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A PLASMA MEMBRANE FRACTION FROM BOVINE ADRENAL MEDULLA: PREPARATION, MARKER ENZYME STUDIES AND PHOSPHOLIPID COMPOSITION

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SUMMARY

A plasma membrane fraction was obtained from the post-mitochondrial supernatant fraction of bovine adrenal medulla by centrifugation through a continuous sucrose gradient in a zonal rotor.

Activities of 5′-nucleotidase (EC 3.1.3.5), (Na^+-K^+)-ATPase (EC 3.6.1.3), Mg^{2+}-ATPase (EC 3.6.1.3), Ca^{2+}-ATPase (EC 3.6.1.3) and acetylcholinesterase (EC 3.1.1.7) were enriched in this fraction and showed a purification of 8-, 6-, 9-, 11- and 14-fold, respectively, over the whole homogenate.

Plasma membranes were reasonably free from mitochondria, microsomes and lysosomes as shown by relatively low activities of cytochrome *c* oxidase (EC 1.9.3.1), glucose-6-phosphatase (EC 3.1.3.9) and acid phosphatase (EC 3.1.3.2), respectively, and by electron microscopy.

The plasma membrane phospholipid fraction contained 4 % lysophosphatidylcholine, 17 % sphingomyelin, 38 % phosphatidylcholine, 28 % phosphatidylethanolamine, 5 % phosphatidylserine and 1 % each of phosphatidylinositol and phosphatidic acid.

INTRODUCTION

Adrenal medulla synthesizes, stores and secretes catecholamines on stimulation by neurotransmitters released from the splanchnic nerve endings on the chromaffin cell surface [1]. The biochemical and morphological evidence indicates that secretion from adrenal medulla occurs by exocytosis [2]. On stimulation, the chromaffin granules containing catecholamines, adenine nucleotides, Ca^{2+} and chromogranins migrate towards the periphery of the cell and fuse with plasma membranes. The membranes break at the point of fusion and the secretory material is released into the lumen. It has been proposed that secretagogues interact with plasma membrane and elevate the levels of messengers, i.e. cyclic AMP and Ca^{2+} which influence the intracellular

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processes concerned with secretion [3, 4]. To elucidate the mechanism of secretion more information is required about the plasma membranes and other subcellular organelles involved in the process [5, 6]. Although many studies have been carried out with chromaffin granules [7], no data are yet available for plasma membranes of adrenal medulla, probably because of difficulties in obtaining a reasonably pure preparation.

Various methods for the isolation of plasma membranes from specific tissues have been described [8–14] but none seem to be generally applicable without modification [15]. This communication describes a procedure for the preparation of a fraction rich in plasma membranes from bovine adrenal medulla using zonal centrifugation.

EXPERIMENTAL

Isolation of plasma membranes

Bovine adrenal glands were obtained from the local slaughter house and cleaned of the extraneous tissue. The medullae were then separated from the cortices and were finely sliced, weighed and homogenized in 8 vols of 0.25 M sucrose in 5 mM Tris–HCl buffer, pH 7.4. All operations were carried out at 2–4 °C unless noted otherwise. The tissue was homogenized by hand in a Potter-Elvehjem homogeniser (0.12 mm radial clearance) and was complete after 4–5 strokes. The homogenate was filtered through 4–5 layers of cheese cloth and the final volume was adjusted to yield 8 ml per g tissue. The homogenate was then centrifuged at $10\,000 \times g_{av}$ for 10 min and the supernatant was carefully withdrawn. The pellet was rehomogenized with 2 vols of the same medium and centrifuged as before. The two supernatants were combined and made 0.5 mM with respect to $MgCl_2$. Zonal centrifugation was then carried out in the BXIV titanium rotor (M.S.E. Ltd., London, U.K.) using a linear gradient prepared from 250 ml 15 % (w/v) sucrose and 250 ml 50 % sucrose. The gradient was formed in an LKB 11 300 Ultragrad gradient mixer (LKB Instruments Ltd., South Croydon, Surrey, U. K.) or in two conical flasks joined by capillary tubing at the base, the solution in the mixer flask being stirred magnetically. The various solutions were pumped at 25 ml/min, using an H.R. Flow Inducer (Watson-Marlow Ltd., Cornwall, U.K.), into the rotor spinning at 2500 rev./min. The sequence was as follows: from the periphery 100 ml 8.5 % sucrose, 500 ml 15 %–50 % sucrose gradient and sufficient 60 % sucrose (150 ml) to remove the original 8.5 % sucrose; from the centre 75 ml adrenal medulla sample followed by an overlay of 25 ml 5 mM Tris-HCl buffer, pH 7.4. After centrifuging at 47 000 rev./min for 3 h the speed was reduced to 2500 rev./min and 16 ml fractions collected in the LKB Ultrorac by pumping 60 % sucrose into the outer edge of the rotor. The fractions were analysed for enzymic activities and chemical composition within one week of their collection.

