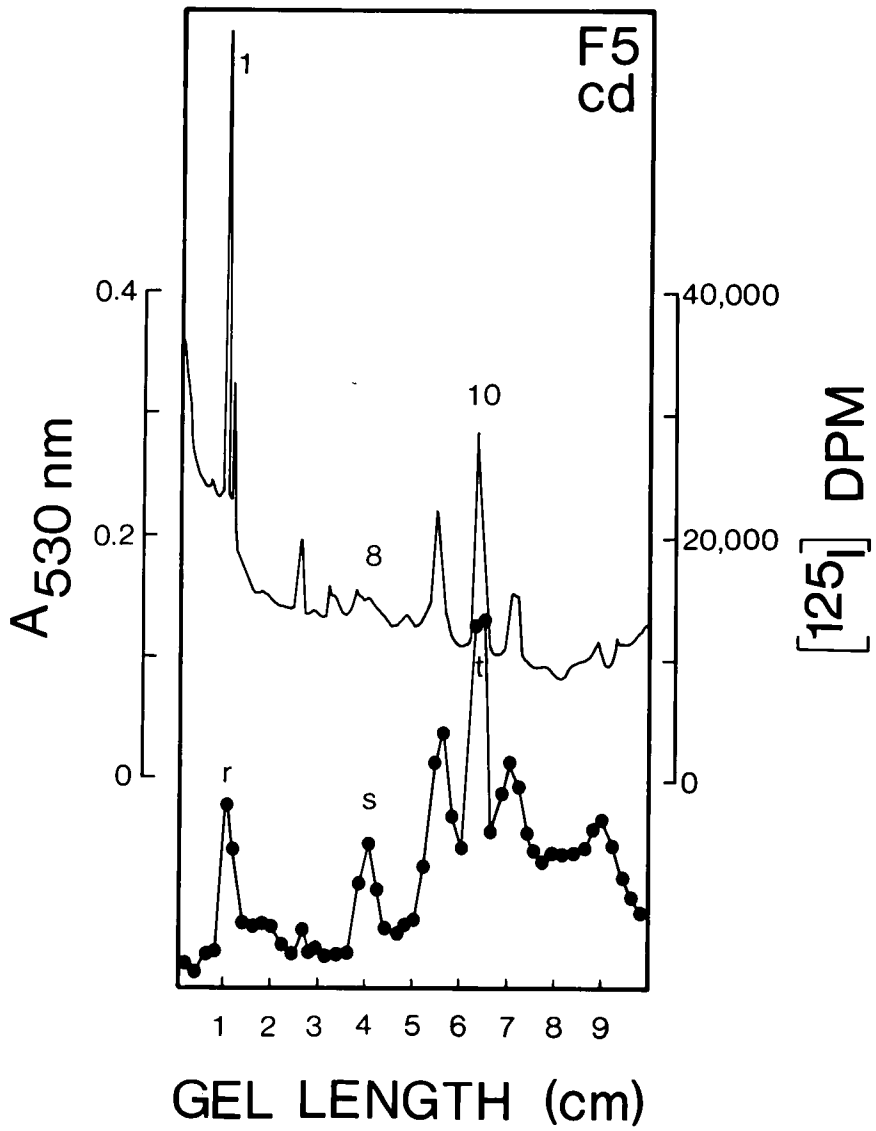


Figure 25. Iodination of Fraction 5. (——), coomassie blue staining profile of iodinated Fraction 5; (●—●), iodination profile of Fraction 5. Fraction 5 was prepared for iodination as described under " Materials and Methods, iodination studies, section B2 ".

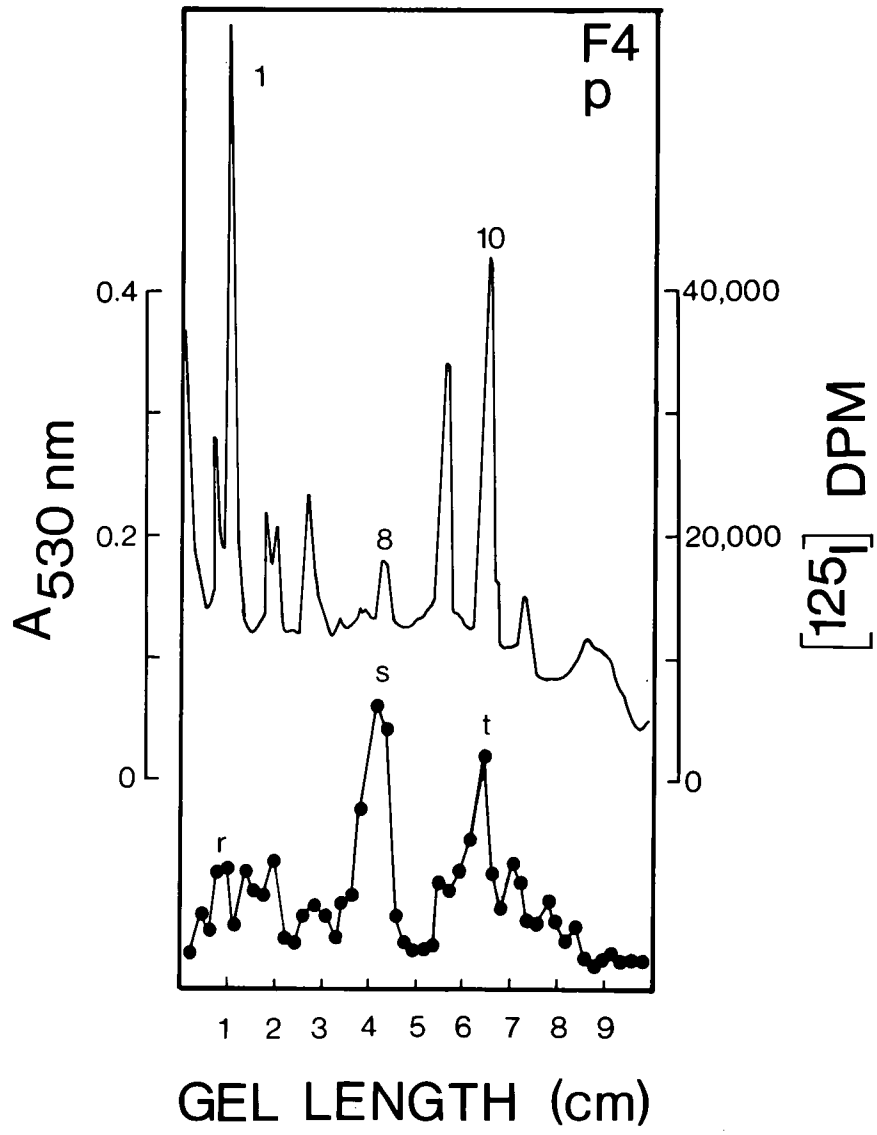


Figure 26. Iodination of sucrose free Fraction 4. (——), coomassie blue staining profile of sucrose free iodinated Fraction 4; (●—●), iodination profile of sucrose free Fraction 4. The procedure used is described under " Materials and Methods, iodination studies, section B3 ".

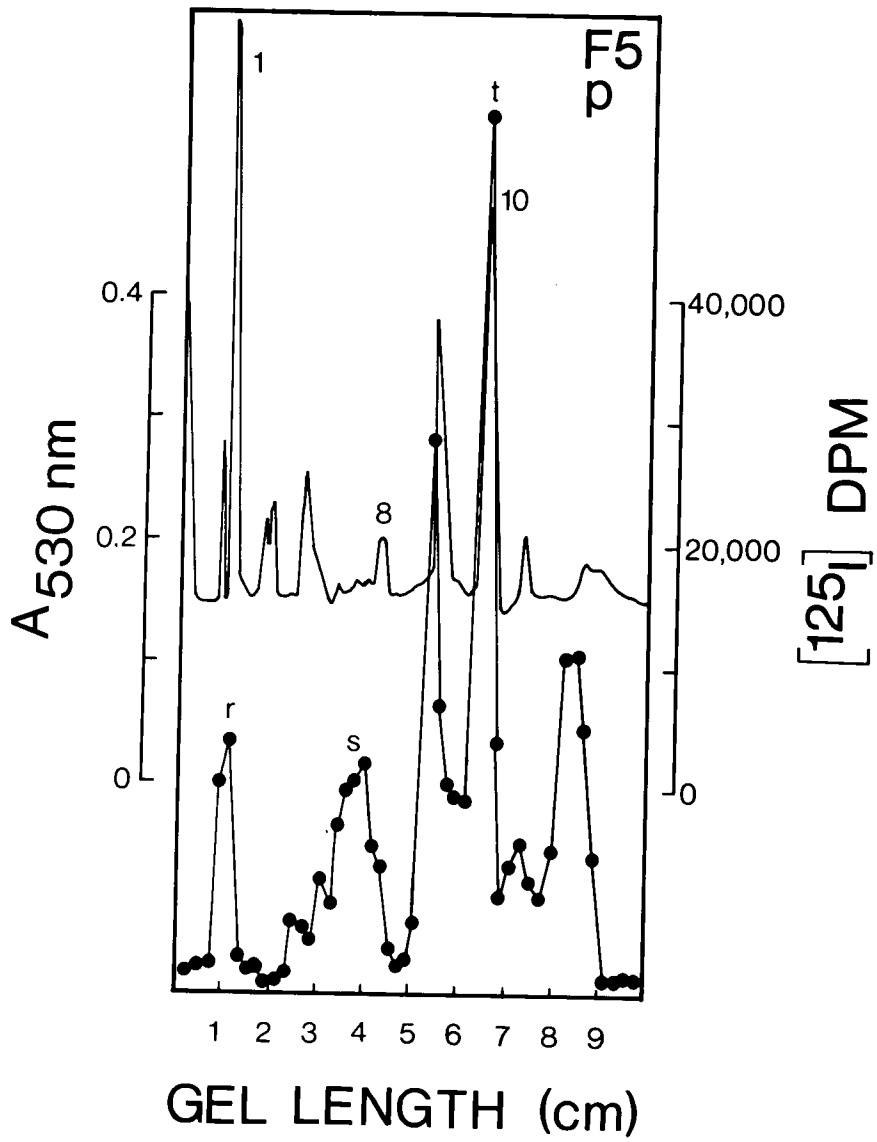


Figure 27. Iodination of sucrose free Fraction 5. (——), coomassie blue staining profile of sucrose free iodinated Fraction 5; (●—●), iodination profile of sucrose free Fraction 5. The procedure used is described under " Materials and Methods, iodination studies, section B3 ".

It can be argued that the labelling seen at 205,000 MW and 100,000 MW is due to self labelling of lactoperoxidase and labelling of a 205,000 MW contaminant in commercial lactoperoxidase preparations. To deal with this potentially serious problem, the following experiments were done. It is well known lactoperoxidase will label itself, especially as the age of the enzyme increases (Figure 28). Fresh lactoperoxidase was iodinated to saturation with non-radioactive iodine, purified and then examined for specific activity, self labelling and purity. Its self labelling ability was reduced to less than 10% of that found for the fresh enzyme, whereas the specific activity of the enzyme was equivilant to that found for the fresh enzyme. The enzyme, on SDS gels, ran as a single band at 98,000 MW.

Using equal amounts of F4 membranes and identical iodination conditions, F4 was labelled with freshly made lactoperoxidase and cold labelled lactoperoxidase (Figure 29, right and left). As well, F4 was labelled with fresh lactoperoxidase in the presence of 0.05% TX-100. The results, tabulated in Table Xa, show that labelling with the two enzymes was identical. The 100,000 MW was mainly labelled. There was also some labelling of the 55,000 MW band. This latter labelling may be due to contamination of F4 by unsealed, RO or leaky membrane vesicles. In the presence of 0.05% TX-100, large increases in the labelling of the 100,000 MW and 55,000 MW bands (Bands 8 and 10 respectively) were noted. We can add here that self-labelling by lactoperoxidase in the presence of 0.05% TX-100 is 50% of that found in the absence

