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THE ISOLATION OF PLASMA MEMBRANE FROM FROG CARDIAC MUSCLE CELLS

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SUMMARY

A vesicular preparation consisting largely of the plasma membrane of frog cardiac cells was isolated and its enzymatic activities and lipid content were investigated.

The enriched plasma membrane preparation was obtained by (1) mildly homogenizing washed ventricles, (2) separating away the cellular debris by low speed differential centrifugation, (3) separating the plasma membrane fraction from other membranous components by centrifugation to equilibrium in various sucrose gradients. The frog cardiac plasma membranes were found to be concentrated between specific gravities of 1.07 and 1.11. In the membrane fraction the specific activities of membrane marker enzymes: 5′-nucleotidase (EC 3.1.3.5), alkaline phosphatase (EC 3.1.3.1) and (Na^+–K^+)-activated ATPase were, respectively, 19, 15, and 14 times greater than in the homogenate. Activities of mitochondrial marker enzymes were either very low or absent. No unusual lipid types were found. Cardiolipin was less than 0.1% (by wt) in the membrane fraction. The molar ratio of phospholipid to cholesterol was approximately 3 : 2.

INTRODUCTION

To understand why an action potential occurs, it is necessary to understand how the permeabilities of an excitable membrane change. In order to understand how this occurs, it is necessary to find out what molecules are in an excitable membrane and how they interact with the ions of importance. Thus one must first isolate, purify and analyze the composition of an excitable membrane. Unfortunately, most nerves have much more Schwann cell membrane than axonal membrane and most types of muscle have more internal membrane, by orders of magnitude, than plasma membrane. Although there have been reports of preparations of plasma membranes from nerve [1], skeletal muscle [2], and mammalian cardiac muscle [3], these preparations all suffer from the difficulties attendant to trying to isolate a minor membrane

fraction of a tissue from the major membrane components. Frog cardiac muscle and perhaps some smooth muscles seem to provide more favorable sources of "excitable" plasma membrane because the muscle cells are by far the predominant cell type in these tissues and it has been reported that these muscle cells have relatively little internal membrane [5, 6].

An additional prerequisite for the correlation of molecular structure to electrophysiological function is that relevant voltage-clamp measurements can be made to determine the kinetics of the voltage-dependent transmembrane ion fluxes underlying excitation. These kinds of measurements have been restricted to relatively few preparations [7]. Fortunately, it appears feasible to voltage-clamp frog atrial bundles [8] and thus there is the possibility at least to proceed with the longer term goal.

In this paper we describe a relatively simple method of isolating a plasma membrane preparation along with some data concerning its lipid composition and complement of enzymatic activities.

MATERIALS AND METHODS

Membrane preparation

Frogs were double pithed and the heart exposed ventrally. The hearts were dissected free, making sure that enough sinus venosus was included such that the hearts continued beating in Ringer's solution at room temperature [9]. The beating hearts were rinsed free of blood by injecting Ringer's solution into ventricles and letting the hearts pump it out. The ventricles were then isolated, trimmed of most loose connective tissue and put into a homogenizing solution (potassium homogenizing solution) at 0 °C which contained 10 mM Tris–malate, 1 mM calcium gluconate and 84 mM K_2SO_4 at pH 7.4. In earlier experiments, 250 mM of sucrose replaced the 84 mM K_2SO_4 to give a homogenizing solution of lower ionic strength.

The ventricles were homogenized in a Teflon pestle hand homogenizer (AH Thomas No. 3431E15 or E20) using 20–30 strokes. The preparations were routinely made using 25–150 frogs. This corresponded to homogenizing suspensions whose volumes varied from 7.5 to 45 ml. The general aspects of the isolation procedure were to (1) homogenize, (2) spin down the cellular debris and (3) separate the membrane from other organelles by suspending them at different places using different density sucrose. We used linear density gradients, layered systems whose density varied in a nearly stepwise fashion, and combinations of the two. Later, we settled on a some what arbitrary, but reproducible, standard procedure.