Enzyme assays

The activity of 5′-nucleotidase was measured by the method of Michell and Hawthorne [16], $(Na^+\text{-}K^+)$-ATPase and Mg^{2+}-ATPase by the procedure of Quigley and Gotterer [17], Ca^{2+}-ATPase by the method of Goz [18] and acetylcholinesterase by that of Hawkins and Knittle [19]. Glucose-6-phosphatase activity was estimated by the method of De Duve et al. [20]. Cytochrome *c* oxidase activity was measured

according to the procedure of Wharton and Tzagoloff [21]. Acid phosphatase activity was measured by the method of Hübscher and West [22].

Activities of 5′-nucleotidase [9, 23], (Na^+-K^+)-ATPase and acetylcholinesterase [10, 23, 24] were used as indicators for plasma membranes. Glucose-6-phosphatase activity served as a marker for microsomes [25], cytochrome *c* oxidase activity as a marker for mitochondria [26] and acid phosphatase activity indicated the lysosomal contamination [9, 20].

Analytical methods

The amount of P_i released into the assay medium by the various phosphatases was estimated by the method of Chen et al. [27]. P_i released in the ATPase assay was measured according to the procedure of Taussky and Shorr [28].

For protein assay tissue fractions were precipitated by the addition of an equal volume of ice cold 10 % (w/v) trichloroacetic acid and the residue after centrifugation was solubilized in 0.25–0.5 ml 1.0 M NaOH. An aliquot of this solution was analysed for protein by the method of Lowry et al. [29].

Sucrose concentration was measured with an Abbe refractometer (Carl Zeiss, Obertrochen, G.F.R.).

Separation and estimation of phospholipids

The tissue fractions were treated with an equal volume of trichloroacetic acid (10 % w/v) and centrifuged in a bench centrifuge for 5 min. The pellets were washed twice with 2 ml 5 % trichloroacetic acid and once with 2 ml water. The washed pellets were then treated with 2 ml chloroform–methanol (1 : 1, v/v) and centrifuged as before. The pellets were then extracted similarly three times with 1 ml chloroform–methanol (2 : 1, v/v). To the combined extracts 1 ml of chloroform and 3 ml 0.15 M NaCl were added to give a two-phase system. The upper phase was withdrawn along with the denatured proteins at the interface and the lower phase was washed three times with the original volume of synthetic upper phase [30].

The phospholipids were separated by two-dimensional thin-layer chromatography and estimated by phosphate analysis after digestion with $HClO_4$. The techniques have all been described previously [30].

Electron microscopy

Peak fractions were combined and centrifuged at 162 000 × *g* for 2 h to produce pellets which were post-fixed in osmium tetroxide and embedded in Araldite for sectioning. Sections were stained in uranyl acetate followed by lead tetraacetate.

RESULTS

Enzyme markers

The proteins of the 10 000 × *g* supernatant were resolved into three distinct peaks (Fig. 1), the lighter Peak I contained soluble proteins along with some lighter particular material, the intermediate Peak II was rich in plasma membranes and the heavier Peak III was heterogenous, containing microsomal membranes along with mitochondria and lysosomes. This classification of protein peaks was based upon the

