The presence of lysophosphatidylcholine in chromaffin granules

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Lysophosphatidylcholine is thought to be a characteristic component of the chromaffin granules in adrenal glands. By the use of a t.l.c. system that resolves minor phospholipids satisfactorily, this subcellular location was confirmed in the present study in bovine glands. However, phospholipid degradation was demonstrated in homogenates of the adrenal medulla and cortex under conditions similar to those of subcellular fractionation (incubation at 4°C for 90 min). Phosphatidylethanolamine and cardiolipin were hydrolysed, but the concentration of lysophosphatidylcholine did not change, indicating that the latter was present in the medulla before this treatment. Attempts were made to decrease the time between death of the animal and the extraction of lipids. Lysophosphatidylcholine was easily demonstrable in lipid extracts of the dissected medulla and even in those of the whole bovine gland. For practical reasons it is not possible to decrease further the time lapse before extraction in the case of this animal. Adrenal glands were obtained from anaesthetized and untreated rabbits. These were frozen immediately in liquid N₂ and the lipids were extracted. In a control experiment, the glands from rabbit were dissected and treated in the same manner as with those of ox, and then the lipids were extracted. No lysophosphatidylcholine was detected in the extracts from glands frozen in liquid N₂ but lysophosphatidylcholine was observed in the controls. These results suggest that lysophosphatidylcholine is not a component of chromaffin granules, but is produced if the period between death of the animal and lipid extraction is unduly prolonged. To discover whether lysophosphatidylcholine affected the permeability barrier properties of chromaffin granules, sonicated liposomes of egg phosphatidylcholine alone or with lysophosphatidylcholine (15 mol/100 mol) were prepared. Both types were shown by electron microscopy to be largely made up of single bilayer vesicles. The exchange diffusion of [¹⁴C]dopamine was measured across their membranes. Both types of liposomes had similar capture volumes (0.5 µl/µmol of phospholipid), and the activation energies of the exchange diffusion of dopamine were also similar (31 kJ/mol). These results indicate that the presence of this proportion of lysophosphatidylcholine in chromaffin-granule membranes is not likely to influence their barrier properties towards catecholamines.

Chromaffin granules in the adrenal medulla contain the catecholamines adrenaline, noradrenaline and dopamine. Under the appropriate stimulus, the granules fuse with the cell-surface membrane releasing their contents outside. Lysophosphatidylcholine has been observed in chromaffin-granule membranes from various animals (up to 17% of the phospholipid in ox; Blaschko *et al.*, 1967; Trifaro, 1969; Dreyfus *et al.*,

1978; 11.3% in pig, 7% in horse, 15.4% in rat; Winkler *et al.*, 1967a; 11.7–23.8% in man, Blaschko *et al.*, 1968). This observation influenced subsequent research in two ways. First, many investigators suggested that lysophosphatidylcholine is intimately involved in the process of fusion (Rubin, 1967; Gutler & Clausen, 1969). For fusion to occur according to Lucy (1969), both membranes should have a relatively high proportion of their phospholipid molecules in the micromicellar configuration. Because of its 'wedge shape', it is thought that the presence of lysophosphatidylcholine will favour the formation of

Abbreviation used: Hepes 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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this configuration in the lipid leaflet. Fusion of the two membranes then occurs by interdigitation of the micelles. Poole *et al.* (1970) did succeed in fusing hen erythrocytes with high concentrations of lysophosphatidylcholine, although the cells quickly lysed. The 'fusogenic ability' of lysophosphatidylcholine has been demonstrated by other workers (Croce *et al.*, 1971; Keoy *et al.*, 1972).

The second influence was somewhat indirect. The synthesis of catecholamines involves the transportation of intermediates across the chromaffin-granule membrane (Kirshner, 1974). Studies of the transport properties of chromaffin granules were carried out with preparations that contained lysophosphatidylcholine in their membranes. No study was, however, carried out to investigate the effect of lysophosphatidylcholine on the barrier properties of the membrane to catecholamines.

The first aim of the present work was therefore to establish whether lysophosphatidylcholine is a true component of the chromaffin granule *in vivo*.

This was achieved by first confirming the presence of high concentrations of lysophosphatidylcholine in chromaffin granules under conditions adopted in previous studies. For this purpose, a reliable system for the extraction of lysophosphatidylcholine and its detection by t.l.c. was developed. A study of the conditions necessary for the production of lyso-compounds during post-homogenization manipulation was then carried out. The phospholipid compositions of adrenal glands from slaughterhouse material and freshly killed laboratory animals were compared.