Electron microscopy

Specimens of myocardium were prepared by dissecting from the ventricular wall, small bundles (50–500 μM) which remained attached at one end to the heart wall including the sinus venosus. Such preparations were allowed to recover 15–90 min in Ringer's solution prior to fixation. All were contracting rhythmically up until the time of fixation with 1.5% glutaraldehyde (Ladd Research Industries) in Ringer's solution for 70 h at 4 °C. Specimens were post-fixed for 2 h in 2% osmic acid in 0.2 M cacodylate, pH 7.3; then in 1% osmic acid in 0.1 M cacodylate with 0.05 M potassium ferricyanide for 1 h. They were dehydrated in graded ethanols with 0.5% uranylacetate in the 70% stage and embedded in Epon–Araldite.

Following initial homogenization, pellets of membrane preparations and cellular debris were fixed overnight in the final centrifuge tube with Karnovsky's fixative [10]. Pellets were rinsed 1 h in 0.2 M cacodylate buffer with 0.2 mM $CaCl_2$, post-fixed in 2% osmic acid in the rinse solution.

All membrane specimens were stained en bloc in 2% aqueous uranyl acetate, dehydrated in graded ethanol solutions, cleared and embedded in a mixture of Epon 812 and Araldite 502.

Thin sections were obtained on an LKB Ultrotome, stained with 2% aqueous uranylacetate followed by Reynolds' [11] lead citrate and viewed with either an Hitachi HU11C or HU12 electron microscope.

Enzyme assays

The following enzymes were assayed: (1) acid phosphatase, alkaline phosphatase and glucose-6-phosphatase according to Hubshen and West [12], (2) lactate dehydrogenase and succinate dehydrogenase according to Ells [13], (3) cytochrome oxidase according to Smith [14], (4) thiamine pyrophosphatase according to Meldolesi et al. [15], (5) 5'-nucleotidase according to Heppel and Helmoe [16], (6) NADH: cytochrome *c* reductase according to Sottocasa et al. [17].

Lipid analyses

Lipids were extracted with chloroform–methanol (2 : 1, v/v). Total cholesterol was determined by the method of Searcy and Berquist [18] and total lipid phosphorus by the procedure of Eng and Noble [19].

Individual phospholipids were determined by two-dimensional thin-layer chromatography. Glass plates (20 cm × 20 cm) were coated with a 250-μM layer of Silica Gel H (Brinkmann Instruments, Inc.). Prior to use, the plates were washed in one dimension with chloroform–methanol–water (65 : 25 : 4, v/v/v). After activation of the absorbent at 110 °C for 30 min, 300–500 μg of lipid was plated under nitrogen. The plates were developed in the first dimension with chloroform–methanol–aqueous ammonia (28% by wt) (65 : 35 : 5, v/v/v) and in the second dimension with chloroform–acetone–methanol–acetic acid–water (10 : 4 : 2 : 2 : 1, by vol.). The lipid spots were identified by the use of standards (Applied Science Laboratories, Inc. and Supelco, Inc.) and with appropriate spray reagents [20]. The lipid spots were removed by aspiration and transferred to test tubes for determination of lipid phosphorus by the method of Rouses et al. [21]. Results were expressed as percent of total lipid phosphorus. All analyses were performed in duplicate.

RESULTS

We set the composition of our homogenizing solutions on the basis of the need to control pH and osmolarity and to provide Ca^{2+} since it is essential for the functioning of excitable membranes. In addition, since it was known that effects of K_2SO_4 on the outside of bundles of frog cardiac muscle were reversible (unreported observations) and that the presence of Cl^- in intracellular perfusion fluids decreases the longevity of squid giant axons [22], and we did not know whether the vesicles would form with the cytoplasmic side of the plasma membrane facing inward or outward, we chose to make up the total osmolarity of our homogenizing solution using K_2SO_4 or sucrose. This also allowed us to check for gross effects of ionic

strength. In early experiments we found little difference in the yield or distribution of material in linear gradient (1.03 to 1.15 specific gravity) experiments according to whether we used the potassium or the sucrose homogenizing solution, but it seemed that there was less aggregation of mitochondrial fragments and plasma membrane vesicles using the potassium homogenizing solution. On this basis and also because the potassium homogenizing solution was closer to physiological conditions, we have used that solution routinely throughout most of the rest of the work reported here.