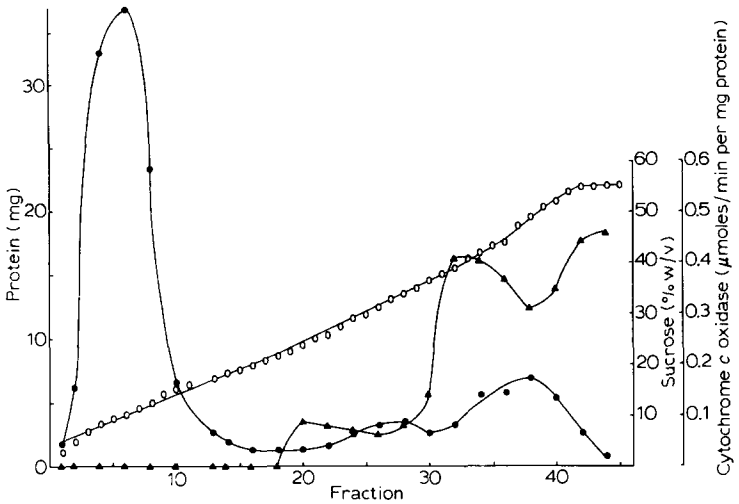


Fig. 1. Profiles of protein and cytochrome *c* oxidase after zonal centrifugation of 10 000 × *g* supernatant of adrenal medulla homogenate, using a sucrose gradient prepared using two conical flasks. ●, protein; ▲, cytochrome *c* oxidase; ○, sucrose concentration. Figs 2–4 refer to the same fractionation. Fractions were combined as follows for protein and lipid analysis: Peak I, Fractions 1–10; Peak II, Fractions 15–26; Peak III, Fractions 27–38.

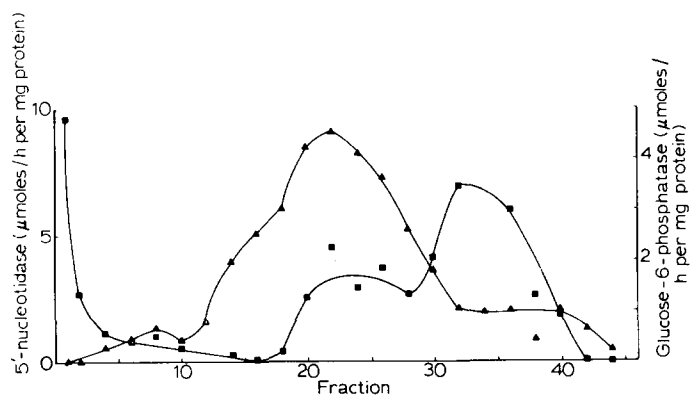


Fig. 2. Profiles of 5′-nucleotidase (▲) and glucose-6-phosphatase (■) in the separation of Fig. 1.

distribution of marker enzymes for different subcellular organelles. The mitochondrial enzyme, cytochrome *c* oxidase showed no activity in Peak I, little in Peak II but considerable activity in Peak III (Fig. 1). The highest enrichment of plasma membrane marker enzymes such as 5′-nucleotidase (Fig. 2), (Na^+-K^+)-ATPase (Fig. 3) and acetylcholinesterase (Fig. 4) was seen in Peak II. Peaks I and III, on the contrary, contained relatively little of these enzymes, the only exception being acetylcholinesterase which appeared in Peak III. No (Na^+-K^+)-ATPase was detectable in Peak I (Fig. 3), though the other two plasma membrane markers showed slight activity.

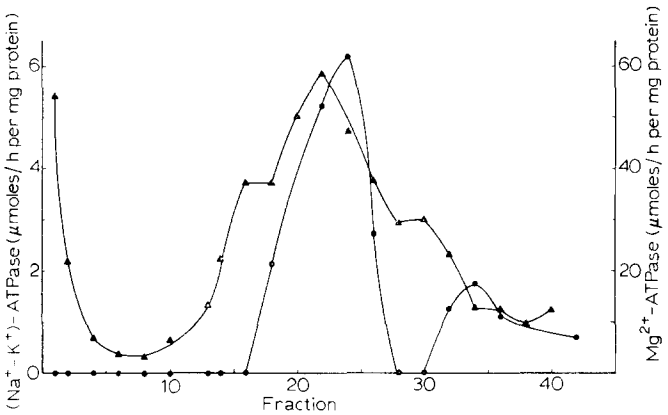


Fig. 3. Profiles of (Na^+-K^+)-ATPase (●) and Mg^{2+}-ATPase (▲) in the separation of Fig. 1.

