Studies on Phospholipids

8. PHOSPHOLIPIDS IN RAT-LIVER MITOCHONDRIA AND MICROSOMES

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(Received 21 September 1960)

Work by Marinetti, Erbland & Stotz (1958) on pig-heart-cell fractions and by Macfarlane, Gray & Wheeldon (1960) on rat-liver-cell fractions have indicated that the mitochondrial and microsomal phospholipids may differ significantly in composition. In view of the results obtained by Collins (1960) and by Collins & Shotlander (1961), showing the widespread occurrence of complex amino-phospholipids, which have not been detected by other workers, it was important to examine the phospholipids in liver-cell fractions with the procedures described by Collins & Shotlander (1961).

EXPERIMENTAL

Preparation of the lipids of rat-liver-cell components. The livers of three male rats (Wistar strain, body wt. 200–250 g.) were treated as described by Hogeboom (1955) and a nuclear, mitochondrial, microsomal and a supernatant fraction were obtained. The lipids were extracted from these fractions and washed with acid as described by Wheeldon & Collins (1957).

Fractionation of phospholipid derivatives. The phospholipids of the mitochondria and microsomes were treated and distributed in the same way as described for whole rat-tissue lipids by Collins & Shotlander (1961). After each distribution the contents of the first and every fifth tube were diluted to 50 ml., by the addition of ethanol, and phosphorus and dinitrophenyl groups were determined.

The contents of tubes 1–35 from the distribution of the acetone-soluble fraction were combined and redistributed in *n*-heptane-methanol for 120 transfers. After this distribution the contents of the first and every fifth tube were diluted to 50 ml. with chloroform. Analyses were made for phosphorus and for dinitrophenyl groups with a value of ϵ_{max} of 16 000 at 340 m μ .

Cardiolipin. A sample (3 mg.) of cardiolipin (obtained from Dr P. Plackett, C.S.I.R.O. Division of Animal Health, Melbourne) was washed with acid and dinitrophenylated and methylated as described above for the rat-liver lipids. The product was completely soluble in acetone, contained no dinitrophenyl groups and was distributed for 120 transfers in *n*-heptane-methanol.

Lysolecithin. A sample (3 mg.) of lysolecithin (obtained from Miss H. Doery, Commonwealth Serum Laboratories, Melbourne) was washed with acid, dinitrophenylated and methylated. The product was completely acetone-insoluble, contained no dinitrophenyl groups and was distributed for 120 transfers in carbon tetrachloride-chloroform-methylene chloride-methanol-water (25:15:10:40:10, by vol.).

Table 1. *Phospholipids of rat-liver-cell fractions*

Four rats (285–295 g. body wt.) yielded livers weighing a total of 42.7 g. Cell fractions were prepared and the lipid contents are shown below.

	Lipid (mg.)	Phosphorus (% of lipid)	Lipid (% of fresh wt. of liver)
Nuclear fraction	474	2.9	1.1
Mitochondria	286	3.5	0.7
Microsomes	529	3.2	1.2
Supernatant	138	0.8	0.3
Total	1427		3.3

Analytical procedure. The analytical procedures for phosphorus and dinitrophenyl groups are those described by Collins (1959).

Solvents. These were redistilled and care was taken that the solvent mixtures used for countercurrent distribution were neutral (Collins & Shotlander, 1961).

RESULTS

Table 1 shows the proportion of total lipid and lipid phosphorus in rat-liver-cell fractions. The lipids from the nuclear fraction were not further investigated as this cell fraction was contaminated with non-nuclear material, and there was insufficient for the study of the lipids in the supernatant fraction.

The distribution of the phospholipids from mitochondria and microsomes was similar to those obtained for whole rat-liver lipids (Collins & Shotlander, 1961) but certain differences between the two cell fractions were noted.