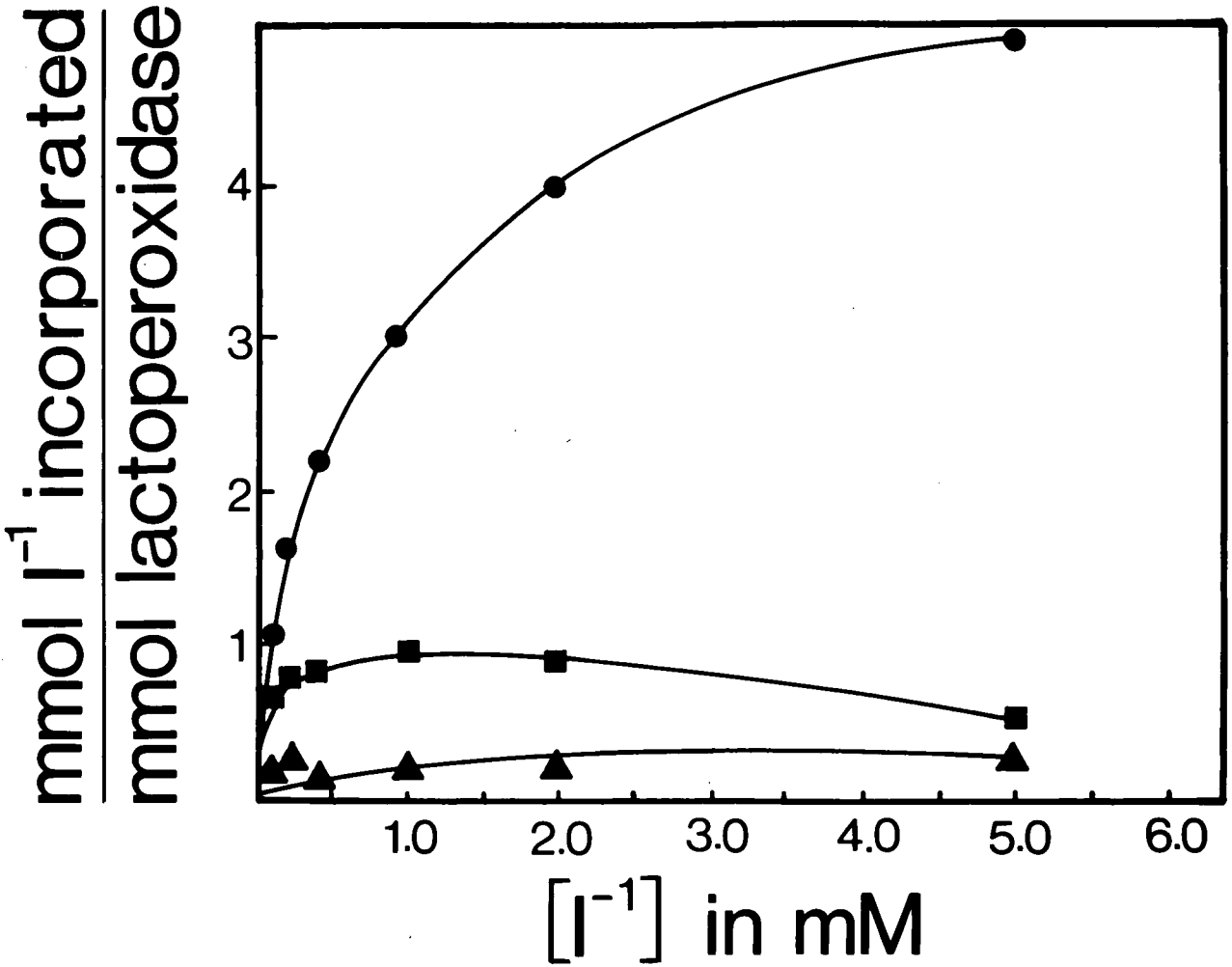
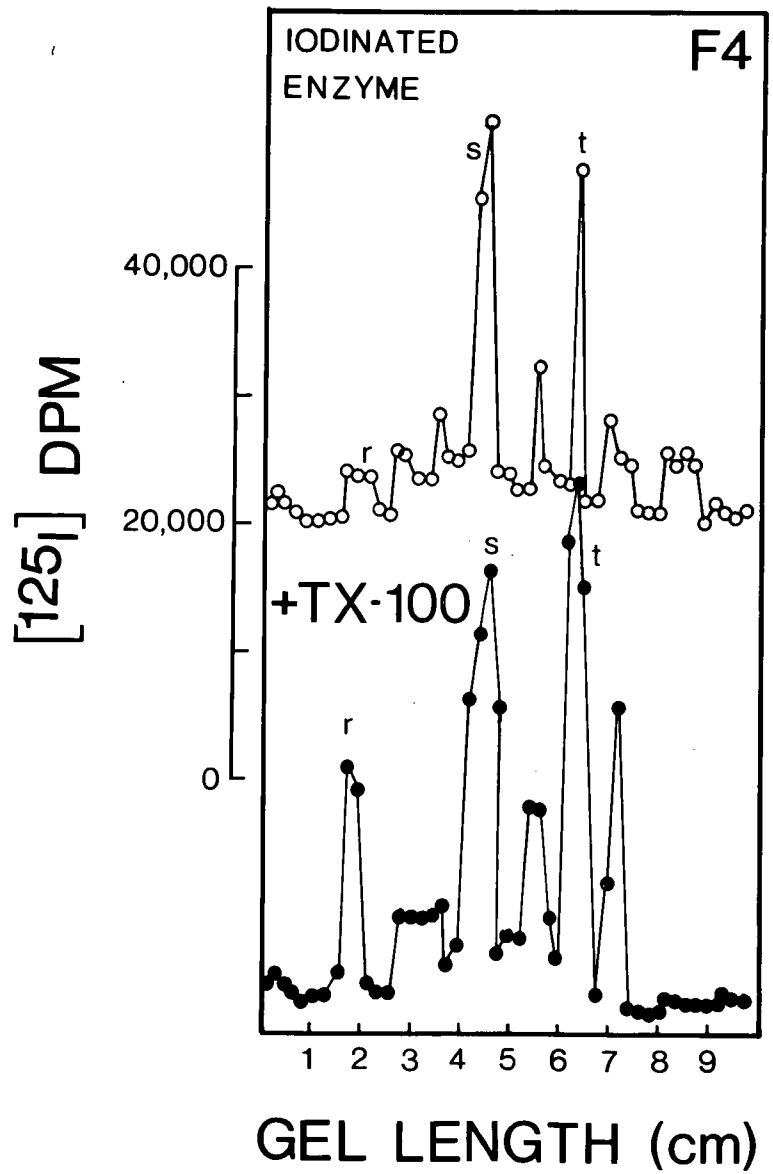


Figure 28. Self iodination of lactoperoxidase. (●—●), three week old enzyme stored frozen; (■—■), freshly prepared enzyme; (▲—▲), previously labelled lactoperoxidase. For preparation of labelled lactoperoxidase see under " Materials and Methods ".

Figure 29. Accessibility of Fraction 4 to iodination using ^{125}I. RIGHT: (——), coomassie blue staining profile of Fraction 4; (●—●), iodination profile of Fraction 4 in the absence of 0.05% Triton X-100. LEFT: (○—○), iodination pattern of Fraction 4 using lactoperoxidase previously labelled with cold iodine; (●—●), iodination of Fraction 4 in the presence of 0.05% Triton X-100.


IODINATED
ENZYME
F4
s
t
r
40,000
20,000
0
+TX-100
s
t
r
[125I] DPM
1 2 3 4 5 6 7 8 9
GEL LENGTH (cm)

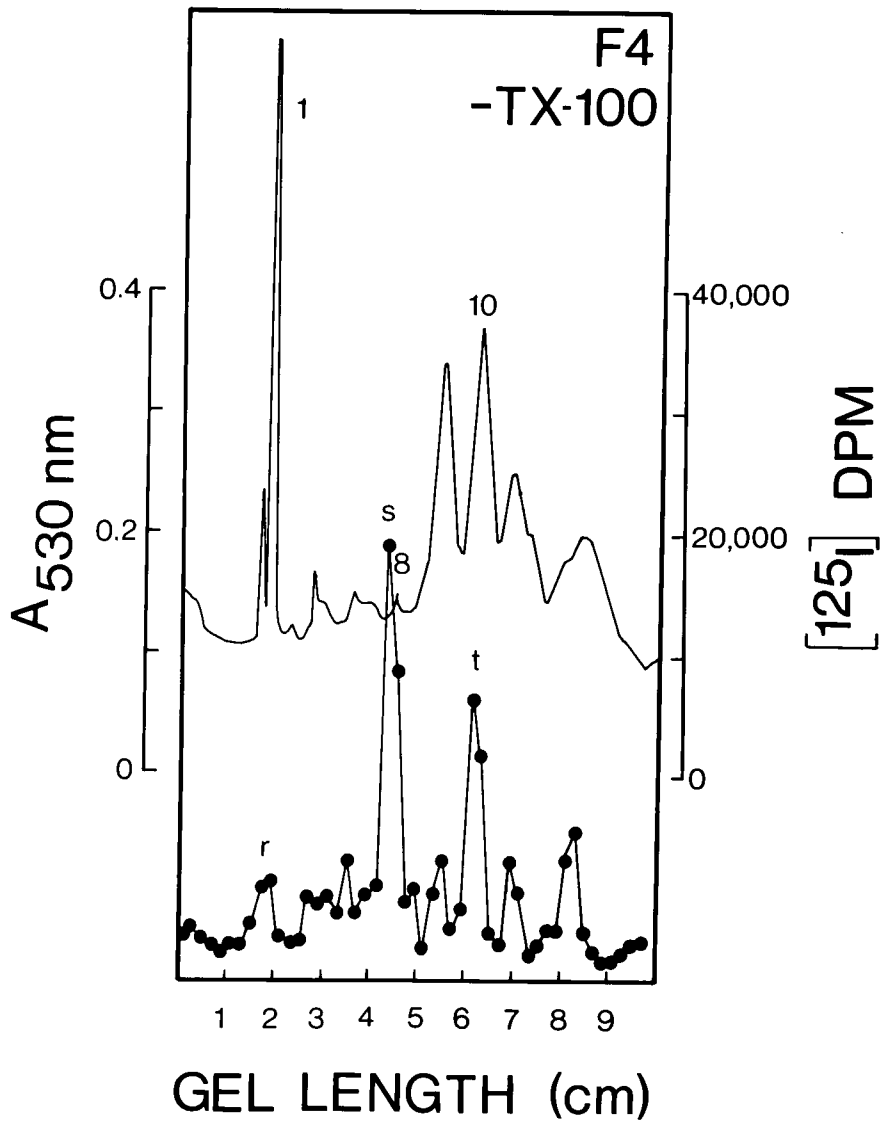
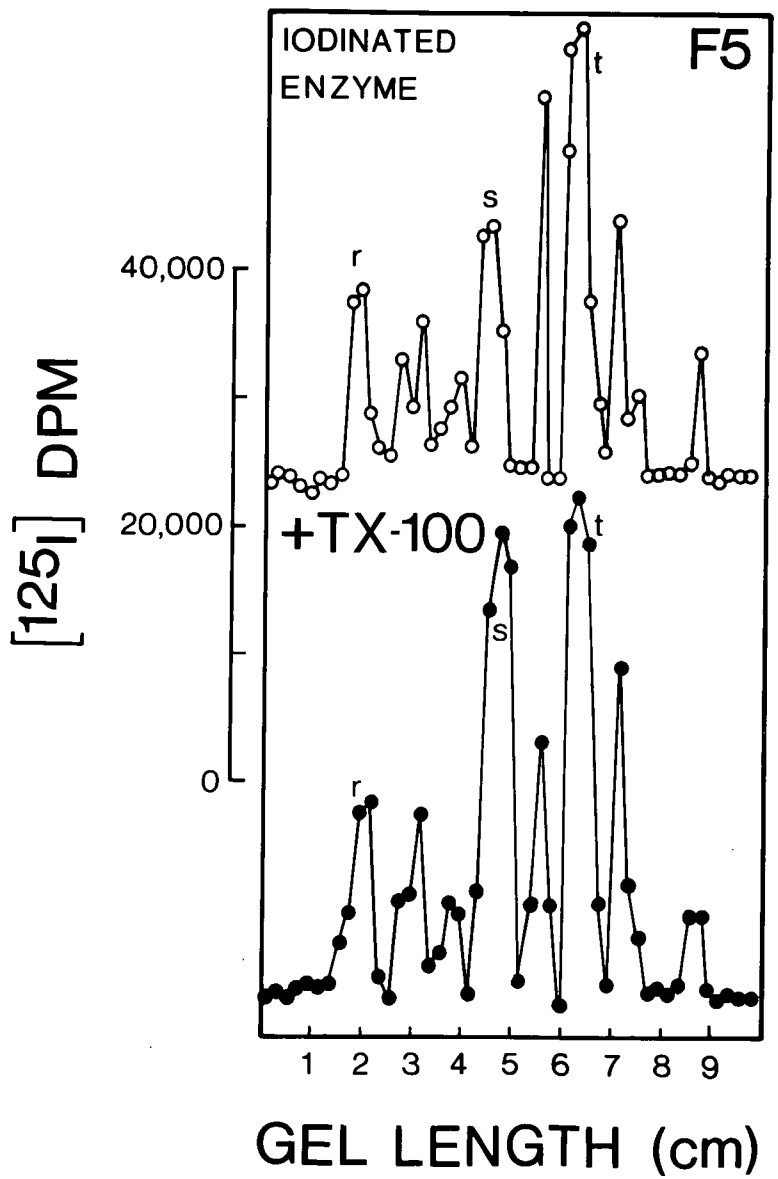
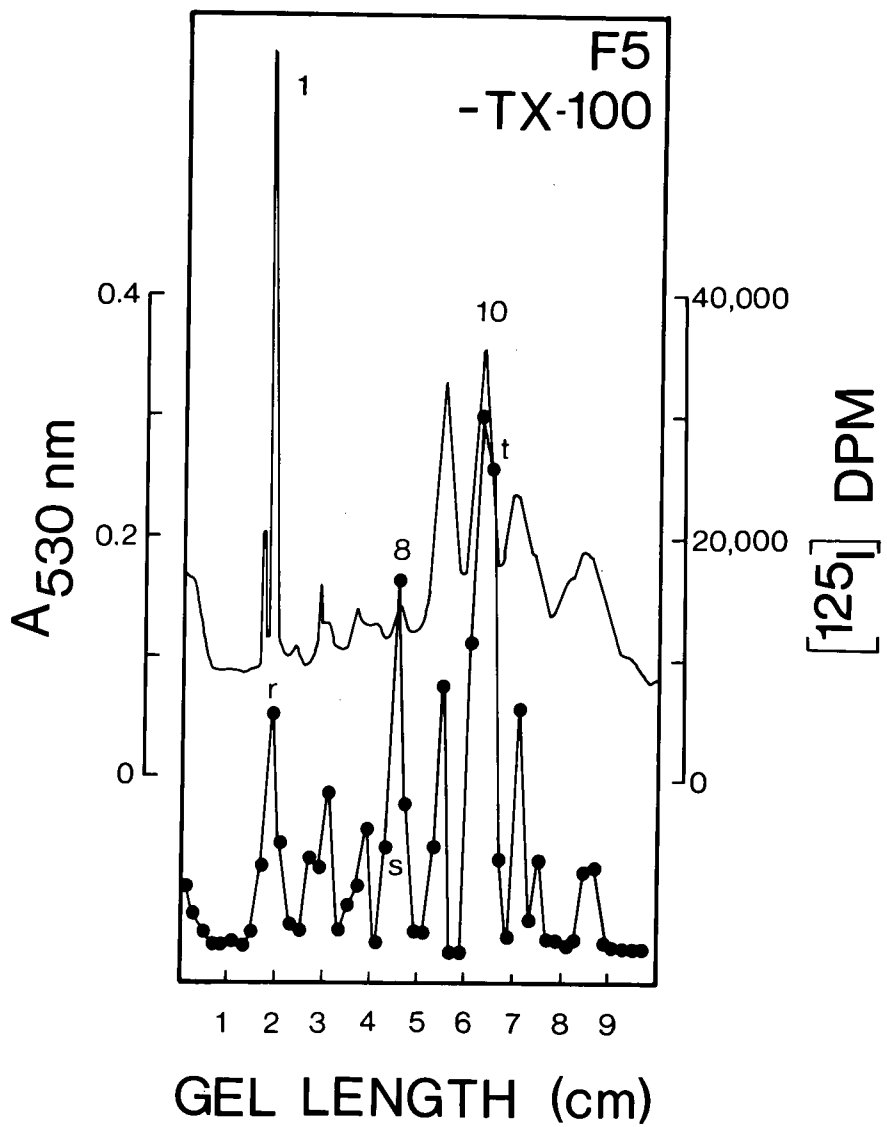