In experiments where fractions were first separated, either by taking parts of a linear density gradient or taking interfaces between layers of different densities, and then rerun using the same density scheme, the fractions always were distributed precisely according to their original specific density position in the centrifuge tubes. If there were large changes of state of aggregation or continuing large losses of protein from the vesicles after our first steps, we would have expected a different redistribution of the vesicles according to density.

Electron microscopy

Frog cardiac muscle consists of long tapering cells 175 μm to 250 μm in length. Cells within a bundle of muscle are interconnected by desmosomes, regions of myofibrillar attachment and nexuses. Within a bundle only muscle cells occur; no connective tissue cells are present. Between bundles unmyelinated nerves may occur and

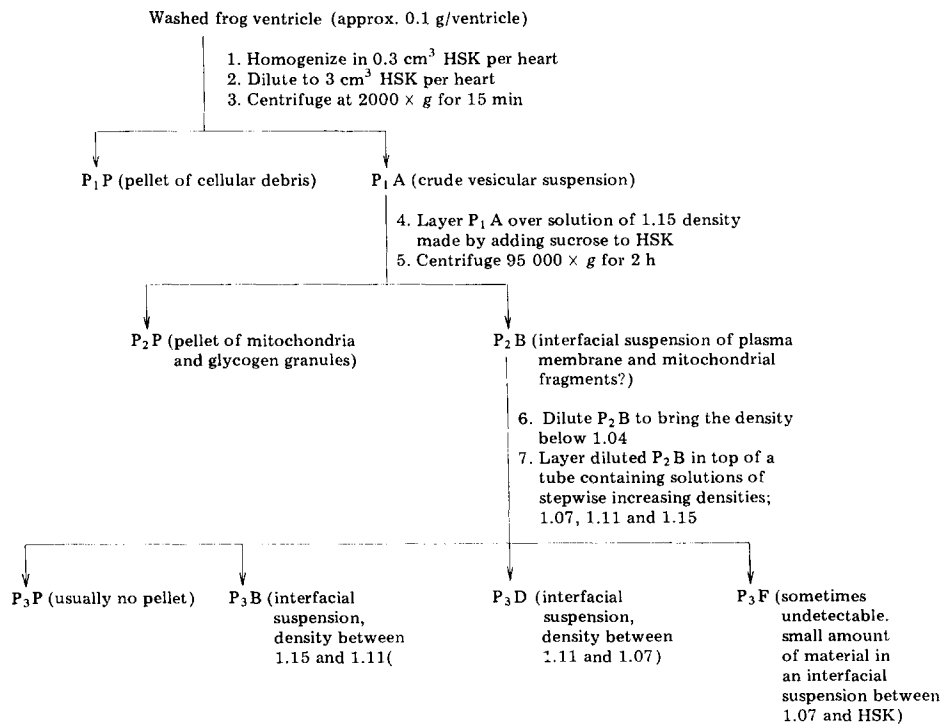

Fig. 1. Standard preparation. HSK, K^+-containing homogenizing solution.

