Lipids of the Adrenal Medulla

LYSOLECITHIN, A CHARACTERISTIC CONSTITUENT OF CHROMAFFIN GRANULES

BY H. BLASCHKO, H. FIREMARK, A. D. SMITH AND H. WINKLER*
Department of Pharmacology, University of Oxford

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1. The lipid composition of microsomes, mitochondria and chromaffin granules, obtained from homogenates of bovine adrenal medulla, has been investigated. 2. The three types of particle showed characteristic differences of phospholipid and cholesterol content. The lipid composition of microsomes and mitochondria resembled that of corresponding particles from other tissues. The chromaffin granules contained 19% of the cholesterol and 14% of the phospholipids of the low-speed supernatant. 3. Thin-layer chromatography indicated the presence of these phospholipids in extracts from each particle: lecithin, lysolecithin, phosphatidylethanolamine (partly plasmalogen), phosphatidylserine, phosphatidylinositol and sphingomyelin. 4. On quantitative analysis of the phospholipids, chromaffin granules were found to contain a high concentration of lysolecithin (17% of the lipid phosphorus). Mitochondria and microsomes, on the other hand, contained very little lysolecithin (less than 2% of the lipid phosphorus).

The chromaffin granules of the adrenal medulla contain large amounts of catecholamines, adenosine triphosphate and water-soluble protein. Much work has been carried out on these constituents in the last few years (see reviews by Weiner, 1964; Blaschko, 1967).

Lipids represents 22% of the dry weight of bovine adrenal chromaffin granules (Hillarp, 1959). Since an earlier report by Hillarp & Nilson (1954*b*) on the lipid content of the particulate material present in homogenates of adrenal medulla, it has been recognized that the so-called large-granule fraction is composed of a mixture of chromaffin granules, mitochondria and lysosomes (Blaschko, Hagen & Hagen, 1957; Smith & Winkler, 1966). In a recent study on the proteins of purified chromaffin granules it was noticed that some material containing phospholipid was present in the soluble fraction prepared from the granules (Smith & Winkler, 1967*b*). This led to the present study of the lipid composition of highly purified chromaffin granules, in comparison with that of other particulate fractions obtained from the bovine adrenal medulla. Some of the results have already been briefly reported (Blaschko, Firemark, Smith & Winkler, 1966).

* Present address: Pharmakologisches Institut der Universität, Innsbruck, Austria.

METHODS

Isolation of subcellular fractions. The procedure used for the isolation, from homogenates of bovine adrenal medulla, of purified chromaffin granules and of fractions containing mitochondria and lysosomes was the same as that described in detail by Smith & Winkler (1967*a*). The procedure involves the preparation of a large-granule fraction, which is further resolved by ultracentrifugation in 1·6M-sucrose. In some of the present experiments the sucrose solutions contained 1mM-sodium EDTA. A microsomal fraction was isolated by centrifugation (110000*g* for 1hr.) of the supernatant remaining after sedimentation of the large granules. The microsomal pellet was resuspended by homogenization in 0·3M-sucrose.

Extraction and analysis of lipids. Lipids were extracted by the method of Folch, Lees & Sloane-Stanley (1957) with analytical grade reagents. The washing procedure was carried out three times, with 'pure solvents upper phase' that contained KCl. Portions of the lower phases were evaporated to dryness for the determination of cholesterol and lipid phosphorus. Total (i.e. free and esterified) cholesterol was determined by the method given by Zlatkis, Zak & Boyle (1963). Lipid phosphorus was determined as inorganic phosphate (Chen, Toribara & Warner, 1956) after digestion by the procedure of Burton & Peterson (1960).

For thin-layer chromatography of phospholipids the lipid extracts were evaporated to dryness under nitrogen and redissolved in chloroform.

The phospholipids were separated by the one-dimensional method of Skipski, Peterson & Barclay (1964), except that Kieselgel H (E. Merck, Darmstadt, Germany) was used. In our hands this system resolved the following classes of

phospholipids: phosphatidylethanolamine, lecithin, sphingomyelin and lysolecithin. The spot containing phosphatidylserine also contained phosphatidylinositol, as could be demonstrated by two-dimensional thin-layer chromatography according to the methods given by Skidmore & Entenman (1962) and Abramson & Blecher (1964). Cardiolipin and phosphatidic acid both moved with the solvent front in the one-dimensional system.