F4
-TX-100
1
10
s
8
t
r
0.4
0.2
0
A530 nm
40,000
20,000
0
[125I] DPM
1 2 3 4 5 6 7 8 9
GEL LENGTH (cm)

Figure 30. Accessibity of Fraction 5 to iodination using ^{125}I. RIGHT: (———), coomassie blue staining profile of Fraction 5; (●—●), iodination profile of Fraction 5 in the absence of 0.05% Triton X-100. LEFT: (O—O), iodination pattern of Fraction 5 using lactoperoxidase previously labelled with cold iodine; (●—●), iodination of Fraction 5 the presence of 0.05% Triton X-100.


IODINATED
ENZYME
F5
+TX-100
r
s
t
40,000
20,000
0
[125I] DPM
1 2 3 4 5 6 7 8 9
GEL LENGTH (cm)


F5
-TX-100
1
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10
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0.4
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A530 nm
40,000
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[125I] DPM
1 2 3 4 5 6 7 8 9
GEL LENGTH (cm)

Table Xa

Accessibity of Fractions 4 and 5 to lactoperoxidase catalyzed iodination.[5]

Sucrose gradient fraction number		^{125}I c.p.m./12 µg protein (55% efficiency) Band 8 - 100,000 daltons	Band 10 -55,000 daltons
4	iodolactoperoxidase	83,000	42,000
	- Triton X-100	85,000	45,000
	+ Triton X-100	149,000	145,000
5	iodolactoperoxidase	61,000	143,000
	- Triton X-100	70,000	153,000
	+ Triton X-100	140,000	155,000

[5] Please see Figures 29 and 30 also.

Table Xb

Self labelling of lactoperoxidase in the presence of Triton X-100. Lactoperoxidase used was one day old.

[Triton X-100]	^{125}I c.p.m./25 µg enzyme
0.00%	1.00×10^5
0.05%	0.50×10^5
0.10%	0.50×10^5
0.20%	0.40×10^5
0.40%	0.30×10^5

of detergent (Table Xb). On the basis of the preceeding results, false peaks due to self-labelling of commercial lactoperoxidase preparations, can be ruled out. The results for F5 are just as revealing. In the absence of detergent, using fresh or cold labelled lactoperoxidase, labelling was observed at 100,000 MW and 55,000 MW mainly. The results using the two different enzymes were identical, thus again ruling out lactoperoxidase self-labelling giving false peaks. Labelling in the presence of TX-100 was shown to be increased only at 100,000 MW. There was no change in the labelling at 55,000 MW. These results provide extremely strong arguements in favour of the hypothesis that F5 PMs are mainly RO oriented and that F4 contains a predominantly IO PM vesicle population with some contamination.

F. Extraction Studies

At this stage, given the indicated orientations of F4 and F5 we had hoped to corroborate this difference in orientation using extraction studies. As well, we had hoped to extract peripheral membrane proteins to simplify the coomassie blue staining profile. Examination of the results yields a rather confusing picture indeed (Figures 31 to 38, Table XI). The agents used were Dimethylmaleic anhydride (DMMA), Ethylenediamine tetraacetate (EDTA), H_2O, Diditonin (DT), Triton X-100 (TX-100), and p-Chloromercuribenzene sulphonic acid (pCMBS). Their use was based on studies by Fairbanks (FAIRBANKS et al., 1971), Steck (STECK & YU, 1973) and Kahlenberg (KAHLENBERG, 1976). DMMA and pCMBS either denature or covalently modify proteins, pCMBS by

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breaking disulphide linkages. In the human red blood cell, these agents selectively solubilize a certain group of membrane polypeptides, leaving the remainder still associated with all the lipid and cabohydrate in the membrane residue. The specific action of the non-ionic detergent TX-100, a Type A amphiphile, is nearly reciprocal to that seen with perturbants such as DMMA. Polypeptides anchored in the memnrane through apolar associations with lipids can be solubilized by TX-100 and hopefully the lipids can be displaced from the hydophobic proteins without denaturation. Digitonin, though classed as a Type B amphile, acts by a different mechanism compared to TX-100 but the end result is the same. EDTA is thought to release membrane fibrillar proteins by chelating membrane bound divalent.

The results are presented band by band. It is important to realixe that F4 may not be entirely homogeneous with respect to IO orientation while F5 is thought to be mainly RO. The behavior of Band 1 (205,000 MW), which may be associated with an ecto Mg^{2+} stimulated ATPase, was quite interesting. We already knew that Band 1 was accessible externally based on the ^{125}I studies. When the membranes were extracted with H_2O, a small amount of the Band 1 protein was extracted from F5 (The appearance of Band 1 in the supernatant of F4 extracted with H_2O is not selective. We are seeing vesicles with a buoyant density sufficient to not allow sedimentation under the conditions used.). DMMA removed Band 1 totally in F4 while 25% was only removed in F5. High concentrations of pCMBS removed 50% of Band 1 in F5 but the band was not touched

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in F4. Interestingly Band 1 was totally removed by TX-100 in F4, but, in F5 the band was not disturbed. Digitonin and EDTA had no effect on Band 1 in F4 or F5. Based on the above we can summarize that Band 1 contains more than one protein, part of the band is peripherally located and part is embedded in the membrane. There is also disulphide bond character in the band proteins.

A rather unusual feature of our membrane preparations was Band 2-3. As a shoulder on Band 1 it appeared at 210,000 daltons or 200,000 daltons. The positon could not be predicted, for example in the TX-100 extraction of F4 (Figure 37) it appeared at 200,000 daltons, whereas in the presence of EDTA and H_2O (Figure 31) it migrated at 210,000 MW. Even when a series of samples from F4 and F5 were subjected to gel electrophoresis under identical conditions, this band appeared randomly at one position or the other. Under treatment with the various extracting media this band behaved like Band 1.

Band 4 was to small to be followed in these studies. Band 5 was partially removed by DT in F4, in contrast Band 5 was extracted by EDTA from F5 only. Using DMMA Band 5 was removed from both F4 and F5. However, the concentration used in the extraction of Band 5 from F5 was lower than for F4. Band 5 in F5 was partially removed at low pCMBS concentrations of 0.01 mM but it was not readily apparent whether Band 5 was extracted in F4 by pCMBS, as there was a large shoulder on Band 1 in which Band 5 may have been present. Triton X-100 totally extracted Band 5 in F4 whereas only a small amount of Band 5 was removed in F5 under the same conditions.

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There might also have been some Band 5 hidden under the shoulder of Band 1 in F5 extracted with 0.05% TX-100. It should be noted that a portion of this band displayed Ca^{2+} dependent increases in phosphorylation and that the binding sites for ATP and Ca^{2+} were accessible from the external surface only. We can thus conclude that Band 5 contains disulphide linkages, may consist of more than one protein component and it lies partially embedded in the hydrophobic region of the membrane. That there must be a peripheral component can be shown by the EDTA extraction of Band 5.

Bands 6.1 and 6.2 were apparently partially removed by H_2O extraction of F4 and F5 but it appeared that there was no actual extraction of 6.1 and 6.2 in F4. As pointed out earlier the gel profile of the supernatant obtained from the H_2O extraction of F4 represents small vesicles that could not be sedimented. In some cases such as the EDTA extraction of F4, the Bands 6.1 and 6.2 could not be identified. DT removed neither 6.1 or 6.2 from F4 or F5 while low concentrations of DMMA partially extracted these bands in F4 and F5. Treatment with pCMBS yielded no extraction of 6.1 and 6.2 but TX-100 removed the bands partially in both F4 and F5. We can therefore conclude that Bands 6.1 and 6.2 also consist of more than one protein and are partly embedded in the hydrophobic part of the membrane.

Band 7 was a very minor band, at times difficult to detect. The only observation that can be made is that TX-100 appeared to selectively remove Band 7 from F5 while Band 7 was only partially removed from F4 by this treatment. It therefore appears that Band 7 may be found in the hydrophobic regions of the membrane.

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Band 8, if a single protein was thought to span the membrane based on the iodination studies. EDTA and DT removed the band from F4 and F5. High concentrations of DMMA removed 100% of Band 8 in F4 but less than 50% in F5. The band was completely extracted by TX-100 in both fractions. Using pCMBS Band 8 was not affected in F4 but appears to have been extracted from F5 (in a broad peak on the shoulder of Band 9). We can summarize that Band 8 appears to span the membrane based on the iodination studies. This is verified by the TX-100 removal of the band in F4 and F5. There may be disulphide character and more than one protein component in this band.

Of all the bands, Band 9 displayed the most variable behavior. EDTA and DT partially removed Band 9 from F4 and F5. DMMA totally removed Band 9 in F4 but only 50% of this band was extracted in F5 using high concentrations of DMMA. The behavior of this band in the presence of pCMBS was again quite different in F4 and F5. This treatment totally removed the band from F5 whereas it was virtually unaffected in F4. Interestingly, the TX-100 extraction of F4 appeared to result in some kind of modification of the Band 9 protein as it became the predominant peak in the gel pattern, possibly at the expense of Band 10. Band 9 in F5 was 75% removed by treatment with TX-100. We can tentatively conclude that Band 9 appears to be one protein with disulphide bond character, and is embedded partly in the hydrophobic regions of the membrane. It can also be added that Bands 9 and 11 were iodinated only from the external membrane surface (Figures 25 to 28), but the extraction studies seem to indicate that Band 9 does have cytoplasmic sites which may not be accessible to iodination.

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Band 10, which had been shown to have externally iodinated sites only, could be extracted by EDTA and DT from F4 and to a lesser degree in F5. DMMA appeared to have modified the band in F4 and extracted 50% of the band in F5. There appeared to have been an increase in Band 9 corresponding to a decrease in Band 10 and possibly implying some type of chemical modification. PCMBS removed part of Band 10 in both F4 and F5 (50%). The only anomalous result was noted in the extraction of F4 by 0.05% TX-100. In this extraction the decrease in Band 10 was again accompanied by an increase in Band 9. Based on iodination studies and these results Band 10 has disulphide bond character, may consist of more than one protein and possesses external sites that can be iodinated but is thought to extend into the hydrophobic region.

Band 11 in both F4 and F5 was not affected by DT, but it was, however, removed from F4 by EDTA. In F5, only 50% of Band 10 was removed by EDTA. DMMA also removed all of Band 10 from F4 but only 50% of the band in F5. Low concentrations of pCMBS totally removed Band 11 in F4 while, in contrast, much higher concentrations of pCMBS were required to elute the band in F5. Again TX-100 selectively removed Band 11 from F4, whereas, in F5 only 50% was removed. Iodination studies (Figures 25 to 28) showed Band 11 could only be iodinated externally. Our results indicate that Band 11 may have disulphide bonds, consist of more than one protein and possesses some cytoplasmic sites in addition to the known externally accessible sites.

There were various anomalies recorded in the extraction

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Table XI

Protein (µg) contained in extraction media (300 µl) and pellet (resuspeded in 150 µl) following extraction procedure. For gel electrophoresis 150 µl of supernatant and 75 µl of pellet were were used unless otherwise indicated. Please see Figures 31 to 38 also.

Extracting Agent	Fraction 4		Fraction 5	
pCMBS	pellet	supernatant	pellet	supernatant
0.01 mM	32	12	38	16
0.10 mM	40	6	32	14
2.00 mM	32	10	20	20
Triton X-100				
0.0]%	24	38[a]	40	30
0.05%	22	60[a]	30	40
0.50%	16	56[a]	42	40
DMMA				
0.1 mg/ml	6	48[a]	20	26
0.4 mg/ml	-	40[a]	20	26
1.0 mg/ml	4	40	16	44
Digitonin				
0.36 mg/ml	24	14	34	10
EDTA				
0.50 mM	30[a]	16[a]	44	32
H_2O	48	12	62	8

[a] Refers to 38 µl used for gel electrophoresis.