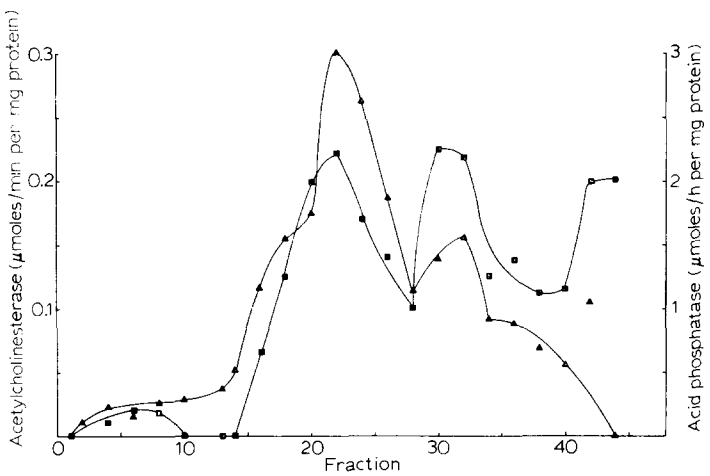


Fig. 4. Profiles of acetylcholinesterase (▲) and acid phosphatase (■) in the separation of Fig. 1.

Unlike the (Na^+-K^+)-ATPase, Mg^{2+}-ATPase was present in the first few fractions (Fig. 3) which probably contained soluble proteins. Most of the Mg^{2+}-ATPase activity was associated with the fractions of Peak II, though the peak for this enzyme was broader than that for (Na^+-K^+)-ATPase.

Glucose-6-phosphatase (Fig. 2) was most active in Peak III, though Peak II showed some activity. The profiles of glucose-6-phosphatase and aryl esterase (unpublished data) were very similar except that aryl esterase activity in Peak III was not as pronounced as that of glucose-6-phosphatase.

Acid phosphatase activity was seen in Peaks II and III (Fig. 4) but the total activity was much greater in Peak III, which contained more protein.

When the sucrose gradient was prepared by means of an Ultrograd gradient mixer, three distinct protein peaks were again obtained (Fig. 5). While Ca^{2+}-ATPase activity was completely absent from Peak I and very low in Peak III, much activity was

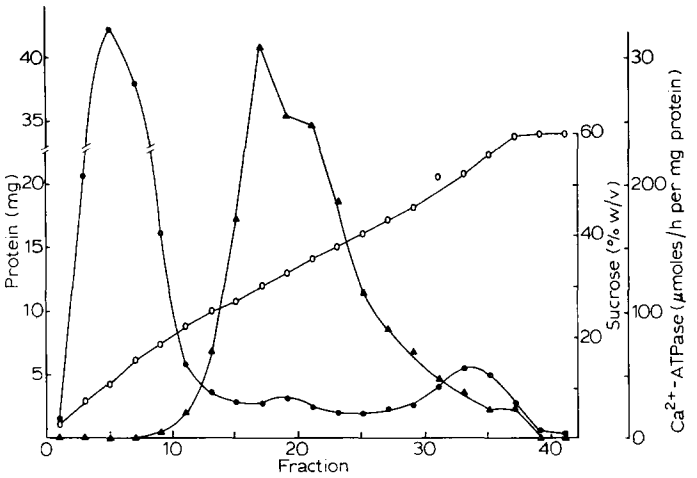


Fig. 5. Profiles of protein (●) and Ca^{2+}-ATPase (▲) after zonal centrifugation as in Fig. 1, but using the Ultragrad gradient mixer (see Experimental). ○, sucrose concentration.

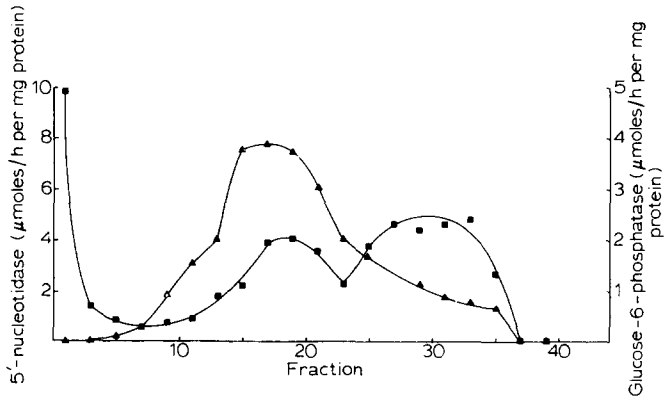


Fig. 6. Profiles of 5′-nucleotidase (▲) and glucose 6-phosphatase (■) in the separation of Fig. 5.