The second aim was to discover whether the presence of high concentrations of lysophosphatidylcholine in membranes influences their permeability barrier properties. Liposomes of lysophosphatidylcholine in lecithin were used to study the exchange diffusion of catecholamines. Mixtures of lysophosphatidylcholine and phosphatidylcholine have been shown to exist as multibilayer structures up to a molar ratio of 1:1 (Mandersloot *et al.*, 1975), and if these are sonicated, a mixed population consisting mainly of single-bilayer structures results (Filgueiras *et al.*, 1977).

Methods

Homogenization of the adrenal glands

Bovine adrenal glands were obtained from the local abattoir and were kept on ice. Fat and connective tissue were quickly removed and the whole glands cross-sectioned. They were either used directly or the medulla was separated from the cortex depending on the experiment performed. The tissue was homogenized in 10 vol. of 0.3 M-sucrose (BDH, Poole, Dorset, U.K.)/25 mM-Tris (Sigma, St. Louis, MO U.S.A.) buffer, pH 7.4. In early experi-

ments, homogenization was carried out by crushing the tissue first in a little sand and then in a motor-driven loose-fitting Elvehjem-Potter homogenizer at 900 rev./min. Six up-and-down strokes were applied. A Silverston homogenizer was used in later experiments, in which case there was no need for prior crushing in sand.

Subcellular fractionation of adrenal medulla

Bovine adrenal medulla was fractionated into three fractions: crude chromaffin granules, mitochondria and supernatant, as described by Phillips (1973).

Lipid extraction of whole tissues

Adrenal glands from oxen or rabbits (New Zealand White females, 2–3 kg) were used. They were either ground in sand or homogenized in a Silverston homogenizer in the presence of chloroform/methanol (1:1, v/v). The lipids were extracted by the method of Folch *et al.* (1957), but partitioned in a solvent system of chloroform/methanol/1 M-HCl (4:2:3, by vol.). The lower chloroform layer was washed finally with 0.5 vol. of 40% methanol in water.

Lipid extraction of homogenates

Lipids were extracted from the homogenates by a modified Bligh & Dyer (1959) method. The proportions of chloroform/methanol/water were changed to give a final ratio of 4:2:3 (by vol.). This gives a quantitative partition of lipids to the organic phase (Sheltawy & Dawson, 1969). To 16 ml of the homogenate, 20 ml of chloroform and 40 ml of methanol were added and shaken for 2 min. A further 60 ml of chloroform and 44 ml of 1 M-HCl were added successively and shaken for 30 s. The upper phase was removed and the lower phase was washed with an equal volume of 40% methanol in water. It is important that this procedure is carried out at temperatures not exceeding room temperature or else degradation of vinyl ether linkages in phospholipids may occur. Subcellular fractions were extracted by the method of Eichberg *et al.* (1964).

T.l.c. of phospholipids

These were fractionated on thin layers (0.5 mm thick for up to 70 µg of lipid phosphorus, and 1 mm thick for 70–120 µg of lipid phosphorus) of Silica gel H (type 60; Merck, Darmstadt, Germany). The suspensions were made in 1% (w/v) potassium oxalate to complex free Ca²⁺. Addition of 0.5–1% (w/v) starch to the suspensions prevented thin layers from flaking off plates and made handling less hazardous. This did not affect the resolution or estimation of phospholipids. Plates were activated for 75 min at 110°C before use. The following

Fig. 1. Two-dimensional t.l.c. of phospholipids

The Figure illustrates the pattern obtained after two-dimensional t.l.c. of the phospholipids listed below. Phospholipids were extracted as described in the Methods section. Developing solvents used were chloroform/methanol/water/acetic acid (65:45:5:0.5, by vol.) in the first dimension and chloroform/methanol/water/15M-ammonia (65:50:4:11, by vol.) in the second. Between developments the plates were dried as described in the text. Abbreviations used: PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; CL, cardiolipin.

combination of solvents was found to give a good resolution of phospholipids including minor and lyso-derivatives (Fig. 1). Chloroform/methanol/water/acetic acid (65:45:4:0.5, by vol.) was used in the first dimension, and chloroform/methanol/water/15M-ammonia (65:50:4:11, by vol.) in the second. Chromatography was carried out at 4°C. After development in the first direction, the plates were dried under reduced pressure and in an atmosphere of N₂ for 90–120 min. Reference phospholipids were chromatographed under the same conditions.

The lipid spots were detected with a spray of Rhodamine 6G (0.2%, w/v) in ethanol/15M-ammonia (99:1, v/v) followed by one of water, and viewed under u.v. light within minutes. Choline phospholipids appeared bright yellow, ethanolamine phospholipids, bluish, and the rest brownish in colour. These differences faded with time. Sprays for amino groups and lipid phosphorus (in this order) were used to aid the identification of phospholipids (Sheltawy & Dawson, 1969a). To confirm the

identity of the lysophosphatidylcholine spot, the spray of Dragendorff (Wagner *et al.*, 1961) was modified to give reproducible and clear detection of choline phospholipids. The solutions 17% (w/v) BiNO₃ in aq. 20% (v/v) acetic acid, and 0.29% (w/v) KI were mixed in the ratio 2:7 (v/v) immediately before use. Choline phospholipids appeared as yellow spots on a grey background.