Figure 31. Extraction of Fraction 4 using H_2O, Ethylenediamine tetraacetate and Digitonin. For µg protein in supernatant (S) and pellet (P) see Table X.

(Right), pellet obtained after extraction procedure.

(Left), supernatant obtained after extraction procedure.

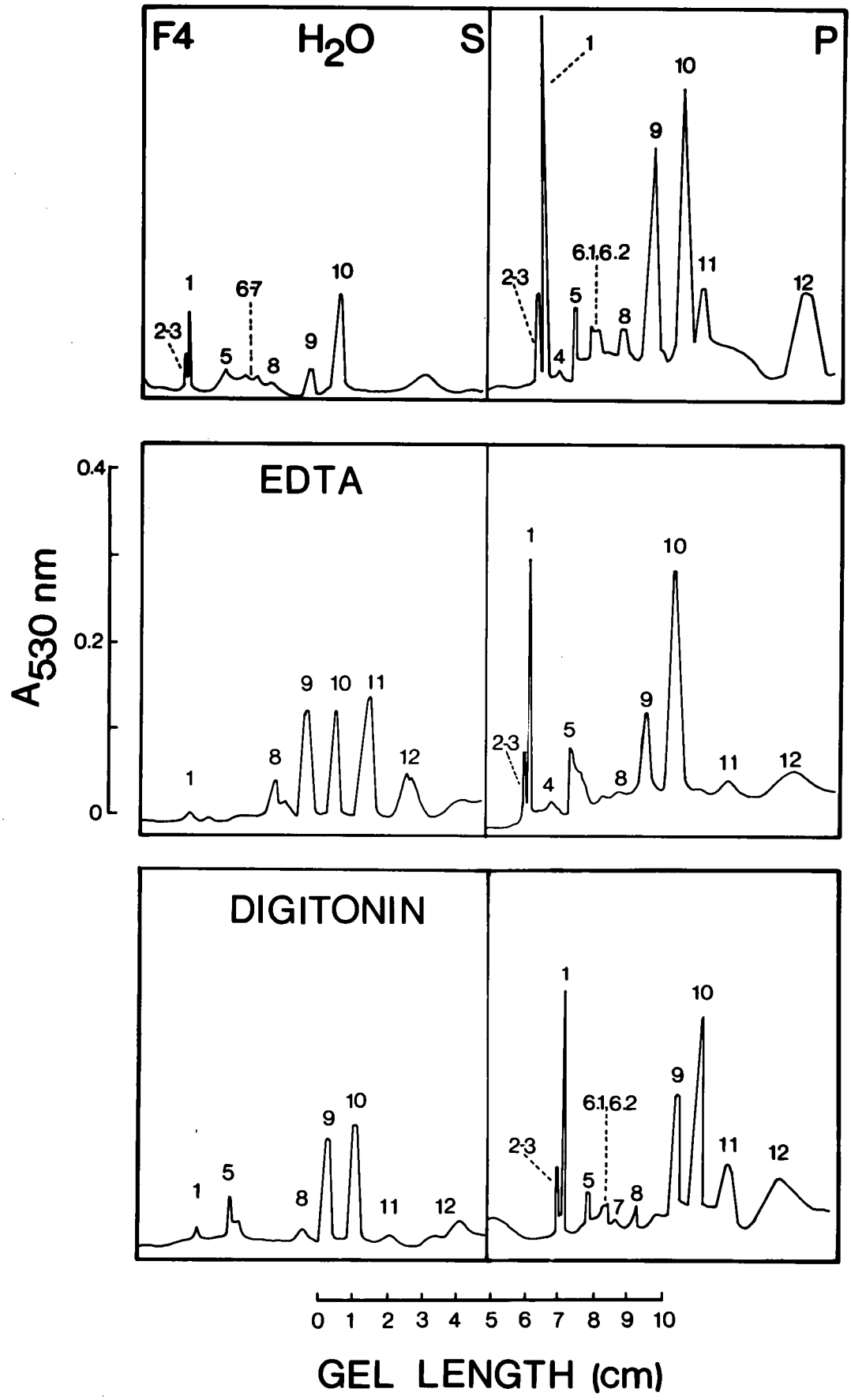

F4
H2O
S
P
EDTA
DIGITONIN
A530 nm
0.4
0.2
0
GEL LENGTH (cm)
0 1 2 3 4 5 6 7 8 9 10

Figure 32. Extraction of Fraction 5 using H_2O, Ethylenediamine tetraacetate and Digitonin. For µg protein in the supernatant (S) pellet (S) see Table X.

(Right), pellet obtained after extraction procedure.

(Left), supernatant obtained after extraction procedure.

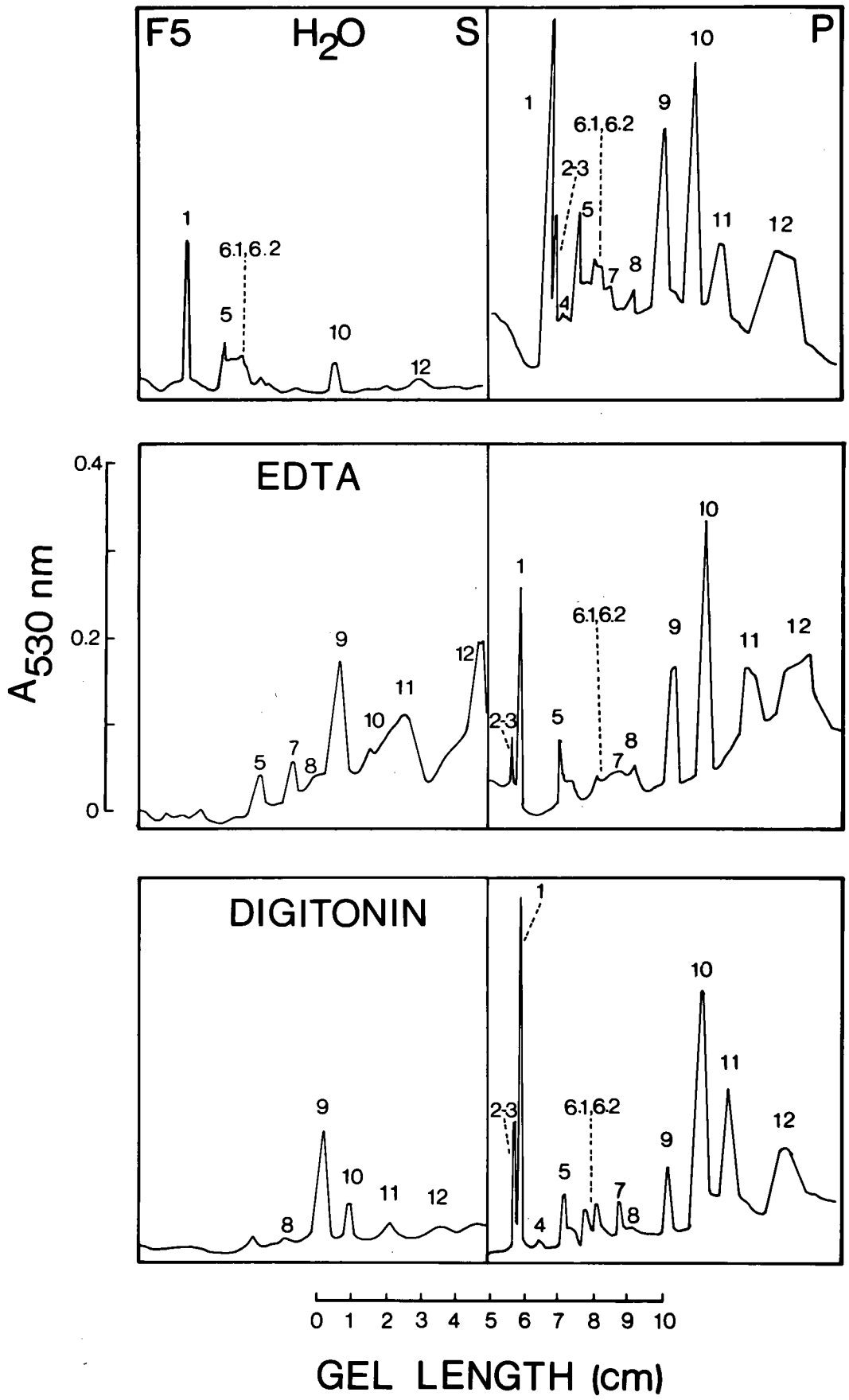

F5
H_2O
S
P
1
6.1,6.2
5
10
12
2-3
4
7
8
9
11
EDTA
DIGITONIN
0.4
0.2
0
$A_{530\ nm}$
0 1 2 3 4 5 6 7 8 9 10
GEL LENGTH (cm)

Figure 33. Extraction of Fraction 4 using Dimethyl maleic anhydride (DMMA). For µg protein in supernatant (S) and pellet (P) see Table X.
(Right), pellet obtained after extraction procedure.
(Left), supernatant obtained after extraction procedure.

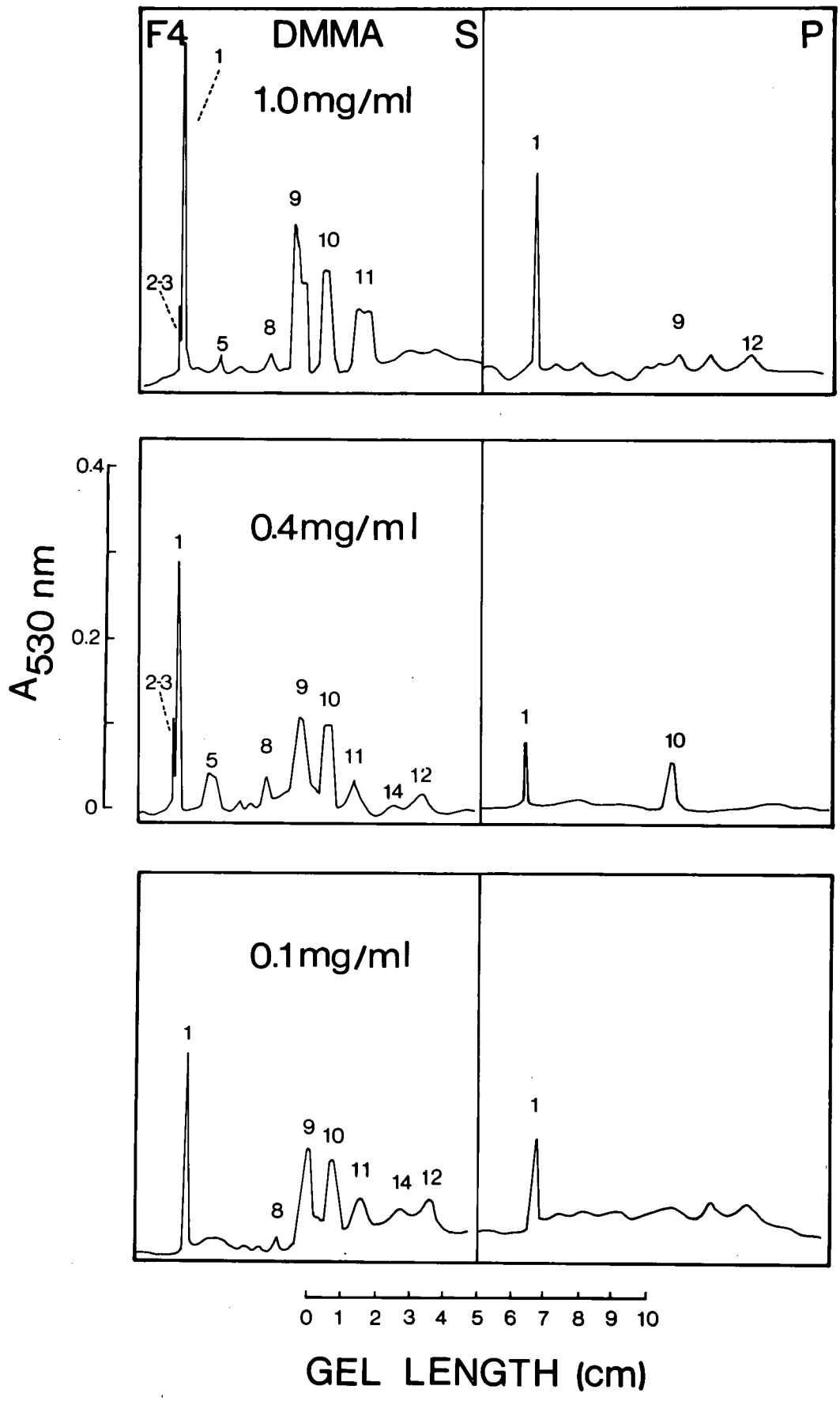

F4
DMMA
S
P
1.0mg/ml
0.4mg/ml
0.1mg/ml
1
2-3
5
8
9
10
11
12
14
$A_{530\ nm}$
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10
GEL LENGTH (cm)

Figure 34. Extraction of Fraction 5 using Dimethyl maleic anhydride (DMMA). For µg protein in supernatant (S) and pellet (P) see Table X.
(Right), pellet obtained after extraction procedure.
(Left), supernatant obtained after extraction procedure.