TABLE I

ENZYME DISTRIBUTION AFTER ORIGINAL 10 000 × *g* CENTRIFUGATION OF ADRENAL MEDULLA HOMOGENATE

The homogenate of adrenal medulla was centrifuged for 10 min at 10 000 × *g* as described in the Experimental section. The figures represent percentages of whole homogenate protein or activity with S.D. The number of preparations is given in parenthesis.

	Protein (3)	5′-Nucleotidase (7)	(Na^+, K^+)-ATPase (3)	(Mg^{2+})-ATPase (3)	(Ca^{2+})-ATPase (1)	Acetylcholinesterase (1)
Supernatant	68.2±4.9	73.9±7.1	76.3±2.6	84.9±5.3	71.3	89.3
Pellet	31.7±4.8	26.2±7.2	24.0±2.5	15.2±5.3	28.7	10.7

found in Peak II. The distribution of Ca^{2+}-ATPase in these fractions resembled that of (Na^+-K^+)-ATPase. The 5′-nucleotidase and glucose-6-phosphatase distributions (Fig. 6) were very similar to those obtained in the first experiments where the gradient was produced by mixing 15 % and 50 % sucrose in two flasks (Fig. 2).

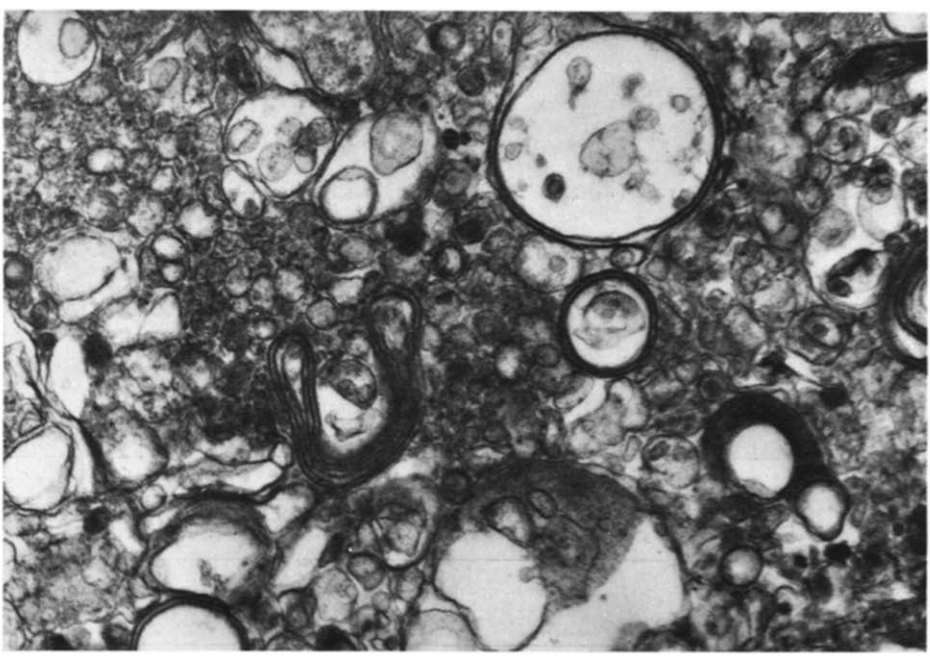
Table I shows the distribution of the plasma membrane enzymes between the pellet and supernatant in the original $10\,000 \times g$ centrifugation.