Determination of lipid phosphorus

This was carried out on lipid samples or spots from thin-layer plates by the method of Bartlett (1959).

Preparation of liposomes

Two types of liposomes were prepared under identical conditions. One consisted of egg phosphatidylcholine (Lipid Products, South Nutfield, Surrey, U.K.), and the other of lyso-phosphatidylcholine (Lipid Products)/phosphatidylcholine (3:17, mol/mol). The method used was similar to that of Brammer & Sheltawy (1975). A total of 4 μmol of phospholipid was dispersed by sonication (water bath, KS 100; Kerry Ultrasonic) for 1 h in 0.75 ml of 'exchange medium'. This was made up of 5 mM-dopamine hydrochloride (BDH) with a specific radioactivity of 1 μCi/mol {3,4-Dihydroxyphenyl}[2-¹⁴C]ethylamine (60 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.), 145 mM-Hepes (Sigma) buffer, pH 7.4, and 0.25% ascorbic acid (BDH). Hepes was used as a buffer, since Tris was observed to catalyse oxidation of dopamine, and ascorbic acid was added to limit this process further. Failure to do this resulted in an orange solution, which when used in exchange-diffusion experiments gave anomalous results. To prevent excessive heating during sonication, the water in the bath was changed every 15 min.

Measurement of diffusion rates of dopamine across liposomal membranes

The liposomes were separated from the external radioactively labelled dopamine by passage through a Sephadex G-50 (coarse; Pharmacia, Uppsala, Sweden) column (16 cm length × 2 cm internal diameter) packed in 'exchange medium', but with unlabelled dopamine. An oxygen trap containing 15% (w/v) pyrogallol (BDH) was attached to the column to prevent oxidation of the dopamine by atmospheric O₂. Chromatography was carried out at 4°C. The radioactivity (LS 200 or LS 230 Beckman Scintillation Counter), and lipid phosphorus were monitored in collected fractions. Each type of liposome (phosphatidylcholine alone or lysophosphatidylcholine/phosphatidylcholine) was completely separated from the external radioactivity. The symmetrical peak of lipid phosphorus coincided well

with a peak of radioactive dopamine that represented the internal pool. The pooled peak measured approx. 6ml and portions were taken for final determination of radioactivity and of lipid phosphorus (Bartlett, 1959).

A portion of the liposomal suspension (4ml) was measured into dialysis bags (diameter 0.125 inches or 3mm), which were tightly sealed and transferred to stoppered tubes containing 20ml of non-radioactive 'exchange medium' previously equilibrated at the required temperature. The tubes were mechanically shaken and 2ml portions transferred at known intervals into scintillation vials. Scintillation fluid [10ml of 0.04% 2,5-diphenyloxazole in Triton X-100 (scintillation grade; BDH)/toluene (2:1, v/v)] was added and the radioactivity was counted for 20min.

Determination of the activation energy of exchange diffusion

By measuring the rates of exchange-diffusion at different temperatures, the activation energy may be calculated by using the Arrhenius equation $P = Ae^{-E_a/RT}$, where P is the rate of exchange diffusion, E_a the activation energy, A a constant, T the absolute temperature and R the gas constant.

Results

Quantitative extraction of lysophosphatidylcholine

Because lysophosphatidylcholine contains only one fatty acid per molecule it is relatively more polar than phosphatidylcholine. Thus it is essential to ensure that large amounts are not distributed into the upper aqueous phase during the extraction procedures (see the Methods section). To investigate this, the following experiments were carried out. A known amount of egg lysophosphatidylcholine was measured into a flask and the solvents evaporated. Chloroform, methanol and 1 M-HCl were then added with shaking to give a final ratio of 4:2:3 (by vol.) as in the modified procedure of Bligh & Dyer (1959) described in the Methods section. The distribution of lysophosphatidylcholine in the two phases was examined by t.l.c. (see the Methods section). A reference sample of lysophosphatidylcholine was chromatographed under the same conditions.

To determine the effect of homogenate proteins on the distribution of lysophosphatidylcholine in this biphasic system, the above experiment was repeated in the presence of a known amount of homogenized bovine adrenal gland, whose total content of lipid phosphorus was known. All the values reported below are means for two experiments agreeing within 2–3%. All analyses were in duplicate.