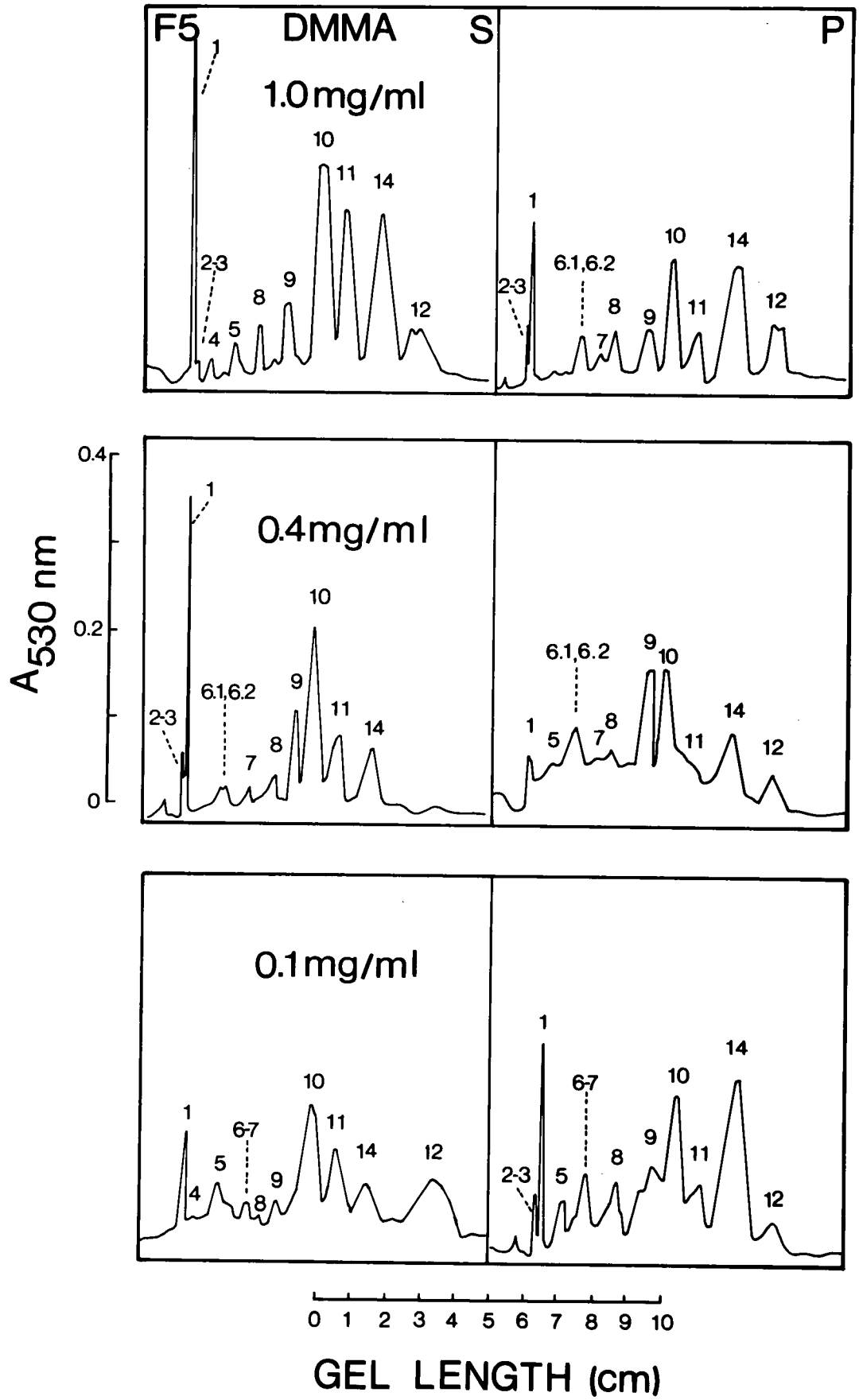

F5
DMMA
S
P
1.0mg/ml
0.4mg/ml
0.1mg/ml
A530 nm
0.4
0.2
0
GEL LENGTH (cm)
0 1 2 3 4 5 6 7 8 9 10

Figure 35. Extraction of Fraction 4 using p-Chloromercuribenzene sulphonic acid (pCMBS). For µg protein in supernatant (S) and pellet (P) see Table X.

(Right), pellet obtained after extraction procedure.

(Left), supernatant obtained after extraction procedure.

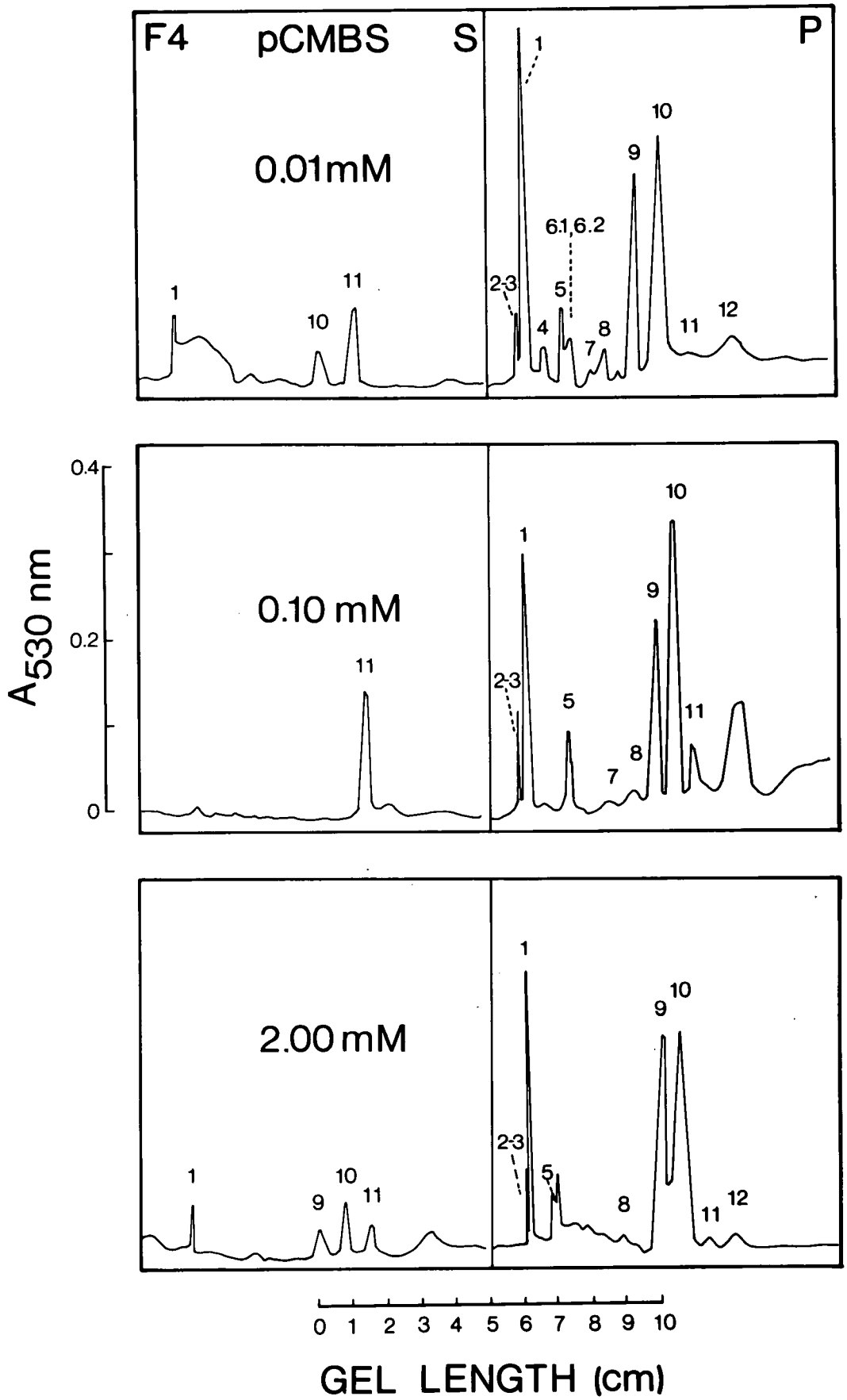

F4
pCMBS
S
P
0.01mM
0.10 mM
2.00 mM
1
2-3
4
5
6.1
6.2
7
8
9
10
11
12
$A_{530\ nm}$
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10
GEL LENGTH (cm)

Figure 36. Extraction of Fraction 5 using p-Chloromercuribenzene sulphonic acid (pCMBS). For µg protein in supernatant (S) and pellet (P) see Table X.

(Right), pellet obtained after extraction procedure.

(Left), supernatant obtained after extraction procedure.

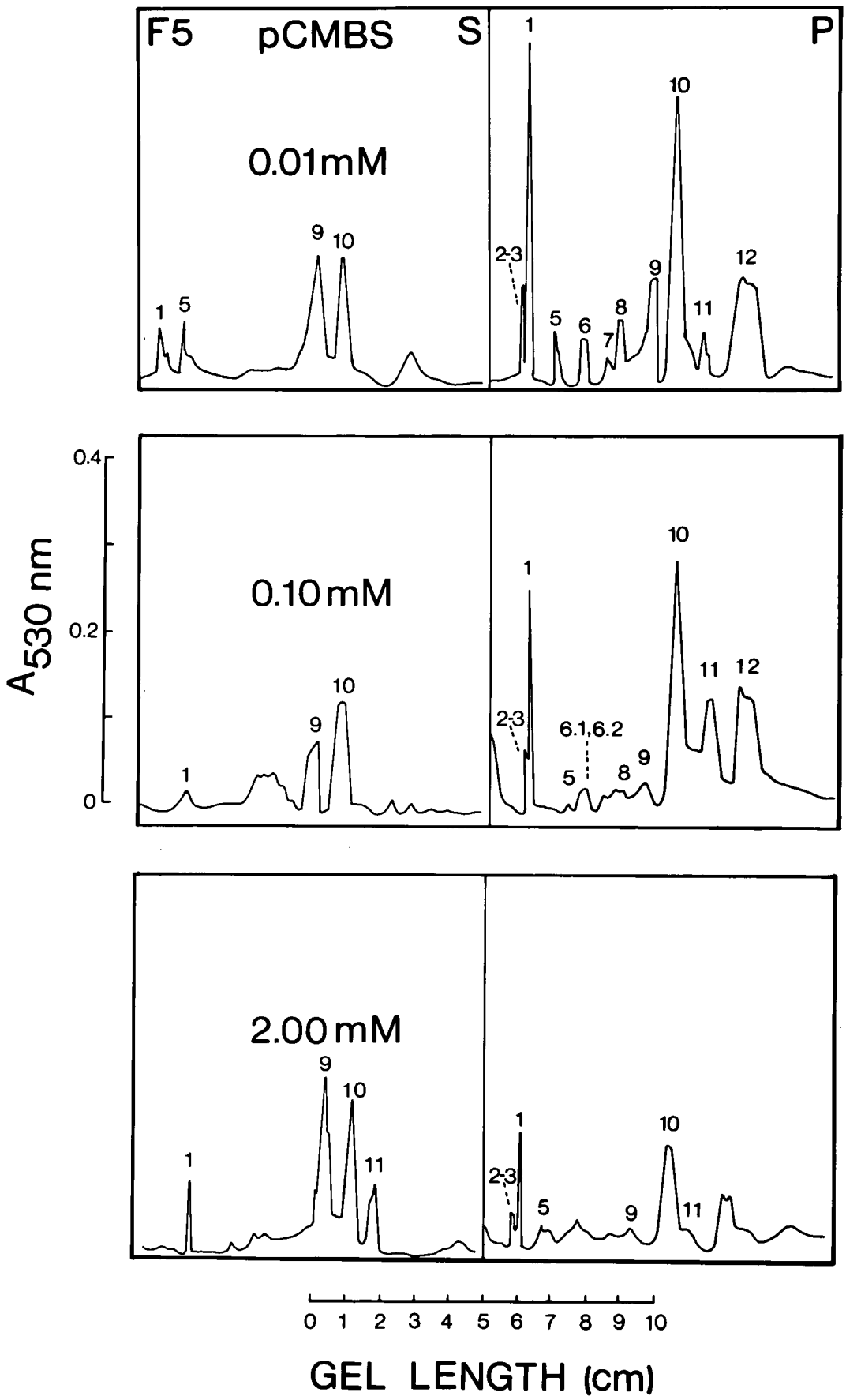

F5
pCMBS
S
P
0.01mM
0.10mM
2.00mM
$A_{530\ nm}$
0.4
0.2
0
1
2-3
5
6
6.1,6.2
7
8
9
10
11
12
0 1 2 3 4 5 6 7 8 9 10
GEL LENGTH (cm)

Figure 37. Extraction of Fraction 4 using Triton X-100 (TX-100). For µg protein in supernatant (S) and pellet (P) see Table X.
(Right), pellet obtained after extraction procedure.
(Left), supernatant obtained after extraction procedure.