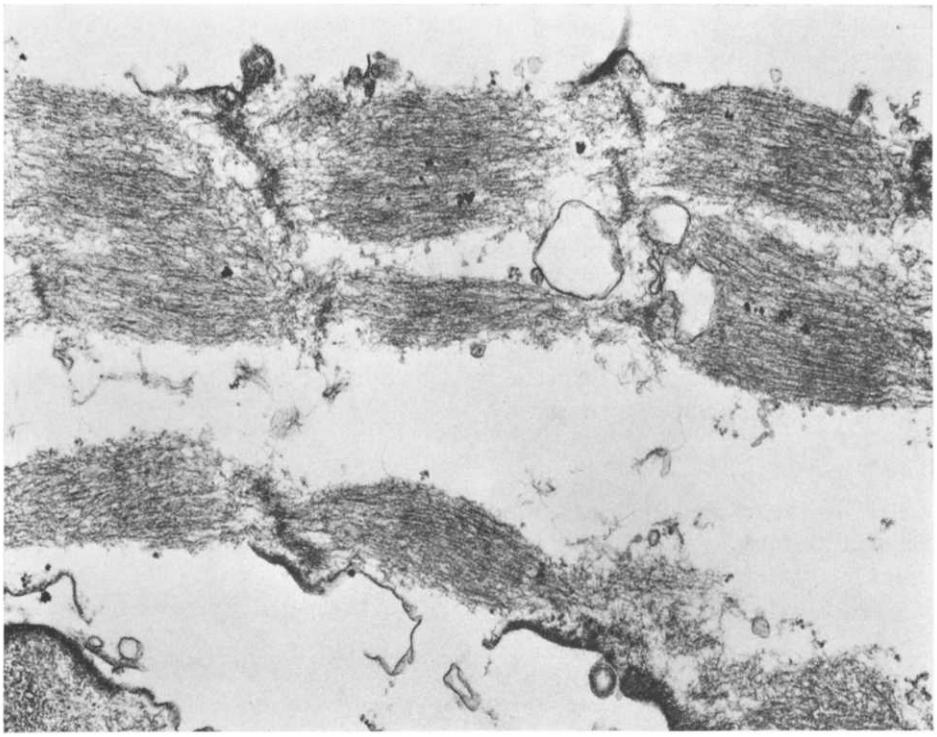

Fig. 2. Electron micrograph of portion of a highly extracted single muscle cell following homogenization and centrifugation into pellet, Fraction P_1P. Sarcomeres exhibit maximal shortening. Portions of sarcolemma remain attached to contractile apparatus at regions of extension of Z band material to the sarcolemma. Remaining portions of the cell are denuded of sarcolemma and most of intermyofibrillar and pericellular sarcoplasma. Magnification, × 28 800.

an occasional fibroblast. Trabecula internal and external surfaces of the ventricular wall are covered by a single layer of simple squamous epithelium. Thus the ventricular wall is nearly a pure preparation of cardiac muscle cells. Membranes isolated from ventricular preparations should contain only a little contamination from non-muscle cells.

Internally, frog cardiac muscle cells contain strikingly few sarcoplasmic membranes. Using horse radish peroxidase as a tracer, no transverse tubule system or its counterpart has been demonstrated (Dewey, Barr and Berger, unpublished results). Only occasional vesicular structures are seen between myofibrils and around the nucleus. Vesicular structures, when seen, most commonly occur just under the sarcolemma. Whatever the function of these vesicles, a sarcoplasmic reticulum comparable to that seen in mammalian cardiac muscle is not present in frog cardiac muscle [5, 23].

Electron micrographs of the pellets of cell debris following homogenization show nearly intact cells partially stripped of their sarcolemma (Fig. 2). The sarcolemma seems to remain attached to the contractile apparatus where Z band materials extend to the sarcolemma. Such pellets clearly demonstrate that many mitochondria

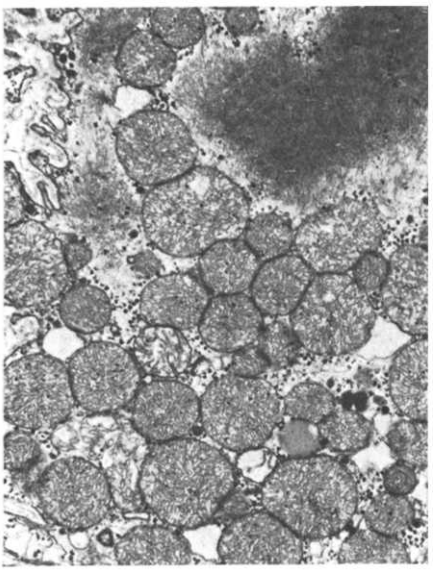

Fig. 3. Electron micrograph of a region of Fraction P_1P which contains a muscle cell cut in cross section near the nucleus. The majority of the mitochondria are normal in appearance. Magnification, ×7200.

TABLE I

VESICLE DISTRIBUTION

Results are expressed as μm.

Prepn. No.	Vesicle distribution (1.11 > density > 1.07)		
	Largest	Smallest	Average
42	0.76	0.03	0.18
47	0.88	0.02	0.18
48	0.83	0.08	0.29
47	1.13	0.02	0.22
49	0.69	0.06	0.21
50	0.74	0.02	0.19
52	0.70	0.02	0.11
53	0.72	0.04	0.19
Grand average	0.81	0.036	0.20

and regions of glycogen remain associated with the cell debris and appear nearly normal in structure (Fig. 3).