Phospholipids in the tissue extracts were identified by comparison of their chromatographic mobility with that of pure standard phospholipids, and by their reaction to various stains. The plates were sprayed with Dragendorff's reagent (Wagner, Hörhammer & Wolff, 1961) to demonstrate choline-containing compounds and with ninhydrin to demonstrate ethanolamine and serine-containing compounds. For the demonstration of plasmalogens the plates were exposed to acetic acid vapour and then sprayed with decolorized fuchsin (Gray & Macfarlane, 1958) or with 2,4-dinitrophenylhydrazine. For quantitative analysis, the phospholipid spots were located by exposure of the plates to iodine vapour; the silica was scraped from the plates and extracted as described by Skipski *et al.* (1964). The extracts were evaporated to dryness with heat and the phosphorus was estimated as described above. Recoveries of the phosphorus applied to the plates averaged 94% (four determinations).

Haemolysis experiments. The haemolytic activity of the lysolecithin was determined by the following method, for which we are grateful to Professor C. Long. Human erythrocytes were washed four times with 0·9% NaCl and then resuspended in 0·9% NaCl to produce a haematocrit of about 12%. Material with chromatographic mobility characteristic of lysolecithin was extracted from the silica with chloroform, and the extract was evaporated to dryness in a small test tube. Saline (0·9% NaCl) was added to the tube, which was agitated at 37° for 5 min. Then 0·05 ml. of the erythrocyte suspension was added and the tube was immediately centrifuged. The extinction of the supernatant was measured at 560 mμ. A control tube contained material extracted from a blank region of silica with the same R_F as that of lysolecithin and in a third tube distilled water was used instead of saline. Haemolytic activity was measured by comparison with the haemolysis produced by the distilled water.

Materials. Lecithin and phosphatidylethanolamine were prepared from hen's eggs by the method of Rhodes & Lea (1957). Lysolecithin was prepared from egg lecithin by Miss M. C. Boadle, by using the procedure given by Saunders (1957). Phosphatidylserine was a gift from Professor C. Long. Phosphatidylinositol, isolated from yeast (Trevelyan 1966), was a gift from Dr W. E. Trevelyan. Methanol (AnalaR), chloroform (AnalaR) and cholesterol were purchased from British Drug Houses Ltd.

RESULTS

Distribution of lipid phosphorus and cholesterol between fractions of the homogenate. The results of these estimations, given in Table 1, have been expressed in two ways: first, as the percentage distribution of lipid phosphorus and of cholesterol between the different fractions obtained from the low-speed supernatant; secondly, as μmoles of each constituent per mg. of nitrogen.

A large proportion of the lipid phosphorus and of the cholesterol was recovered in the microsomal fraction, and this fraction also had a high ratio of lipids per mg. of nitrogen. Mitochondria had a phospholipid content similar to that of the microsomes, but they contained much less cholesterol. The chromaffin granules were relatively rich in cholesterol but their phospholipid content was lower than that of the mitochondria and microsomes. These three types of cell particle are therefore readily distinguished on the basis of their lipid composition. The molar ratio of cholesterol to lipid phosphorus also differed according to the particle: the ratio is 0·23 for mitochondria, 0·44 for microsomes and 0·58 for chromaffin granules. The lysosomal fraction, which was by no means pure, contained much less phospholipid and cholesterol

Table 1. *Distribution of lipid phosphorus and of cholesterol between fractions of the low-speed supernatant of bovine adrenal medulla*

Figures for the distribution of lipid phosphorus and of cholesterol are given as a percentage of the amount recovered. The actual recoveries were 96% for lipid phosphorus and 102% for cholesterol. Figures for μmoles of constituent per mg. of nitrogen are means ± S.D., and the number of experiments is given in parentheses. The fraction called residues is that remaining in the tube after the large-granule fractions have been resuspended: it is composed mainly of erythrocytes and some large granules.

	Lipid phosphorus		Cholesterol	
Fraction	% of total	μmoles/mg. of N	% of total	μmoles/mg. of N
Mitochondrial fraction	27·5	4·3 ± 0·47 (5)	14·3	0·99 ± 0·09 (5)
Microsomal fraction	42·8	4·40 ± 0·30 (3)	41·7	1·92 ± 0·10 (3)
Chromaffin granules	13·8	2·83 ± 0·30 (5)	19·4	1·6 ± 0·16 (5)
'Lysosomal fraction'	3·9	1·23 ± 0·32 (4)	4·8	0·58 ± 0·14 (4)
Final supernatant	8·1	0·33 (2)	15·7	0·28 (2)
Residues	3·9	2·56 (2)	4·1	1·14 (2)

Table 2. *Phospholipids of mitochondria, microsomes and chromaffin granules from the bovine adrenal medulla*

Figures represent the percentage of the total lipid phosphorus recovered from the chromatography plate, and are the means ± S.D. of six analyses of mitochondria, five of microsomes and nine of chromaffin granules.