In the absence of the adrenal homogenate, 97% of the lysophosphatidylcholine was recovered in the lower organic phase, with only 3% in the upper

aqueous phase. In the presence of proteins from the homogenate, no phospholipids were detected in the upper phase. The recovery of phospholipids (exogenous lysophosphatidylcholine plus phospholipids from the tissue) was 96% of the amount added. The quantity of lysophosphatidylcholine in the lower phase was determined by two-dimensional t.l.c. and, after making corrections for lysophosphatidylcholine contributed by the homogenate, the recovery of exogenous lysophosphatidylcholine was 98% of the amount added.

These experiments demonstrate that the present modification of the method of Bligh & Dyer (see the Methods section) results in almost complete recovery of lysophosphatidylcholine in the chloroform layer together with other phospholipids.

Phospholipid composition of subcellular fractions of bovine adrenal medulla

Bovine adrenal medulla was fractionated into crude chromaffin granules, mitochondria and supernatant as described by Phillips (1973). The results obtained confirmed previous observations that lysophosphatidylcholine is confined to the chromaffin-granule fraction (Trifaro, 1969; Blaschko *et al.*, 1967; Dreyfus *et al.*, 1978). Lysophosphatidylcholine made up 13% of chromaffin granule phospholipid phosphorus. The other subcellular fractions contained small amounts of lysophosphatidylcholine, but these may have been due to contamination with the granules or to the type of general degradation described later. As expected, mitochondria were enriched in cardiolipin (6% of lipid phosphorus) and the supernatant contained none.

Phospholipid composition of bovine adrenal medulla and cortex

To study the degradation of phospholipids during post-homogenization manipulation, the following experiments were carried out on homogenates of the bovine adrenal medulla or cortex, and not their subcellular fractions. The control experiment involved immediate extraction of the tissue with organic solvents as described in the Methods section. This is expected to denature most enzymes and the phospholipid composition (Table 1a) would be that of the tissue before homogenization in buffer. Homogenates (10%, w/v) of the medulla or cortex were prepared in sucrose/Tris buffer and incubated for 90min at 4°C, conditions similar to those encountered during subcellular fractionation. These were then extracted by the modified method of Bligh & Dyer (see the Methods section), and the phospholipid composition shown in Table 1(b).

In the control experiments, the cortex was found to be richer in phospholipids (1.3 ± 0.1) than the medulla (0.83 ± 0.1 mg of lipid phosphorus/g fresh

Table 1. *Phospholipid composition of bovine adrenal medulla and cortex*

Lipids were extracted and analysed by two-dimensional t.l.c. as described in the Methods section. Values represent means $\pm$ S.D. for three experiments.

Phospholipid	Medulla		Cortex	
	(μ g of phosphorus/g of wet tissue)	(% of total lipid phosphorus recovered)	(μ g of phosphorus/g of wet tissue)	(% of total lipid phosphorus recovered)
(a) Tissues extracted immediately				
Phosphatidylcholine	348 $\pm$ 22	39.7 $\pm$ 1	673 $\pm$ 13	51 $\pm$ 4
Lysophosphatidylcholine	54 $\pm$ 14	6.2 $\pm$ 2	None	None
Phosphatidylethanolamine	216 $\pm$ 61	24 $\pm$ 6	384 $\pm$ 56	29 $\pm$ 2
Lysophosphatidylethanolamine	None	None	None	None
Cardiolipin	22 $\pm$ 10	2.6 $\pm$ 1	70 $\pm$ 27	5 $\pm$ 2
Phosphatidylserine	55 $\pm$ 6	6.1 $\pm$ 1.2	44 $\pm$ 3	3.4 $\pm$ 0.6
Sphingomyelin	91 $\pm$ 10	11 $\pm$ 1.5	63 $\pm$ 5	5.0 $\pm$ 1.3
*Phosphatidylinositol + phosphatidic acid	93 $\pm$ 25	10.5 $\pm$ 3	83 $\pm$ 9	5.5 $\pm$ 1
(b) Tissues homogenized and incubated for 90 min at 4°C				
Phosphatidylcholine	339 $\pm$ 10	39.5 $\pm$ 1.5	601 $\pm$ 20	50 $\pm$ 1
Lysophosphatidylcholine	47 $\pm$ 8	5.4 $\pm$ 1	25 $\pm$ 9	2 $\pm$ 0.7
Phosphatidylethanolamine	208 $\pm$ 35	24 $\pm$ 4	310 $\pm$ 15	26 $\pm$ 0.5
Lysophosphatidylethanolamine	44 $\pm$ 16	5 $\pm$ 2	82 $\pm$ 28	7 $\pm$ 3
Cardiolipin	8 $\pm$ 4	1 $\pm$ 0.4	23 $\pm$ 21	3 $\pm$ 1
Phosphatidylserine	64 $\pm$ 8	7.8 $\pm$ 1	47 $\pm$ 5	4 $\pm$ 1
Sphingomyelin	104 $\pm$ 13	11.6 $\pm$ 2	71 $\pm$ 8	5.8 $\pm$ 1.6
*Phosphatidylinositol + phosphatidic acid	46 $\pm$ 15	6 $\pm$ 2	54 $\pm$ 21	4 $\pm$ 1