Electron micrographs of the purified membrane preparations show vesicles ranging in diameter from 0.2 μm to 2 μm (Table I). These preparations would seem to be especially useful for spectroscopic uses because of the small diameter and stability of the vesicles. Preliminary scattering data show the vesicles to be osmotically active. Few contaminating structures are present. In high resolution micrographs the membranes appear as triple-layered structures and are approximately 7.5 nm in

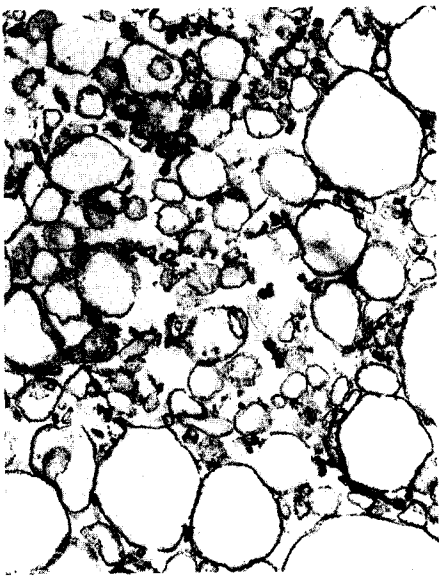

Fig. 4. Electron micrograph of sarcolemmal preparation (P_3D). Membrane appears as intact vesicles; ruptures or breaks in membrane are rare and where they occur are assumed to be artifacts resulting from mechanical stresses engendered during fixation, dehydration and final manipulation of pellet. Few intercellular organelles and inclusions (mitochondria, glycogen) and/or extracellular material are present. Membranes measure approximately 7.5 nm in width and appear triple layered at high magnification. Magnification, ×34 800.

thickness (Fig. 4). The similarity of the size distributions of vesicles in different plasma membrane preparations is shown in Table I. The smallest vesicles are near the lower limit that one might expect for the formation of vesicles, while the largest vesicles are far smaller than the plasma membrane sheets derived from liver epithelial cells. This indicates also that only small pieces of membrane are probably coming off during the mild homogenization instead of a big breakup of cells.

Enzymatic activities

The enzymatic complements of the different membrane organelles are presumably characteristic. It is often assumed that some enzymes are so well localized that they may be used for markers indicating the presence of a particular membrane in a subcellular fraction. Clearly, however, some enzymes may be used for this purpose more reliably than others [24, 25]. We have assayed for a number of marker enzymes in order to decide whether they might provide consistent information about the components of our imputed plasma membrane fraction. The results of these assays are shown in Table II.

Once washed, P_3D preparations (see Fig. 1) contained approximately 0.5% of the total tissue protein. The total enzymatic activities in our P_3D fraction of 5′-nucleotidase, alkaline phosphatase, (Na^+–K^+)-ATP ase, NADH : cytochrome *c* reductase, and thiamine pyrophosphatase ranged in the neighborhood of 10% of the total activities in the homogenate. In general, this was more characteristic for the 5′-nucleotidase