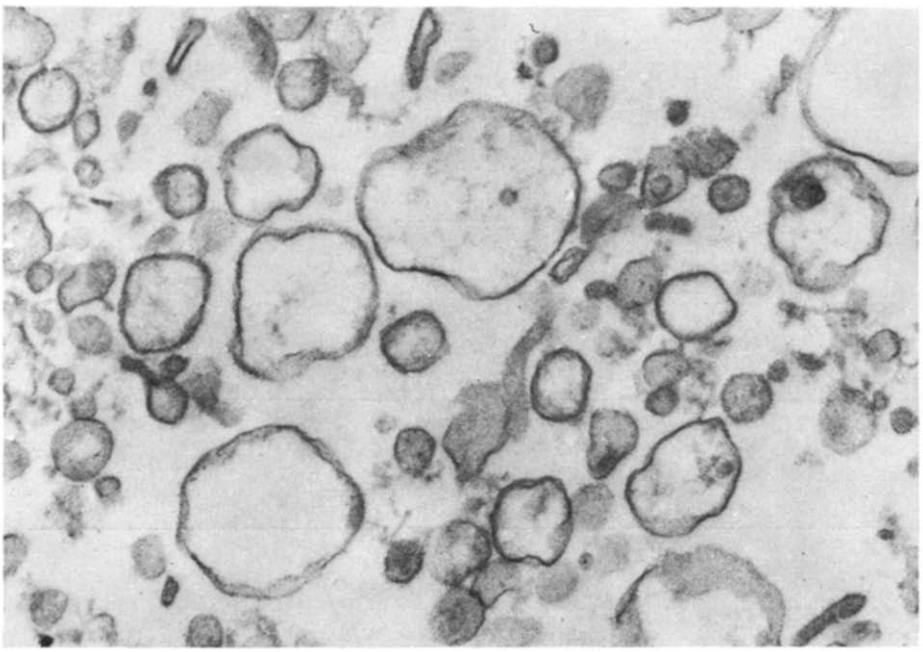
Electron microscopy

Electron microscopy of material from the three peaks confirmed the conclusions deduced from the distribution of marker enzyme activities. Pellets prepared by centrifuging fractions from the peaks at $162\,000 \times g$ for 2 h were examined. The pellet from Peak I was very small, most of the protein being soluble. It contained many small smooth vesicles which may have originated in the Golgi region of the chromaffin cell, together with non-vesicular membranes similar to those of the Golgi (Fig. 7A). Myelin fragments can also be seen. Peak II gave the most homogeneous pellet. The membranes were in the form of smooth vesicles (Fig. 7B). Peak III was more heterogeneous (Fig. 7C), showing lysosomes and damaged mitochondria as well as smooth vesicles.

Phospholipid composition

The sphingomyelin, phosphatidylserine and lysophosphatidylcholine contents of Peaks I, II and III were different, but phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidic acid showed no differences (Table II). The phospholipid fraction of Peak II contained roughly twice as much sphingomyelin as that of Peak I. Peak I contained relatively little phosphatidylserine but was richest in lysophosphatidylcholine.





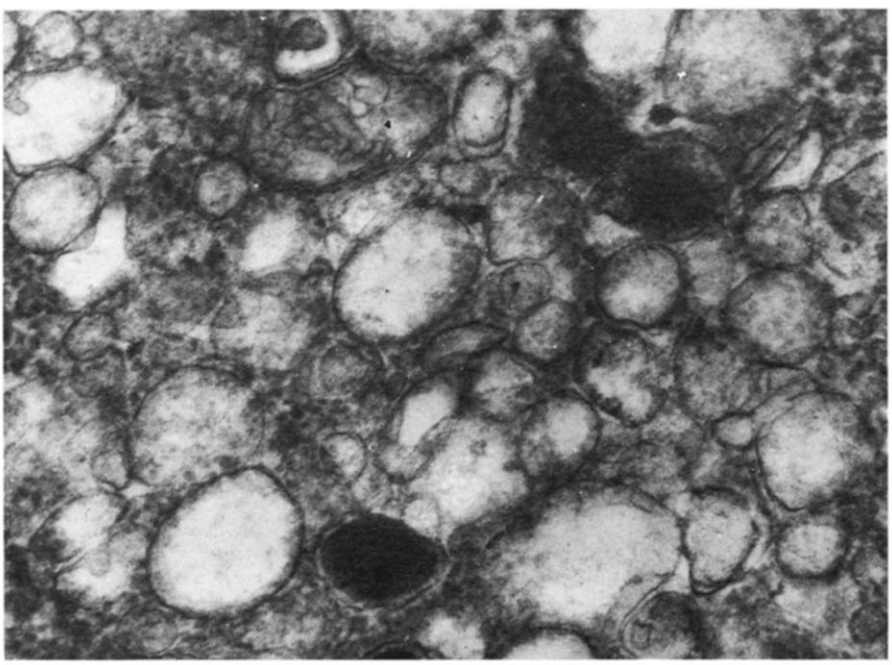


Fig. 7. Electron micrographs of the pelleted fractions from Peaks I–III (see text). Fractions were combined as indicated in the legend to Fig. 1. Magnification $\times 64\,000$. A, Peak I; B, Peak II; C, Peak III.