* Although these phospholipids were almost completely resolved in this two-dimensional t.l.c. system, their distinction was not always consistent. They were combined and analysed together.

weight; these results represent means $\pm$ S.D. for seven experiments). Lysophosphatidylcholine was confined exclusively to the medulla and its proportion did not vary with incubation (Table 1). This confirmed that lysophosphatidylcholine is indeed a constituent of the medulla from bovine adrenal glands obtained freshly from the abattoir.

When the tissues were homogenized and incubated at 4°C, general hydrolysis of phospholipids was observed and a small amount of lysophosphatidylcholine even appeared in the cortex. Other phospholipids were hydrolysed on incubation leading to the appearance of the corresponding lyso-compounds. The proportion of phosphatidylethanolamine was decreased and the lyso-compound was detected (Table 1b). The proportion of cardiolipin was similarly decreased and in some experiments (both medulla and cortex) this phospholipid was almost completely hydrolysed. Simultaneously a new spot, presumably its degradation product, was observed.

Phospholipid composition of bovine adrenal glands

Although lysophosphatidylcholine was conclusively demonstrated in bovine adrenal medulla and its chromaffin granules, it may well be produced

during the period of tissue dissection. To eliminate this, the above experiments described for the medulla were repeated with whole adrenal glands (Table 2).

Lysophosphatidylcholine was again observed in the whole gland and can all be attributed to the medulla content. This experiment shows that lysophosphatidylcholine may be easily detected in the whole tissue by the t.l.c. system developed in the present study. When the tissue was homogenized and incubated at 4°C, general hydrolysis of phospholipids was again observed, but the lysophosphatidylcholine concentration did not change. This is similar to the results obtained with the medulla.

Phospholipid composition of rabbit adrenals

Adrenal glands in abundant supply were usually obtained from the local abattoir; this practice is necessary to obtain a reasonable yield of chromaffin granules. An indeterminate period (30–60 min) occurs between the killing of the animal and the acquisition of tissue for experimentation. Although laboratory animals do not yield enough material to be used as a routine source of this subcellular fraction, they do offer the opportunity of obtaining adrenals immediately after death. Experiments in

Table 2. *Phospholipid composition of bovine adrenal glands*

The glands were extracted immediately with organic solvents or after incubation of homogenates at 4°C for 90 min. T.l.c. was carried out as described in the Methods section. Values represent means $\pm$ S.D. for five experiments

	Lipid extracted immediately		Lipid extracted after incubation	
	(μg of phosphorus/g of wet tissue)	(% of total lipid phosphorus recovered)	(μg of phosphorus/g of wet tissue)	(% of total lipid phosphorus recovered)
Phosphatidylcholine	473 $\pm$ 19	43 $\pm$ 1	418 $\pm$ 5	43 $\pm$ 1.5
Lysophosphatidylcholine	27 $\pm$ 4	2.5 $\pm$ 0.6	21.1 $\pm$ 5	2.2 $\pm$ 0.2
Phosphatidylethanolamine	360 $\pm$ 17	32.5 $\pm$ 1	282 $\pm$ 15	29.4 $\pm$ 1
Lysophosphatidylethanolamine	None	None	36.6 $\pm$ 15	3.7 $\pm$ 1
Cardiolipin	69.5 $\pm$ 6	6.3 $\pm$ 1	45.4 $\pm$ 0.2	4.7 $\pm$ 0.7
Phosphatidylserine	44.0 $\pm$ 3	4.0 $\pm$ 0.5	44.4 $\pm$ 4	4.6 $\pm$ 0.2
Sphingomyelin	63 $\pm$ 4	5.7 $\pm$ 0.6	63 $\pm$ 5	6.5 $\pm$ 0.5
*Phosphatidylinositol + phosphatidic acid	72 $\pm$ 8	6.5 $\pm$ 0.6	62.5 $\pm$ 16	6.4 $\pm$ 0.9

* See Table 1.

Table 3. *Phospholipid composition of rabbit adrenal glands*

Rabbit adrenals were removed from anaesthetized and non-anaesthetized animals and frozen immediately in liquid N₂. In the case of control animals the glands were dissected 30 min after death and left on ice for a further 30 min, as for slaughterhouse material. Lipids were extracted from both sets of glands and analysed as described in the Methods section. Values are means $\pm$ S.D. for three different experiments.