TABLE II

ENZYME ACTIVITIES

Enzyme	Homogenate		Fraction P_2P (density > 1.15) Mitochondria + glycogen granules		Fraction P_3D (1.07 < density < 1.11) Plasma membranes	
5'-Nucleotidase ($\mu M\ P_i \cdot min^{-1} \cdot mg^{-1}$)	0.11	(18)	0.077	(14)	2.08	(8)
Alkaline phosphatase ($\mu M\ P_i \cdot min^{-1} \cdot mg^{-1}$)	0.206	(5)	0.123	(5)	3.16	(5)
(Na^+-K^+)-ATPase ($\mu M\ P_i \cdot h^{-1} \cdot mg^{-1}$)	0.31	(1)	0.22	(1)	4.5	(2)
Thiamine pyrophosphatase ($\mu M\ P_i \cdot min^{-1} \cdot mg^{-1}$)	0.009	(6)	0.0072	(6)	0.154	(6)
NADH: cytochrome *c* reductase (rotenone insensitive) (μM cytochrome $c \cdot min^{-1} \cdot mg^{-1}$)	1.3	(2)	3.4	(2)	12.1	(2)
Acid phosphatase ($\mu M\ P_i \cdot min^{-1} \cdot mg^{-1}$)	0.010	(2)	0.03	(2)	0.04	(2)
Glucose-6-phosphatase ($\mu M\ P_i \cdot min^{-1} \cdot mg^{-1}$)	0.12		"0"	(2)	"0"	(2)
Lactic acid dehydrogenase (arbitrary units)	4.0	(2)	"0"	(2)	"0"	(2)
Succinic acid dehydrogenase (μM succinic acid $\cdot min^{-1} \cdot mg^{-1}$)	0.41	(2)	1.0	(1)	"0"	(2)
Cytochrome oxidase (in $A_{[\text{cytochrome } c]} \cdot min^{-1} \cdot mg^{-1}$)	77.0	(3)	400.0	(3)	12.0	(3)

while the enzymes showing somewhat smaller increases of specific activity likewise have lower percent recovery. The impressive feature of the fractionation procedure, relative to enzymatic activity, is that the forementioned five enzymes underwent roughly the same increase in specific activity.

There was no evidence of the existence of a membrane bound glucose-6-phosphatase in this tissue, although there was a considerable amount in the soluble phases. Substrate specificity was not determined.

Attempts to increase 5'-nucleotidase activity

We adopted the hypothesis that 5'-nucleotidase activity should be higher in the plasma membrane than in the internal membranes of frog cardiac cells. On this basis, we attempted to increase the specific 5'-nucleotidase activity in our preparations in a variety of ways short of destroying the vesicles. Exposure of the vesicles to either distilled water or to 0.6 M KCl at various stages of the isolation procedure including the terminal P_3D stage did not significantly increase the specific activity of the final preparations. Increases of ionic strength tended to decrease the yield of total activity but decreases of ionic strength with or without concomitant decreases in osmotic pressure had little effect one way or the other. The sequestration of divalent cations by 1 mM EDTA added to the original potassium homogenizing solution and following solutions likewise decreased the total yield but did not increase the specific 5'-nucleotidase activity of preparations.

The isolation of vesicles at pH 9 instead of the usual 7 or 7.4 caused no significant changes in the distribution or total amount of the activities of cytochrome *e* oxidase, NADH : cytochrome *c* reductase or 5'-nucleotidase. However, reduction to pH 5 caused apparent aggregation of material and a large increase in the protein of pelleted material and a concomitant decrease in the activities of the P_3D and P_3F fractions. The lower pH values were clearly deleterious to the vesicular integrity.

Lipid composition

The plasma membrane fraction (P_3D) was found to have a molar phospholipid to cholesterol ratio of 1.4 : 1. The cruder (P_2B) fractions which were heavily contaminated with mitochondrial components had about the same molar phospholipid to cholesterol ratio but had considerable cardiolipin content, 6.4% total lipid (by

TABLE III

PHOSPHOLIPID COMPOSITION

Phospholipid	Plasma membrane fraction (P_3D) (% total)	Crude fraction (P_2B) (%)
Phosphatidylcholine	45.0	47.5
Phosphatidylethanolamine	22.6	23.4
Phosphatidylinositol +sphingomyelin	22.2	20.4
Phosphatidylserine	8.6	2.3
Phosphatidic acid	1.5	Trace
Cardiolipin	—	6.4

wt). The mitochondrial glycogen pellet (P_2P) had a phospholipid to cholesterol ratio of 3.34.

The protein to total lipid ratio of the plasma membrane fraction (P_3D) was 3 : 2 (by wt). As may be seen from Table III, the plasma membrane fraction has relatively more phosphatidylserine and phosphatidic acid and a trifle less of other major phospholipids than the cruder P_2B fraction. As can also be seen from Table III and the comparable data reported for whole frog heart [26] (phosphatidylcholine 36.5%, phosphatidylethanolamine 29.2%, phosphatidylserine 4.7%, phosphatidylinositol 4.1%, sphingomyelin 7.2%) the lipid distributions by class may simply reflect the fact that most of the phospholipid in frog heart is in plasma membrane and mitochondria.