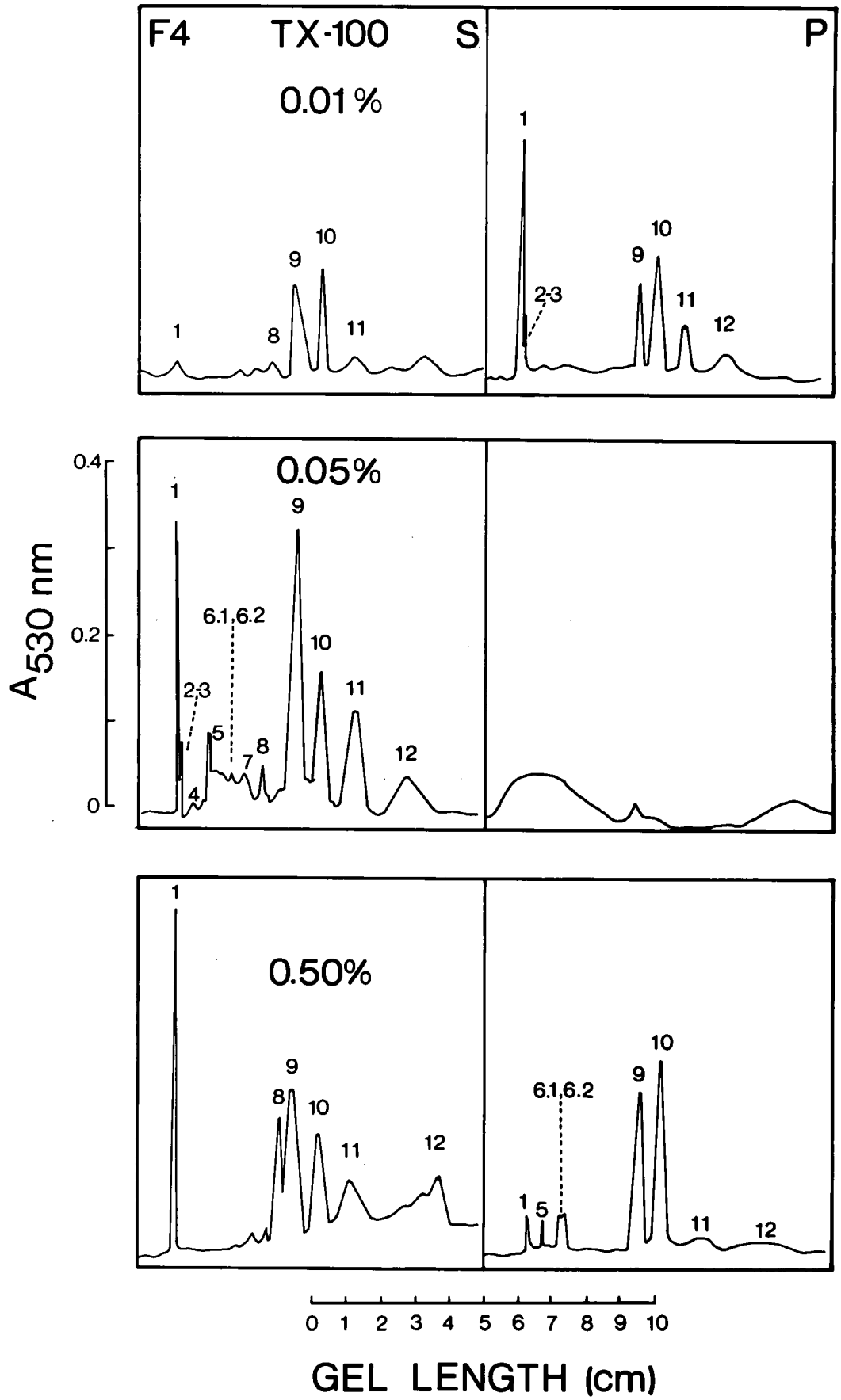
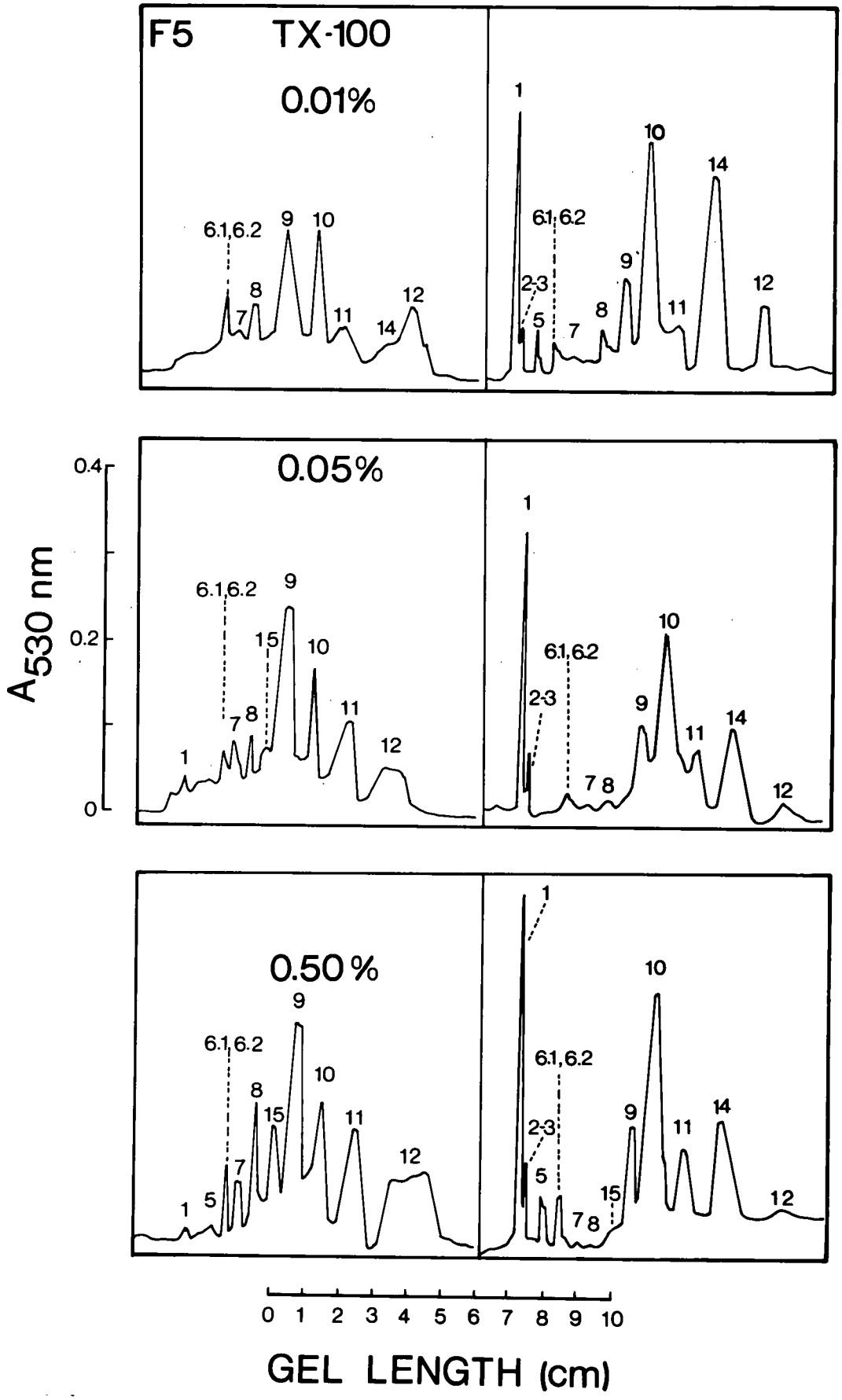

F4
TX-100
S
P
0.01%
0.05%
0.50%
A530 nm
0.4
0.2
0
GEL LENGTH (cm)
0 1 2 3 4 5 6 7 8 9 10

Figure 38. Extraction of Fraction 5 using Triton X-100 (TX-100). For µg protein in supernatant (S) and pellet (P) see Table X.

(Right), pellet obtained after extraction procedure.

(Left), supernatant obtained after extraction procedure.


F5 TX-100
0.01%
0.05%
0.50 %
$A_{530\ nm}$
0.4
0.2
0
6.1,6.2
1
2-3
5
7
8
9
10
11
12
14
15
0 1 2 3 4 5 6 7 8 9 10
GEL LENGTH (cm)

studies; two of these being Bands 14 and 15. Band 14 (31,000 MW) appeared both in the DMMA and TX-100 extractions of F4 (Figure 33). Whether this band represents chemical modification is unclear at this point but since it is present it must be reported. Similarily, treatment of F5 with 0.05% and 0.50% TX-100 resulted in the formation of Band 15 (93,000 MW). It can be argued that Band 15's assignment should really be designated as Band 8 and as a result the designated Bands 7 and 8 should be designated 6.2 and 7 respectively. Based on Rf values however, this designation though desirable could not be made.

Even though the results of the extraction studies were biased by unsealed vesicles in F4 some information was gained by these studies. The results at times appeared to be indicative of differences in membrane orientation of the two fractions F4 and F5. Further pursuit of the extraction studies, however, was terminated as the additional information to be gained was questionable.

G. Affinity Chromatography

It seemed desirable to determine as accurately as possible how pure F4 and F5 were with respect to their orientations. We also intended to further purify the membranes as we were aware of the possible contamination of F4 by vesicles of differing orientation. Con A - sepharose affinity chromatography was carried out using F4 and F5 in the hopes of further purifying and characterizing the fractions. When F4 membranes, labelled with ^{125}I, were applied to the affinity column, 2 peaks were

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obtained. An additional 2 peaks being obtained with eluting buffer containing α methyl-D-mannoside (Figure 39). When the first 2 peaks were analyzed for ^{125}I profiles (Figures 40a and 40b), labelling was observed exclusively at 100,000 daltons, this fact being consistent only with IO vesicles but, an increase in AchE accessibilty was surprisingly not noted, though there was an increase in the specific activity (see Discussion). The peak at fraction 17 was indicative of F4 being a population of heterogeneous vesicles with respect to thier size. Analysis of column fraction 35 for iodination and AchE specific activities showed rather interesting results. The ^{125}I profile showed the same number of counts in Band 8 as in Band 10, this fact being consistent with unsealed vesicles (Table Xa). The AchE accessibilty results agree with the above interpretation (Table XII). Fraction 52, eluted with higher α methyl-D-mannoside concentrations, yielded results similar to fraction 35, again indicating unsealed vesicles. In all, 90% of the F4 protein applied to the column could be eluted from the column.

F5 when applied to the affinity column yielded only two elutable peaks and these peaks represented only 5-10% of the total protein originally applied to the column. A full 90% could not be eluted even in the presence of high concentrations of eluting sugar and borate buffer. Column fraction 6 when examined for ^{125}I profile and AchE specific activities yielded results consistent only with this fraction being mainly IO vesicles (Figure 41). Fraction 73 studies indicate that this fraction consisted of unsealed membranes.

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It should be pointed out that the results presented on pages 134 to 139 for the affinity chromatography experiments represent the mean values for three to four experiments. Similar data was obtained for Con A - Agarose affinity chromatography. This results are not presented here for that reason. Extensive controls were run with each column used. These are dealt with at length in the Discussion section.

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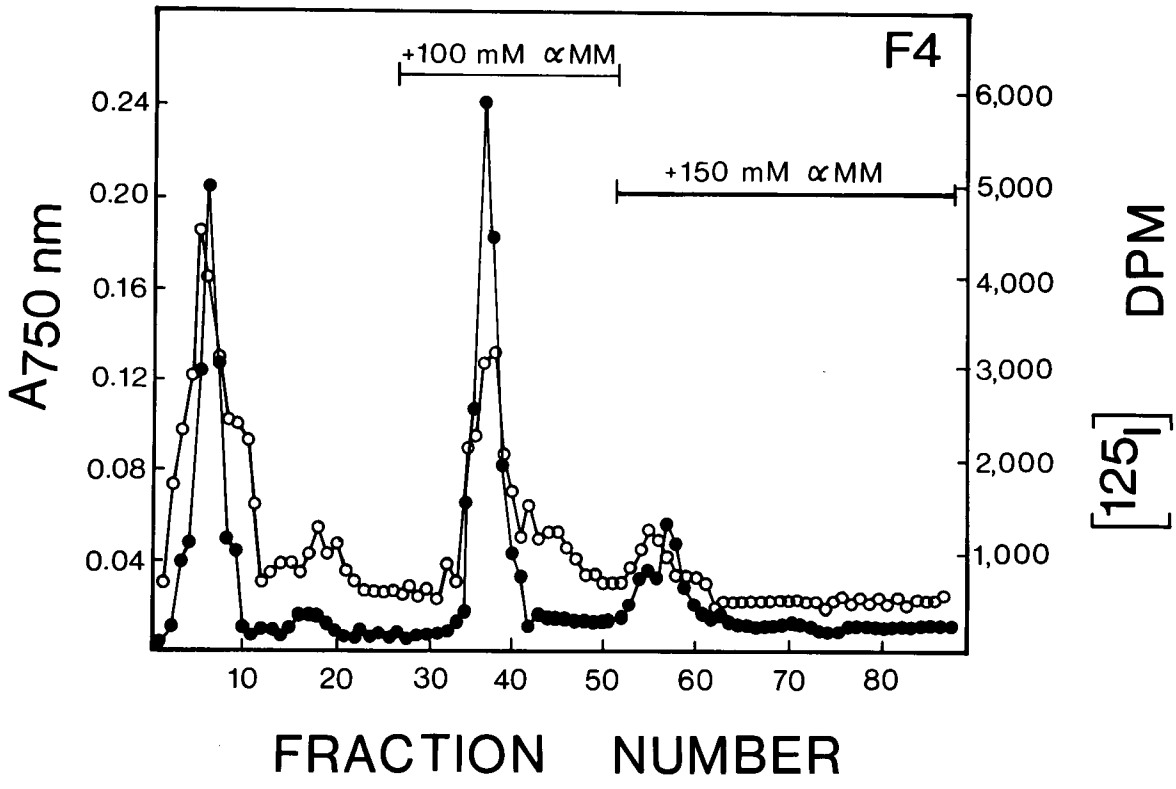
Figure 39. Con A - Sepharose 4B affinity chromatography of Fraction 4 (top) and Fraction 5 (bottom). (●—●), ^{125}I d.p.m. of labelled membranes eluted from the column; (○—○), protein profile of column effluent using Lowry protein assay. α MM refers to α -methyl - D - mannoside. Borate refers to the buffer used in place of the normal eluting buffer.