TABLE II

PHOSPHOLIPID COMPOSITION OF SUBCELLULAR FRACTIONS OBTAINED FROM BOVINE ADRENAL MEDULLA

The figures represent percentage of total lipid P and are means (±S.D.) of results from three fractionations.

Phospholipid	Subcellular fraction			
	Whole homogenate	Peak I	Peak II	Peak III
Lysophosphatidylcholine	8.2±0.6	8.5± 1.8	4.3±0.2	6.3±2.2
Sphingomyelin	11.1±1.1	9.0± 2.7	17.1±4.0	14.3±3.9
Phosphatidylcholine	33.6±1.7	38.9±10.2	38.1±6.8	37.6±9.8
Phosphatidylethanolamine	34.4±3.4	29.2± 3.8	28.5±1.8	27.1±1.4
Phosphatidylserine	5.4±0.9	1.1± 0.2	4.9±0.5	9.2±2.7
Phosphatidylinositol	4.1±0.1	2.2± 1.3	1.1±0.7	1.2±0.1
Phosphatidic acid	1.7±1.4	1.7± 1.3	1.0±0.8	0.8±0.5
Other lipids	1.3±0.5	7.3± 0.6	3.8±0.5	2.1±0.3

When the phospholipid composition of the three fractions was compared with that of the whole homogenate, sphingomyelin and phosphatidylcholine showed some enrichment in the plasma membrane fraction (Peak II). In contrast, there was less phosphatidylethanolamine.

DISCUSSION

The results in Table I show that only 25–30 % of the plasma membrane marker enzyme activity was recovered in the 10 000 × *g*–10 min pellet. Thus the method of Neville [8] for the isolation of plasma membranes from the nuclear pellet could not be used with the adrenal medulla. The methods of Bosmann et al. [11] and of House et al. [14] described for the isolation of plasma membranes from the post-mitochondrial supernatant of HeLa cells and liver respectively, were then attempted. Neither of these procedures resolved 5′-nucleotidase activity from glucose-6-phosphatase activity (unpublished data). A good separation of these enzyme activities was achieved however, by treating the supernatant with 0.5 mM $MgCl_2$ prior to its centrifugation over a continuous sucrose gradient in the BXIV Zonal rotor. This method also gave good resolution of other enzyme activities (Figs 1–4). Similar resolution of marker enzymes from the $Pb(NO_3)_2$-treated post-lysosomal supernatant of rat liver was reported by Hinton et al. [31].

The distributions of plasma membrane marker enzymes such as 5′-nucleotidase, (Na^+-K^+)-ATPase, acetylcholinesterase, Mg^{2+}-ATPase and Ca^{2+}-ATPase demonstrate the enrichment of plasma membranes in Peak II (Table III). The low activities of 5′-nucleotidase, acetylcholinesterase, Mg^{2+}-ATPase, acid phosphatase and glucose-6-phosphatase in Peak I suggest the presence of material originating from a variety of subcellular membranes. This peak also contained soluble protein. Electron microscopy of this fraction revealed small smooth vesicles, myelin fragments and membranes resembling those seen in the Golgi region of the chromaffin cell. Peak III appeared to

TABLE III

SPECIFIC ACTIVITIES OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS OBTAINED FROM BOVINE ADRENAL MEDULLA BY ZONAL CENTRIFUGATION

The method of Fig. 1 was used to prepare the gradient. The homogenate figures correspond to 10 g fresh adrenal medulla. About 68 % of homogenate protein was found in the 10 000 × *g* supernatant applied to the zonal gradient. Enzyme activities correspond to the peak fraction in each case and are given in μmoles substrate converted /h per mg protein except for acetylcholinesterase and cytochrome *c* oxidase, where the units are μmoles/min per mg protein. The figures represent means from the number of fractionations indicated in parenthesis. Protein figures represent recoveries from the whole of each peak.