	Mitochondria	Microsomes	Chromaffin granules
Lecithin	44·3±3·3	42·2±2·3	26·0±1·7
Lysolecithin	1·7±0·6	2·0±0·6	16·8±1·4
Phosphatidylethanolamine	34·9±2·0	33·0±2·2	36·1±3·3
Phosphatidylserine+phosphatidylinositol	7·6±1·7	12·1±1·5	9·2±2·0
Sphingomyelin	3·6±2·3	9·3±0·4	10·9±1·1
Cardiolipin and/or phosphatidic acid	7·6±1·8	1·2±1·1	0·6±0·2

per mg. of nitrogen than did the other particulate fractions.

Phospholipids of mitochondria, microsomes and chromaffin granules. In each of these three particulate fractions the following phospholipids were identified by one- and two-dimensional thin-layer chromatography: phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, lecithin, lysophosphatidylcholine and sphingomyelin. Only the phosphatidylethanolamine fraction from each type of particle gave a strong positive test for plasmalogen.

For quantitative analysis, one-dimensional thin-layer chromatography was used and the results are given in Table 2. Lecithin and phosphatidylethanolamine are the major components of the phospholipids of each cell particle. However, whereas lecithin comprises more than 40% of the lipid phosphorus of mitochondria and of microsomes, only 26% of the lipid phosphorus of chromaffin granules is present as lecithin. On the other hand, in the chromaffin granules there was a relatively high concentration (16·8% of the lipid phosphorus) of lysolecithin.

The fraction containing cardiolipin and phosphatidic acid represented 7·6% of the lipid phosphorus of mitochondria. This is probably due to the presence of cardiolipin, a phospholipid characteristic of mitochondria (Strickland & Benson, 1960). The other two particulate fractions contained only traces of cardiolipin or phosphatidic acid.

Lysolecithin of chromaffin granules. In one-dimensional thin-layer chromatography (see Table 2) lysolecithin was identified by comparing its mobility with a sample prepared from egg lecithin.

Since the lysolecithin content of chromaffin granules was high, other criteria of identification were applied. The chromaffin granule phospholipids were co-chromatographed with egg lysolecithin in two-dimensional thin-layer chromatography. The solvent systems described by Abramson & Blecher (1964) and Skidmore & Entenman (1962) were used. In each system, the lysolecithin from chromaffin granules moved to the same position as egg lysolecithin.

In both one-dimensional and two-dimensional thin-layer chromatography the lysolecithin spot gave a positive test with Dragendorff's reagent but did not react with ninhydrin, indicating the presence of a quaternary nitrogen (e.g. in choline) but the absence of an amino group. When the chromatography plate was sprayed with alkaline bromothymol blue solution (Jatzkewitz & Mehl, 1960), only the lysolecithin spot gave a 'halo' characteristic of lysophospholipids (Habermann, Bandtlow & Krusche, 1961).

The ability of lysolecithin to haemolyse red cells was used as a further test for the identity of this compound. It was found that the material eluted from the lysolecithin spot caused immediate haemolysis of human erythrocytes. The degree of haemolytic activity per mole of phosphorus was of the same order as that obtained with a sample of egg lysolecithin that had also been eluted from a thin-layer chromatography plate. Under the conditions of the experiment no haemolysis was obtained with the material extracted from the blank region of silica.

To test for lysolecithin formation in isolated granules, purified chromaffin granules were resuspended in 0·3 M-sucrose and incubated for 3 hr. at 37°. After incubation, the lysolecithin content (17·0% of fresh lipid phosphorus) was not significantly different from that of fresh granules.

DISCUSSION

The particle typical of the adrenal medullary cell is the chromaffin granule. The experiments just described were undertaken to obtain information on the lipids of these granules and to compare them with the lipids of the other cell particles.

Mitochondria, microsomes and chromaffin granules contained most of the phospholipid and cholesterol. The high phospholipid and cholesterol

content of the microsomes is similar to that of microsomes from other tissues (for review see Fleischer & Rouser, 1965). The mitochondrial fraction, which was not highly purified, was also rich in phospholipids but contained relatively little cholesterol. Chromaffin granules are distinguished from mitochondria by their high content of cholesterol, and from both mitochondria and microsomes by a high molar ratio of cholesterol to lipid phosphorus. The conclusion of Hillarp & Nilson (1954*b*) that the lipids of chromaffin granules were similar to those of mitochondria is explained by the fact that, at that time, the separation of chromaffin granules from mitochondria had not been achieved. The chromaffin granule fraction used in the present work was highly purified and contained less than 3% of the mitochondria (Smith & Winkler, 1967*a*). Chromaffin granules from pig and horse adrenals, prepared by the same method, could likewise be distinguished from mitochondria on the basis of their cholesterol and phospholipid content (Winkler, Strieder & Ziegler, 1966, 1967*b*).