F4
+100 mM αMM
+150 mM αMM
A750 nm
DPM
[125I]
0.24
0.20
0.16
0.12
0.08
0.04
6,000
5,000
4,000
3,000
2,000
1,000
10
20
30
40
50
60
70
80
FRACTION NUMBER

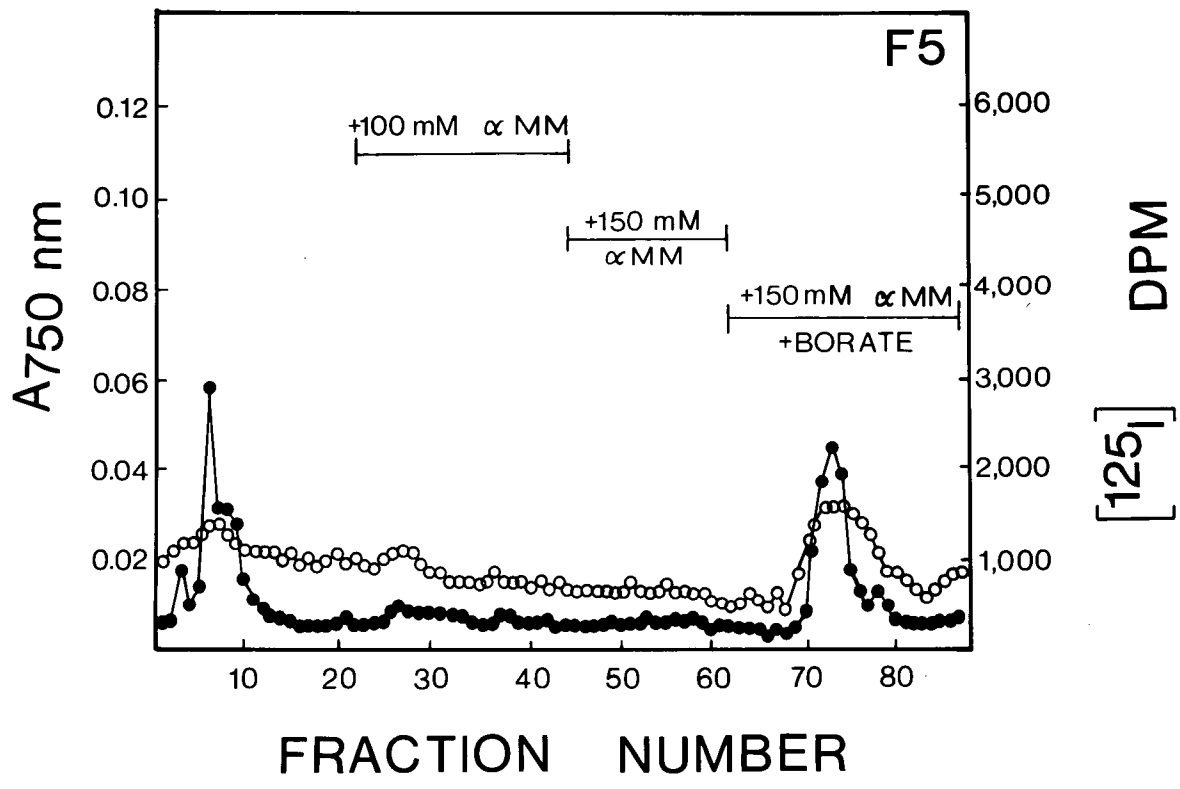

F5
+100 mM α MM
+150 mM
αMM
+150mM αMM
+BORATE
A750 nm
DPM
[125I]
0.12
0.10
0.08
0.06
0.04
0.02
6,000
5,000
4,000
3,000
2,000
1,000
10
20
30
40
50
60
70
80
FRACTION NUMBER

Table XII

Characterization of column fractions eluted from Con A - Sepharose affinity columns. Acetylcholinesterase sp. activity expressed as nm/mg protein/min.

	Fraction 4	Fraction 5
Total ^{125}I d.p.m. applied	3.6×10^7	4.0×10^7
Recovery	90 - 95%	10 - 15%
Total protein on column	0.735 mg	0.850 mg
Recovery	90 - 95%	8 - 10%

Fraction Number	Column Fraction Number	acetylcholinesterase - Triton X-100	acetylcholinesterase + Triton X-100
4	stock	2.72	15.43
	5	8.61	26.11
	17	22.96	42.13
	35	30.64	30.64
	52	29.01	30.10
5	stock	14.16	13.50
	6	7.00	22.50
	28	-	-
	73	26.70	27.09

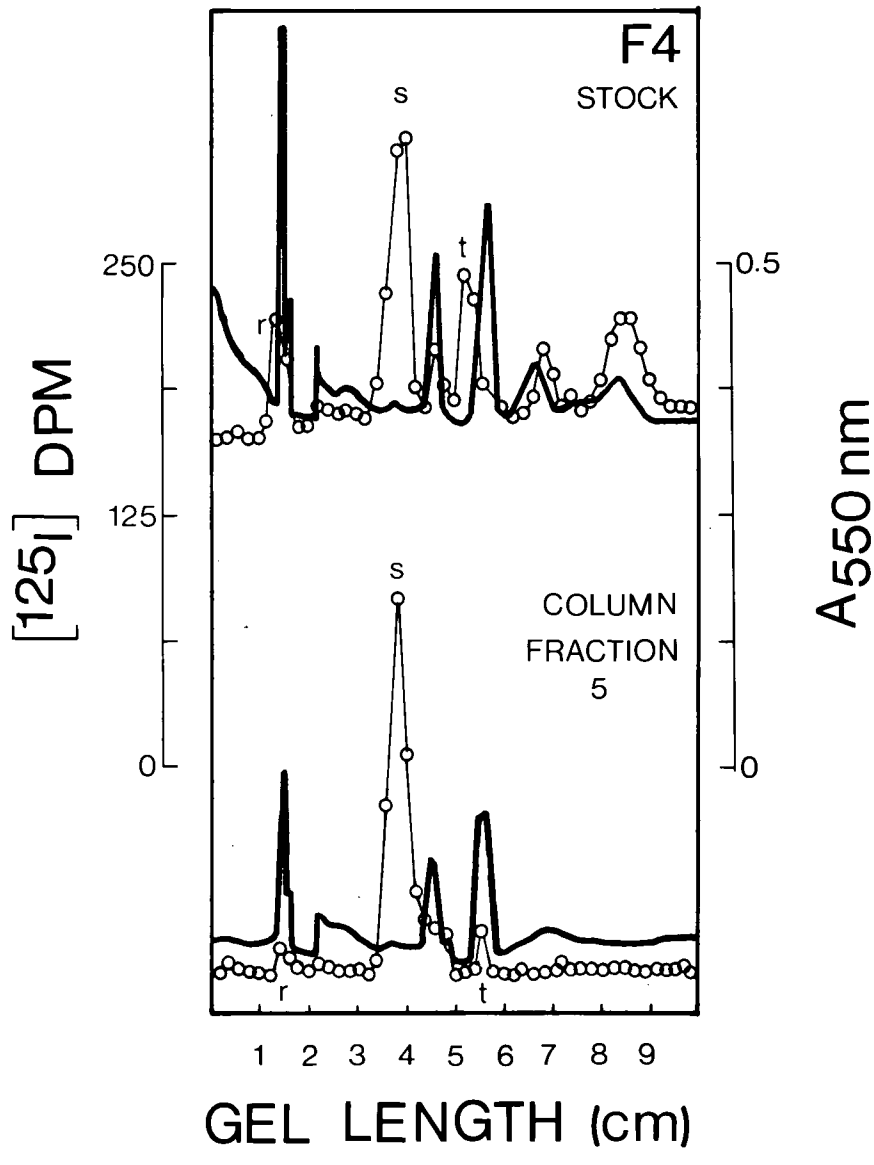


Figure 40a. Analysis of peak fractions obtained by Con A - Sepharose affinity chromatography of Fraction 4. (——), coomassie blue staining profile of peak fractions; (O—O), iodination pattern of protein eluted in peak fractions. Fraction 4 stock refers to the membranes originally applied to the column. Greater than 85% of the protein applied to the column was eluted. See also Figure 40b for peak fractions 17,37 and 54.

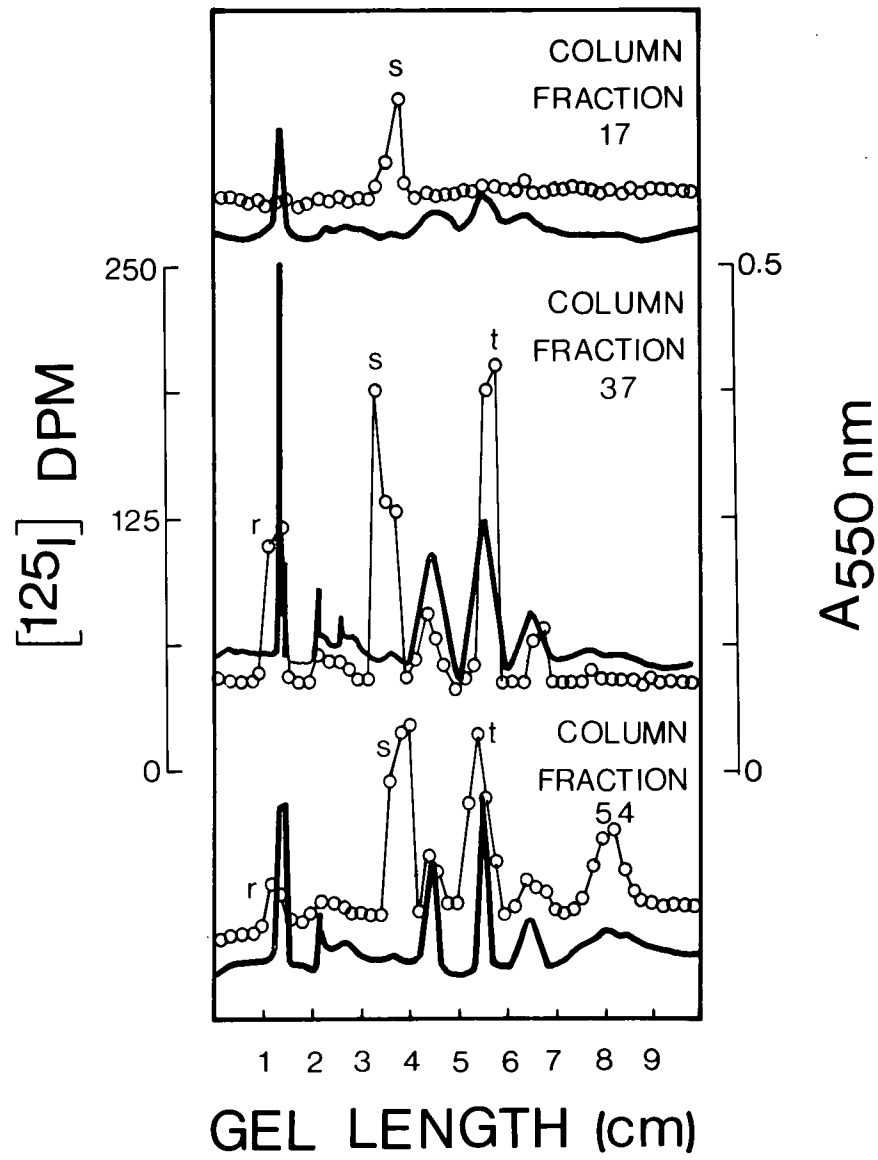


Figure 40b. Analysis of peak fractions obtained by Con A - Sepharose affinity chromatography of Fraction 4. (——), coomassie blue staining profile of peak fractions; (O—O), iodination pattern of protein eluted in peak fractions. Greater than 85% of the protein applied to the column was eluted. See also Figure 40a for peak fraction 5 and Fraction 4 stock membranes originally applied to the column.