Phospholipid	Glands frozen immediately in liquid N ₂		Glands collected as for slaughterhouse material	
	(μg of phosphorus/g of wet tissue)	(% of total lipid phosphorus recovered)	(μg of phosphorus/g of wet tissue)	(% of total lipid phosphorus recovered)
Phosphatidylcholine	756 $\pm$ 15	51 $\pm$ 1	737 $\pm$ 58	50 $\pm$ 1
Lysophosphatidylcholine	None	None	18 $\pm$ 1	1.2 $\pm$ 0.2
Phosphatidylethanolamine	411 $\pm$ 10	28 $\pm$ 0.5	369 $\pm$ 13	25 $\pm$ 1
Lysophosphatidylethanolamine	None	None	30 $\pm$ 6	2 $\pm$ 0.4
Cardiolipin	45 $\pm$ 4	3 $\pm$ 0.3	49 $\pm$ 7	3.3 $\pm$ 0.6
Phosphatidylserine	48 $\pm$ 5	3 $\pm$ 0.3	47 $\pm$ 3	3.2 $\pm$ 0.1
Sphingomyelin	80 $\pm$ 8	5 $\pm$ 0.5	72 $\pm$ 3	5 $\pm$ 0.5
*Phosphatidylinositol + phosphatidic acid	134 $\pm$ 9	9 $\pm$ 1	160 $\pm$ 19	11 $\pm$ 1

* See Table 1.

Table 2 show that this type of analysis is possible, since lysophosphatidylcholine from chromaffin granules is easily detected in the whole gland (2.5% of total lipid phosphorus).

Whole adrenals were obtained from one rabbit, anaesthetized with a calculated dose of Sagatal (May and Baker, Dagenham, Essex, U.K.) injected intravenously. The adrenal glands were immediately frozen in liquid N₂. Two other animals were killed by a blow on the head and similarly treated. The glands were crushed to a fine powder and the lipids extracted and analysed as described in the Methods section. Control animals were killed, and as in the case of slaughterhouse material, the glands were collected 30 min after death of the animal and put on

ice for another 30 min before the lipids were extracted.

No lysophosphatidylcholine was detected in the lipid extracts of glands that were removed immediately and frozen in liquid N₂ and no difference was due to anaesthetic treatment (Table 3). On the other hand, lysophosphatidylcholine and lysophosphatidylethanolamine appeared in the extracts from the control animals, but apart from this, the phospholipid composition of the glands of both the experimental and control groups was identical and similar to that obtained from slaughterhouse oxen. Each thin-layer plate was loaded with more than 60 μg of whole-gland lipid phosphorus and even when loaded with 120 μg , lysophosphatidylcholine

EXPLANATION OF PLATE 1

Electron micrograph of phosphatidylcholine vesicles

Phosphatidylcholine ($4\mu\text{mol}$) was dispersed in 0.75 ml of buffer containing dopamine hydrochloride, sonicated for 1 h as described in the Methods section, and negatively stained. The bar represents 100 nm.

EXPLANATION OF PLATE 2

Electron micrograph of phosphatidylcholine/lysophosphatidylcholine vesicles

A mixture of phosphatidylcholine and lysophosphatidylcholine (17:3 molar ratio; total amount $4\mu\text{mol}$) was dispersed in buffer containing dopamine hydrochloride, sonicated for 1 h as described in the Methods section, and negatively stained. The bar represents 100 nm.

was still not detected in the extracts from the glands frozen in liquid N₂. When an external source of lysophosphatidylcholine (from egg) was included in one extract, this was completely resolved as demonstrated above (Fig. 1) and the recovery was 99% of that loaded.

This experiment conclusively demonstrates that lysophosphatidylcholine is produced in adrenals if delay is experienced after killing the animal or dissection of the tissues, as is inevitable in slaughterhouse material.

The exchange diffusion of liposomal dopamine

To investigate the effect of lysophosphatidylcholine on the permeability properties of liposomes, two types were compared. These were made up of phosphatidylcholine and lysophosphatidylcholine/phosphatidylcholine (see the Methods section), and contained radioactive dopamine inside. The exchange diffusion was measured against the same concentration of non-radioactive dopamine as described in the Methods section.

Table 4 compares the capture of dopamine by the two types of liposomes used. The phosphatidylcholine vesicles appeared to have a slightly larger capture volume than those containing lysophosphatidylcholine, but this was not statistically significant (Student's *t* test, *P* > 0.5). Assuming uniform lamellar spacing and the same radioactive compound used, capture volume can be taken as an indication of the size of the vesicles (Papahadjopoulos & Watkin, 1967). Hence the phosphatidylcholine and lysophosphatidylcholine/phosphatidylcholine liposomes may be considered of similar

average sizes. Negative-staining electron microscopy (Plates 1 and 2) of the two types of liposomes showed them to be intact and of two size ranges (uni- or multi-lamellar).