DISCUSSION

The mildness of the homogenizing process is indicated by the presence of considerable amounts of sarcolemma and the relative integrity of cytoplasmic structures in the cellular debris pellet (P_1P). In particular, the presence in the debris of perinuclear interfilamentary columns of mitochondria indicates that the amount of mitochondrial material in the supernatant was considerably less than might have resulted from a more exhaustive homogenization designed to disrupt more of the sarcolemma. As seen in Table I the vesicles in the fraction (P_3D) identified as plasma membrane differed considerably from the large sheet-like collapsed vesicles characteristic of plasma membrane preparations from erythrocytes, various epithelia and various cultured cell types [25]. On the other hand, the results here are more comparable to those of Kidwai et al. [2, 4] obtained from other muscle types. Perhaps muscle plasma membranes are somewhat less mechanically rigid than other cell types [27].

Four enzymatic activities: 5′-nucleotidase, alkaline phosphatase, (Na^+–K^+)-ATPase and thiamine pyrophosphatase, all are concentrated in our plasma membrane fraction, P_3D, relative to the tissue homogenate by from 14 to 19 times. This is not an impossible range of membrane to cell protein ratios. The relative concentration of rotenone-insensitive NADH : cytochrome *c* reductase in Fraction P_3P was only slightly less, being about 9 times that in the homogenate. Three of these enzymes, 5′-nucleotidase, alkaline phosphatase and (Na^+–K^+)-ATPase, have often been used as plasma membrane markers [24] and therefore might be expected to be increased in specific activity to the same extent if the plasma membrane vesicles were being isolated without significant extraction losses. However, in other cell types NADH : cytochrome *c* reductase has been associated both with internal membranes [15, 24] and with plasma membranes [1, 15]. Thus, it is possible that the somewhat lower purification ratio (spec. act. P_3D/spec. act. homogenate) of NADH : cytochrome *c* reductase is due to the presence in the cell of another site for this enzyme. The presence of a high purification ratio for TTPase is more surprising because of the widespread evidence that it is associated with the Golgi membranes. Although Golgi membranes have been found which have densities in the range of our plasma membrane fraction, P_3D, the electron micrographs show the perinuclear regions are usually fairly well intact. This alone would have led one to expect a lower purification ratio even if TTPase was in the plasma membrane also. Perhaps there is a complication of a high activity non-specific TTPase activity in our membrane preparation.

In any event, although it is possible that these enzymes have been associated with the plasma membrane fraction by virtue of contaminating vesicles of other organelle membranes or by the formation of hybrid vesicles, the lack of complete disruption of the cell interiors during homogenization and the high specific enzymatic activities make the notion that they are part of the plasma membrane enzymatic complement seem more favorable. The amount of acid phosphatase in the plasma membrane fraction could have come from contamination of the fraction by lysosomes. The lack of a membrane bound glucose-6-phosphatase should not be surprising because it is consistent with the metabolic demands of a tissue that is a sink for and not a source of plasma glucose. As is more conventional, the activity of lactate acid dehydrogenase was also distributed as a completely soluble entity. Finally, although it is unwarranted from our data to conclude that there are no mitochondria in P_3D, it seems likely that they constitute only a few percent by weight.

The phosphatidylcholine and phosphatidylethanolamine content are well within the range found in other plasma membrane preparations. There was relatively more phosphatidylcholine than Chako et al. [28] found in garfish olfactory axolemma, but as has been pointed out [27, 29], there are considerable differences between even the same cell types from different species. The frog cardiac plasma membrane is also similar to the garfish olfactory axolemma in its high phosphatidylserine content. The same is likely to be true for sphingomyelin. The latter point is interesting in terms of the reported specific requirement of sphingomyelin for 5'-nucleotidase activity [30]. These four common phospholipid classes accounted for approximately 98% of the total phospholipid in our plasma membrane preparation. The absence of detectable cardiolipin in this preparation also indicates a paucity of mitochondrial contamination.

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