A further, and striking, difference between the lipids of chromaffin granules and those of the other cell particles was revealed on analysis of the phospholipids: lysolecithin was one of the main components of the phospholipids of chromaffin granules, but only a very small amount was present in mitochondria and microsomes. Evidence has been given in the Results section that this compound behaves exactly like lysolecithin in chromatography and also has the characteristic haemolytic property of lysolecithin. The high concentration of lysolecithin is unusual; in other tissues, e.g. rat brain (Webster & Thompson, 1962) and rat liver (Skipski *et al.* 1964; van den Bosch & van Deenen, 1965), it is present only in trace amounts. In a survey of several tissues from the ox, Hadju, Weiss & Titus (1957) have already found that the adrenal medulla was a rich source of a cardioactive compound which was identified as lysolecithin. Thus the present work has confirmed this finding and shown that the lysolecithin is concentrated in the chromaffin granules. Mitochondria and microsomes contain only small amounts of lysolecithin, and it is probable that some of this is derived from contamination of these fractions by chromaffin granules. It is of interest that the sums of lecithin plus lysolecithin are equal in each of the three particles examined.

It has to be considered whether the lysolecithin was formed in the gland *post mortem*, during isolation of the granules, or during extraction of the lipids. The last possibility is excluded since the other cell particles were treated in the same way as were the chromaffin granules, but they contained only traces of lysolecithin. During isolation, the cell fractions were kept between 0° and 2° and enzymic formation of lysolecithin is therefore not likely. This conclusion is further supported by the observation that incubation of isolated granules at 37° did not increase their lysolecithin content. Lysolecithin could have been formed only between the death of the animal and homogenization of the gland. However, chromaffin granules isolated from rat adrenal glands (Winkler *et al.* 1966; 1967) and from three tumours of the human adrenal medulla (H. Blaschko, A. H. T. Robb-Smith, A. D. Smith & H. Winkler, unpublished observations) were rich in lysolecithin; the rat glands were placed in ice-cold 0·3 M-sucrose within 2 min. of death, and the tumours were also cooled immediately after removal. This makes it very unlikely that the high lysolecithin content of chromaffin granules is an artifact that arises *post mortem*. We therefore conclude that lysolecithin is a characteristic constituent of chromaffin granules.

The specific localization of lysolecithin in the particles that store the catecholamines raises the question whether the membrane-lytic properties of this substance have anything to do with the release of the hormones from the cell. Feldberg (1940) demonstrated that small amounts of lysolecithin cause a release of adrenaline from the cat adrenal gland, and both he and Hillarp & Nilson (1954*a*) have shown that lysolecithin will liberate catecholamines from what is now known to have been isolated chromaffin granules. These early observations do not provide evidence for a physiological role of lysolecithin but they do indicate how sensitive the chromaffin cell is to this compound.

Morphological studies (de Robertis & Vaz Ferreira, 1957; Coupland, 1965) and biochemical evidence (Banks & Helle, 1965; Douglas & Poisner, 1966; Kirshner, Sage, Smith & Kirshner, 1966) favour the idea that the secretion of catecholamines is preceded by fusion of the chromaffin granule membrane with the plasma membrane of the cell. If such a mechanism is operative then lysolecithin is the kind of compound that might well be involved. Either the formation of lysolecithin from lecithin, or the 'activation' of protein-bound lysolecithin, may precede the secretion of catecholamines, adenosine triphosphate and protein from the chromaffin cell. Suggestions have been made by Högberg & Uvnäs (1957) and by Keller (1962) that release of histamine from mast cells is brought about by the action of phospholipase A resulting in the formation of lysolecithin. Similarly, an activation of a phospholipase might be involved in secretion from the adrenal medulla. There is an old observation on the presence of phospholipase A in the bovine adrenal gland (Francioli, 1935). Recently this enzyme has been found in the bovine adrenal medulla (Winkler, Strieder & Ziegler, 1967*a*).

Further work is necessary to elucidate the possible

role of lysolecithin, and of enzymes concerned in its metabolism, in the function of the adrenal medulla.

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