The exchange diffusion of dopamine out of the vesicles was followed for 1 h. In all cases the initial rate of release was constant up to 30 min, indicating that the liposomes used were mostly of the single bilayer type. This is confirmed by electron microscopy (Plates 1 and 2). Exchange diffusion from multi-bilayer structures is of course complicated by the large number of compartments involved. The rates of exchange diffusion of dopamine from liposomes with or without lysophosphatidylcholine were similar (Table 4).

Activation energy of the exchange diffusion

As might be expected, increase in temperature increased the rate of diffusion. The activation energies for the two liposomes were similar and the values obtained were 30 and 32 kJ/mol for phosphatidylcholine and lysophosphatidylcholine/phosphatidylcholine liposomes respectively. In the temperature range examined (22–48°C), it is not expected to observe a change in activation energy for either type of liposomes (with phospholipids of a heterogeneous fatty-acid composition) and indeed none was observed.

Importance of ascorbic acid in the medium containing dopamine

Dopamine solutions are easily oxidized especially in the alkaline range. Ascorbic acid is added to prevent this process, and experiments carried out in

Table 4. *The rates and activation energies for the diffusion of dopamine across phosphatidylcholine and lysophosphatidylcholine/phosphatidylcholine liposomes*

Liposomes composed purely of phosphatidylcholine and of phosphatidylcholine/lysophosphatidylcholine (17:3, mol/mol) containing radioactive dopamine were prepared as described in the Methods section. The diffusion rates of radioactive dopamine and activation energies were compared (see the Methods section). Values represent means for two closely agreeing experiments. Results for capture are averages at different temperatures ± s.d.

Phospholipid composition	Temperature (°C)	Capture (μl/10 μmol)	Diffusion rate (% diffused/min)	Activation energy (kJ/mol)
Phosphatidylcholine	22.5	5.4 ± 0.6	0.16	30
	28		0.25	
	31.5		0.24	
	37		0.30	
	40.5		0.40	
	48		0.42	
Phosphatidylcholine/ lysophosphatidylcholine (17:3 mol/mol)	22.5	4.6 ± 0.6	0.14	32
	28		0.20	
	31.5		0.22	
	37		0.34	
	40.5		0.36	
	48		0.45	

its absence gave anomalous results. By measuring the radioactivity of dopamine solutions, it is possible to calculate the capture volume of the liposomes. This was apparently greater for those containing oxidized dopamine than for the 'fresh' (2.1 and 2.5 times for phosphatidylcholine and lysophosphatidylcholine/phosphatidylcholine liposomes respectively). By contrast the diffusion rates were much less (e.g. at 37°C rates were three times less) and the activation energies higher (1.5 times higher). These results pointed to a tightly bound pool of oxidized dopamine that does not exchange readily.

Discussion

Previous reports (see Winkler, 1976) indicated that chromaffin granules from the adrenal medulla of various animals are atypical among subcellular fractions in containing high concentrations of lysophosphatidylcholine. We have confirmed this for ox chromaffin granules. With a two-dimensional t.l.c. system, we have also demonstrated that this component of the chromaffin granule is easily detected in lipid extracts of the medulla and even in those of the whole gland. Homogenates of whole glands were therefore used to study the possibility of hydrolysis of endogenous phospholipids during manipulations at 4°C. Not unexpectedly, general hydrolysis was observed even at this temperature (Tables 1 and 2), and for times usually required for subcellular fractionation (90 min). Phosphatidylethanolamine and cardiolipin were degraded and their products detected. The content of lysophosphatidylcholine remained constant.

Although Smith & Winkler (1968) demonstrated that adrenal lysosomes contain soluble phospholipases of the A₁ and A₂ types, they argued (Blaschko *et al.*, 1967) that these were unlikely to affect the endogenous phospholipids at the low temperatures of subcellular fractionation (4°C). They may have been justified in their conclusions by two coincidences. On the one hand, many t.l.c. systems do not resolve lysophosphatidylethanolamine from phosphatidylserine, or cardiolipin breakdown products from lysophosphatidylcholine or sphingomyelin, and these degradation products may have thus escaped undetected. On the other hand, as we also demonstrated, no increase in the proportion of lysophosphatidylcholine on incubation of homogenates at 4°C was observed (Blaschko *et al.*, 1967). It was therefore believed for a long time that lysophosphatidylcholine was a genuine constituent of chromaffin granules from bovine adrenals.