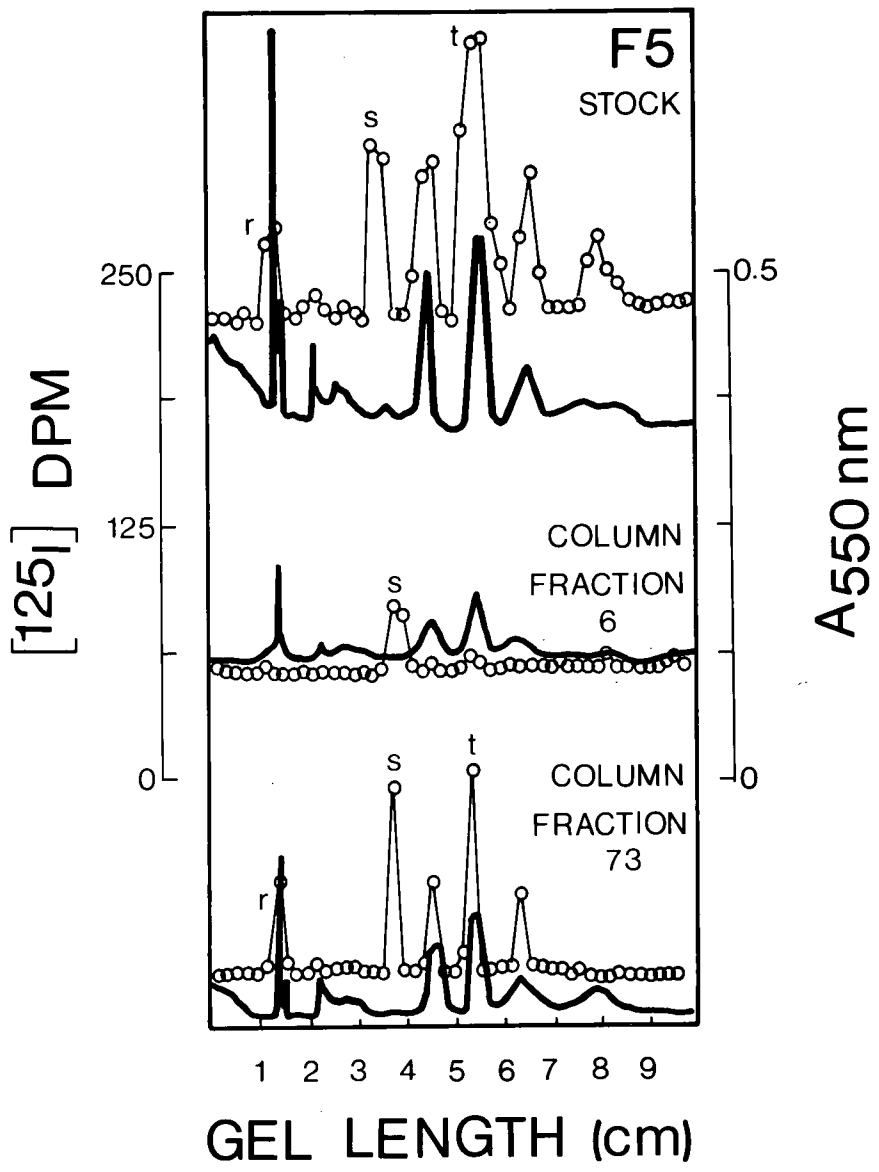


Figure 41. Analysis of peak fractions obtained by Con A - Sepharose affinity chromatography of Fraction 5. (——), coomassie blue staining profile of peak fractions; (O—O), iodination pattern of protein eluted in peak fractions. Fraction 5 stock refers to the membranes originally applied to the column. Less than 10% of the protein applied to the column was eluted.

H. Summary

In summary we can say that F4 was found to contain 20-25% unsealed membrane vesicles and that these were the contaminating components in the preparations used for sidedness studies. F5 represents a membrane preparation already 90% right-side-out. Any attempt to improve on this would be unlikely to succeed. The problem of eluting the 90% of protein bound (in F5) was not solved. The binding to the affinity column appears to be non-specific adsorption. Perhaps conditions could be modified for the elution of these membranes but further work would be required.

In conclusion, we have prepared two sets of membrane vesicles with enriched orientations. At the same time we have demonstrated the nature of some of the proteins in the membrane. Methods usable for the identification of RO and IO membranes were also presented and indications were given of a possible technique for preparing PM vesicles of pure defined orientation.

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Discussion

The chicken gizzard, although known to be rich in smooth muscle, has not been used extensively in membrane work. However it was felt that this was an ideal source of smooth muscle for an isolated pure plasma membrane preparation. A Polytron was used for the initial homogenization since the tissue is difficult to homogenize by other methods. Homogenization times chosen for muscle cubes and cell sheets were optimized to yield maximal specific activities and total activities of 5' nucleotidase, a plasma membrane marker. No other marker studies were performed during this procedure. The Polytron, the most commonly used homogenizer in membrane preparations from visceral smooth muscle, was found to be quite successful in our work.

Differential centrifugation was quite successful in enrichming the 100,000 g pellet with plasma membranes, as 40% of the 5' nucleotidase activity was retained from the residue and crude filtrate. There was a 10 fold purification of the plasma membranes based on the marker 5' nucleotidase. This latter result being similar to those noted elsewhere in the literature (see Table I, gradient preparations). No increase in the specific activity of NADPH cytochrome c reductase was noted, this result being in contrast to other studies which show increases in the specific activities of markers for the SR in 100,000 g pellets. Slight increases in the specific activities of acid phosphatase and succinic dehydrogenase. What is more important is to note that there was a decrease in the total units of these enzymes. At the same time it must be pointed

out that no orientation studies were done on the pellets and supernatants prior to sucrose gradient centrifugation. In retrospect this should have been done to check if the values for total units of activity were indeed correct. It must be remembered, however, that results applicable to one type of smooth need not necessarily apply to smooth muscle obtained from another source.

The fractionation of the various cell membrane types achieved by the use of the sucrose gradient centrifugation was similar to that observed in various other plasma membrane preparations but the distribution of the different membranes was in disagreement with these other studies. In our case, mitochondria were found in the region between 36 and 40% sucrose, which was in agreement with the literature. The SR was distributed throughout the gradient with the specific activity of marker enzymes for the SR being highest in Fraction 2 (27% sucrose).

Three peaks of activity were observed using the marker NADPH cyt c reductase, but these results did not agree with the distribution of the markers glucose-6-phosphatase and NADH cyt c reductase. The results of MATLIB (MATLIB et al., 1979) showed NADH cyt c reductase to be an unacceptable specific marker of the SR. Our results seem to bear this out. It may be that we are seeing the effects of differing orientation or a non specific enzyme distribution of glucose-6-phosphatase or NADH cyt c reductase. The plasma membranes were generally

found at lower densities, however, SR was also present in certain fractions. The distribution observed was in agreement with the results of Moore (MOORE et al., 1975) and Hurwitz (1974). There were no indications that the plasma membranes were binding higher density membrane fragments. On the other hand, most separations on gradients yield results dissimilar to these above as the SR is found at higher densities than plasma membrane in these studies.

A rather interesting feature of our plasma membrane fractions was the Mg^{2+} stimulated ATPase observed on the external membrane surface of F5 and single cells. Labelling with [^{32}P] ATP seemed to indicate that the ATPase was localized to an apparent M.W. of 205,000 daltons. Mg^{2+} stimulated ATPases have been found associated with the plasma membrane of aortic smooth muscle, myometrial and intestinal smooth muscle, but their function is currently under considerable dispute. A Mg^{2+} stimulated ATPase similar to that observed here has reportedly been found on the external surface of the red blood cell plasma membrane. This enzyme exhibits high substrate inhibition and other similar properties to our enzyme (SMOLEN & WEISSMAN, 1978). The authors speculate that Mg^{2+} stimulated ATPases may have some role in chemotaxis, phagocytosis and superoxide anion generation but it remains to be seen whether these are really true. When investigating any Mg^{2+} stimulated ATPase in muscle there is the added danger of myosin ATPase contaminating the preparation, as myosin may be absorbed to the membrane surface during homogenization. However, we feel that we can rule this out as the Mg^{2+} stimulated

ATPase activity noted in F5 was the same as that found in free cells. Another problem with the high Mg^{2+} stimulated ATPase activity observed in our Fraction 5 was that it prohibited the measurement of any Na+/K+ ATPase or Ca^{2+} ATPase activity. It was only through [^{32}P] ATP labelling that we became aware of the possibility that more than one ATPase may be present. It is were to be demonstrated convincingly that the 165,000 MW peak represents a Ca^{2+} ATPase, selective extraction of these bands or selective removal of the Mg^{2+} stimulated ATPase would have to be achieved first (JORGENSON, 1974). It might be further added that the phosphorylation conditions used by other authors (see Methods section) were not found to work in our studies whatsoever, and our conditions were chosen as the result of many different variations. Not only did the phosphorylation experiments results yield information of possible ATPases but they provided further evidence for the different orientations of F4 and F5.

Perhaps the most interesting results were obtained from the iodination experiments for the latter provided another means for determining orientation and degree of purification of plasma membranes. The inherent assumption in all such studies is that the labelling species will not permeate the membrane (MORRISON & SCHONBAUM, 1976). Though the exact mechanism of lactoperoxidase catalyzed iodination is not fully known, it is felt that the iodinating species satisfies this condition. We might further point out that extensive controls must be run to account for any inherent peroxidase activity and non specific ^{125}I binding.

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A simple washing of the membranes after iodination is quite inadequate, therefore, controls deleting H_2O_2 or lactoperoxidase must be run. Free $^{125}I^-$ can be removed by gel electrophoresis, if the latter is done properly. Our results have taken all these factors into account and they represent the ^{125}I incorporated after background controls have been subtracted. The treatment of membranes with Triton X-100 to increase accessibility of the substrate to enzymes located on the inner membrane surface was reported by Steck (1974a) but until recently this has not been applied to the labelling of membranes (HARTIG & RAFTERY, 1977). Hartig and Raftery used Emulphogene as the detergent since TX-100 was found to inhibit the enzyme, but, our experiments showed contrary results. Our investigation showed that TX-100 inhibited self labelling of the enzyme but, not labelling of the membranes, much to our surprise. It was only at concentrations above 0.40% TX-100 that we observed decreased labelling of the membranes. The use of self-labelled lactoperoxidase could now be easily avoided by use of lactoperoxidase covalently linked to sepharose beads. This would greatly facilitate dealing with contamination due toself labelling. All in all, the iodination studies unequivocally demonstrated the preferential orientation of the two sets of membranes. They also provided a convenient means of following the two sets of membranes in any further purification procedure.

The extraction results were quite unsuccessful in many ways. We had hoped to selectively remove certain bands and investigate them as well as the residual bands but there were no clear cut results as were seen in the extraction studies performed on red

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blood cells (rbc). Our results, however, seemed to reflect differences in membrane orientation between F4 and F5. This was not seen in studies done on rbc by Fairbanks (FAIRBANKS et al., 1971 b) and Steck (STECK & YU, 1973). These studies using the red blood cells have shown that TX-100 removes glycoproteins embedded partially in the hydrophobic region of the membrane and that protein perturbants remove the inner cytoplasmic proteins. However, whether these agents act in the same way on membranes other than the rbc is questionable based on our results. A good example of this is Band 1. Band 1 has been shown to be externally localized, but it was removed totally by TX-100 in F4 and remained unchanged in F5. pCMBS on the other hand, removed Band 1 in F5 but not in F4. It would seem then that accessibility is the limiting factor in extraction. The results are further complicated by the lack of knowledge of the exact mechanism of extraction of the various agents used. A desirable addition to these experiments would be to measure the ATPase activities of the extracted membranes in conjunction with electron microscopy and marker assay studies. This however, was beyond the scope of our work.

The final step in our membrane isolation procedure was purification of the membranes using affinity chromatography. The results, presented for Fractions 4 and 5 in Table XII and Figures 39-41, are similar to those obtained by Walsh (WALSH et al., 1976). Based on the iodination studies alone, the labelling pattern observed for the first two peaks eluted when F4 was applied to the column is consistent only with the membranes

being inside-out. As a control, non-iodinated membranes (F4) eluted from the column were iodinated. The pattern observed was similar to that in Figures 40a and 40b. However, labelling to a minor degree was also seen at 205,000 and 55,000 daltons. These control experiments would seem to indicate that the membranes, though initially pure, the IO vesicles, may have become leaky or due to their unstable nature, RO or unsealed. The latter two peaks, eluted in the presence of α methyl-D-mannoside, based on the iodination studies appeared to consist of unsealed membrane vesicles. Control studies using non-iodinated membranes yielded similar results.

The above results wre confirmed using AchE sidedness assays of the various eluted membrane fractions. There was no increase in accessibility of column fractions 5 and 17 when compared to the initially applied F4 stock membranes. In actuality, there was a decrease in the accessibility. What was noted was a 1-2 fold increase in the specific activity of AchE. This may have been due to removal of mitochondrial contamination or contamination from the SR. This would be plausible as the sialic acid determinations showed the presence of sialoglycoproteins in fractions enriched with the SR and mitochondria (see Tables VIIa, VIIb and VIII). If these contaminating organelle membranes were RO oriented they may have been bound to the column. It is also plausible that there may have been some modification of the membranes due to capping which is known to occur in the presence of lectins. The decrease in AchE accessibility may be attributed to either

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leaky IO vesicles or IO vesicles becoming RO and/or unsealed. The high osmolarities of the buffers used would seem to rule out osmotic effects being responsible for the increase in permeability. What would be necessar is to do further marker assays, lectin binding studies and maybe some cross-linking studies to determine the exact cause of the apparent increase in membrane permeability.

Based on the AchE assays the latter two eluted peaks from the F4 column appeared to contain unsealed membrane vesicles. These latter results being in agreement with the iodination studies. The increase in the specific activity of the AchE may have been due to the reasons discussed earlier. On the basis of the increase in specific activity of AchE, it may be stated that the membranes in F4 were purified 1-2 fold. This, when combined with the purification of F4 based on 5'-nucleotidase studies, would make an overall purification of 40-60 fold of the plasma membranes. Though this is considerably higher than most plasma membrane preparations, a certain degree of caution is required as the final "IO" membrane preparation appears to be very unstable or permeable. These latter issues may be resolved before using these membranes for membrane transport investigations. It should also be pointed out that yields of membrane protein using the earlier described procedure are low and would require a batch preparation of plasma membranes for proper investigation.

The behaviour of F5 membranes was rather expected based on the results of Walsh (WALSH et al., 1976). Only two peaks

were eluted, these representing 8-10% of the total applied protein. The initial peak was assessed to consist of IO plasma membrane vesicles, while the second peak, eluted in the presence of α methyl-D-mannoside, found to contain unsealed membrane vesicles. These conclusions were based on the iodination and AchE sidedness studies. As seen using F4, the IO fraction was found to be either permeable or contain unstable IO vesicles. Since 90% of the applied protein could not be eluted the applicability of this method for further purification of a set of RO membrane vesicles must be suspected. Further, it must be questioned as to why a set of RO plasma membrane vesicles, already 90% pure, would require further purification.

The two sets of membranes isolated (F4 and F5) possess many desirable features for the investigation of membrane transport but as is apparent, further work is required to ascertain the exact characteristics of F5 and more importantly F4.

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