		Whole homogenate	Peak I	Peak II	Peak III
Proteins	(4)	666.2 (100 %)	330.6 (49.6 %)	21.9 (3.3 %)	98.3 (14.7 %)
5′-Nucleotidase	(4)	1.05	0.38	8.84	1.32
(Na^+-K^+)-ATPase	(3)	1.31	0.45	7.79	1.17
Mg^{2+}-ATPase	(3)	9.57	6.96	81.60	23.35
Acetylcholinesterase	(2)	0.02	0.02	0.35	0.07
Ca^{2+}-ATPase	(2)	23.36	0	260.95	32.35
Glucose-6-phosphatase	(3)	0.40	0.61	2.16	3.66
Acid phosphatase	(2)	0.56	0.11	2.28	2.61
Cytochrome *c* oxidase	(2)	0.06	0	0.07	0.39

contain microsomes, lysosomes and light or broken mitochondria. In general, electron microscopy confirmed the marker enzyme results.

In a recent review [15], Solyom and Trams have surveyed the various marker enzymes in many plasma membrane preparations obtained from different tissues by different techniques and have discussed the validity of their use as markers. Specific and relative specific activities of marker enzymes quoted in this study would appear to compare reasonably well with the average values reported therein except that of 5′-nucleotidase. Specific activity of 5′-nucleotidase in adrenal medulla plasma membranes was rather low when compared with the values reported by other investigators [15]. However, an enrichment of 8–10-fold over the original homogenate compared well with the reported average value (Table III). The relative specific activities of other plasma membrane enzymes were similar to the average values reported by other authors [15].

Acetylcholinesterase is abundant in nervous tissue though it is present in the plasma membranes of many other tissues [15, 24]. Peak II will probably contain some plasma membrane fragments from the cholinergic endings of the splanchnic nerve which innervates the adrenal medulla [32]. Since the plasma membrane of the chromaffin cell will also contain acetylcholinesterase, particularly at the junctions with nerve endings, it is impossible to estimate how much of the activity is due to nerve membranes.

Although Mg^{2+}-ATPase has been considered a poor marker for plasma membranes in other tissues [15], the present results (Fig. 3 and Table III) show its high activity in adrenal medulla plasma membrane and its suitability as a marker. This view is consistent with histological observations on the adrenal medulla of cat [23] and hamster [33]. Mg^{2+}-ATPase activity in Peak I was partly soluble and partly mem-

brane-bound since centrifugation for 2 h at 162 000 × *g* only sedimented 70–75 % of the activity (unpublished data).

Most of the Ca^{2+}-ATPase activity was recovered in the plasma membrane fraction (Fig. 5). This is consistent with the findings of others [15] who reported high activity in plasma membranes obtained from heart, intestinal brush border and erythrocytes. The association of Ca^{2+}-ATPase with plasma membranes of adrenal medulla has also been demonstrated histochemically [33].

Some glucose-6-phosphatase activity has been reported in many plasma membrane preparations [9, 15] and the plasma membrane enzyme differs from the microsomal one [34]. Thus the glucose-6-phosphatase of Peak II need not indicate microsomal contamination [34].

Acid phosphatase in Peak II represented only 11 % of the total activity of homogenate and its wide subcellular distribution in adrenal medulla has been demonstrated histochemically [26]. Electron microscopy revealed no lysosomes in the material of Peak II and acid phosphatase has been found in several plasma membranes [15]. Thus its presence cannot be attributed simply to lysosomal contamination of Peak II.

Phospholipid composition of adrenal medulla plasma membrane differed little from that of plasma membranes obtained from pancreas [35], submaxilllary glands [13], cerebral cortex [36, 37] and liver [12]. The adrenal medulla plasma membranes were enriched with sphingomyelin as has been shown for other plasma membrane preparations except those from cerebral cortex [36, 37].

The phospholipid compositions of synaptic vesicles and synaptosomal plasma membranes from rat brain seem almost identical [37]. The adrenal medulla plasma membrane fraction, on the other hand, has a phospholipid composition different from that of chromaffin granules (ref. 38 and Table II). The granules contain much more lysophosphatidylcholine, more phosphatidylethanolamine, but less sphingomyelin and phosphatidylcholine. While the synaptosomal plasma membrane represents a small proportion of the neuronal cell membrane closely concerned with exocytosis, our adrenal plasma membrane fraction arises from the whole chromaffin cell and may include plasma membrane from other cell types as well.

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