There was one possibility still to be investigated, however. Considering the constancy of phospholipid composition in similar membranes from different species, it is justifiable to ask whether

lysophosphatidylcholine is also present in adrenals freshly obtained from small laboratory animals. The results of experiments in the present work (Table 3) conclusively demonstrate that this phospholipid is absent from, or undetectable in, rabbit adrenals (t.l.c. loading of up to 120 µg of lipid phosphorus). When the rabbit glands were treated in a manner similar to that of the bovine glands, lysophosphatidylcholine was easily detected in the tissue. The phospholipid composition of the two animals is similar, confirming the dogma of interspecies constancy described above.

Since chromaffin granules are the main source of this phospholipid in extracts of whole adrenals (see the Results section and Table 2), we conclude by implication that lysophosphatidylcholine is not a true constituent of these granules. It is produced *post mortem*, but conclusive proof will only be obtained on complete inhibition of the hydrolytic activity. Two difficulties arise in this field. (1) Lysophosphatidylcholine will have already formed in the adrenals on acquisition of the tissue or formed during the dissection and fractionation. (2) Phospholipases are somewhat difficult to inhibit specifically.

Meldolesi *et al.* (1971) also observed rapid hydrolysis of phospholipids in pancreas even at 4°C. Polyphosphoinositides in brain are similarly hydrolysed rapidly *post mortem* (Sheltawy & Dawson, 1969b), and it was only after inhibition of the phosphomonoesterases (Hauser & Eichberg, 1973) that their subcellular distribution in the brain was truly understood.

Lysophosphatidylcholine was postulated to play an important role in the fusion of chromaffin granules with the cell-surface membrane (Lucy, 1969). We cannot on the basis of our present evidence concur with this view. Indeed, doubts as to lysophosphatidylcholine playing a general role in fusion (De Robertis & Vaz Ferreira, 1957; Schneider *et al.*, 1967; Diner, 1967; Douglas, 1968) are enhanced by its low concentrations in the various secretory vesicles examined: 1.5% in brain synaptic vesicles (Breckenridge *et al.*, 1973), none in 5-hydroxytryptamine vesicles of rabbit blood platelets (Da Prada *et al.*, 1972) and 1% in neurosecretory vesicles from the bovine posterior hypophysis (Vilhardt & Holmer, 1972). Meldolesi *et al.* (1971) observed 9.1% lysophosphatidylcholine in the zymogen granules from guinea-pig pancreas, but they showed that this was due to enzymic breakdown during the homogenization and fractionation procedures. Preliminary observations in this laboratory indicate that if delay is avoided in obtaining the parotid, pituitary, thyroid and pancreas from rabbits, and if the glands are treated in a similar fashion to the rabbit adrenals, lysophospholipids are not observed.

Because chromaffin granules *in vivo* do not

appear to have any lysophosphatidylcholine (Table 3), it was of interest to find out if its presence in the membrane affects the diffusion of catecholamines across them.

The concentration of lysophosphatidylcholine incorporated into our liposomes (15 mol/100 mol of phospholipid) was selected for two reasons. (i) The detected concentration of lysophosphatidylcholine in chromaffin granules is around 15% of the total phospholipids. (ii) It has been demonstrated by negative-staining electron microscopy (Inoue *et al.*, 1977), light-scattering birefringence, and X-ray studies (Mandersloot *et al.*, 1975) that mixtures of lysophosphatidylcholine and egg phosphatidylcholine up to a molar ratio of 1:1 consist of intact bilayers.

Results of the diffusion studies showed that both liposome preparations had intact vesicles of mixed population sizes consisting mainly of single bilayer structures (Figs. 3a and 3b), thus confirming the observations mentioned above. The two preparations showed no differences in the diffusion rates of dopamine or the activation energies of the process (Table 4). Mandersloot *et al.* (1975) reported no differences in the rate of leakage of KCNS for preparations of up to 22.5% lysophosphatidylcholine in phosphatidylcholine, whereas Kitagawa *et al.* (1976) reported an increase in glucose permeability with increase in lysophosphatidylcholine content.

The dopamine molecule may be calculated to have a charge of +0.94 at pH 7.4. The activation energy was found to be 31 kJ/mol. Papahadjopoulos & Watkins (1967) obtained a value of 62 kJ/mol for the activation energy of the exchange diffusion of K⁺. It would thus appear that the presence of a hydrophobic ring over-compensates for the larger molecular size of the dopamine. This is well within theoretical expectations of lipid-bilayer membranes (Stein, 1967).

We conclude, therefore, that the presence of lysophosphatidylcholine in chromaffin-granule membranes is not likely to affect the passive diffusion of dopamine and their properties may thus be unimpaired. However, it is not known at present whether these 'artificial' lyso-derivatives affect the conformation and hence the function of membrane proteins, not least of which are those involved in transport.

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