The distribution of the acetone-soluble portions (Fig. 1) of mitochondrial lipids shows a peak at tube 5, which is not apparent in the microsomal distribution, whereas the main peak in the complex phospholipid fraction is at tube 81 for mitochondria and at tube 70 for microsomes. The position of the maximum in the distribution of the phosphatidylethanolamine and phosphatidylserine derivatives is at tube 17 in both cases, which demonstrates that the other differences are not due to small variations in the composition of the solvent systems or to temperature effects.

The contents of tubes 1-35 were combined and redistributed in *n*-heptane-methanol (Fig. 2). The phosphatidylethanolamine and phosphatidylserine were maximal at tube 38 in both cases. The two distributions differed mainly in the amount of lipid phosphorus maximal at tube 95; there was little or no lipid phosphorus in this region in the microsome lipids but substantial amounts with the mitochondrial lipids.

When a sample of cardiolipin was dinitrophenylated and methylated and then distributed in heptane-methanol the material was maximal at tube 89. Hence it may be concluded that the material present in mitochondrial lipids, but present only in trace amounts in microsomes, is probably cardiolipin.

A comparison of the distribution of the acetone-insoluble lipids is shown in Fig. 3. Here there are differences in the minor phospholipids (tubes 40-120) but their importance is difficult to assess owing to the small amounts of material involved. In both cases the main peak, due to lecithin (Collins & Shotlander, 1961), is at the same position. After treatment with 1-fluoro-2:4-dinitrobenzene, and

with diazomethane, lysolecithin was insoluble in acetone and, on distribution in carbon tetrachloride-chloroform-methylene chloride-methanol-water, occurred maximally at tube 85, as shown in Fig. 3 (b).

The proportions of the various fractions were computed from the area under the curves (Collins & Shotlander, 1961) and the results are shown in Table 2.

DISCUSSION

Marinetti *et al.* (1958), in a study of the phospholipids of pig-heart-cell fractions, showed the presence of lysolecithin in the microsomes, although this material was not detected in the mitochondria and Macfarlane *et al.* (1960) failed to detect it in rat-liver-cell fractions. Marinetti *et al.* (1958) obtained lysolecithin in the proportion of 8% of the microsomal-lipid phosphorus, and in Fig. 3 the broken line represents distribution which would be expected if 8% of the lipid phosphorus had been lysolecithin. The experimentally determined curve has no peak in the precise position of lysolecithin

Fig. 1. Countercurrent distribution of the acetone-soluble-phospholipid derivatives in carbon tetrachloride-methanol-water. Every fifth tube was analysed for phosphorus (—) and dinitrophenyl groups (----). (a) Mitochondrial lipids (160 $\mu\text{g. atoms of phosphorus}$); (b) microsomal lipids (227 $\mu\text{g. atoms of phosphorus}$).

Fig. 2. Countercurrent distribution of the contents of tubes 1-35 of the distribution shown in Fig. 1. The solvent mixture was *n*-heptane-methanol and every fifth tube was analysed for phosphorus (—) and dinitrophenyl groups (----). (a) Mitochondrial lipids; (b) microsomal lipids. Results have been corrected for the material used in the analyses shown in Fig. 1.

and even if the small peak at tube 80 were lysolecithin it would represent no more than 0.5% of the total lipid phosphorus. The present work therefore does not support claims for the occurrence of

lysolecithin in microsomal lipids although species differences cannot be ruled out.

Collins (1959) found that three compounds occurred in small amounts in rat-liver lipids which resembled phosphatidic acid in that they contained neither choline nor amino bases. It was shown that one of these compounds (fraction Y) had a high radioactivity after incorporation of inorganic ^{32}P *in vitro*, but had a fatty acid to phosphorus molar ratio of 4. Hokin & Hokin (1958) and Hübscher & Clark (1960) found phosphatidic acid in animal tissues, although in amounts of less than 1%. Marinetti, Erbland, Albrecht & Stotz (1957) also presented evidence for the occurrence of a phosphatidic acid-like material but with an acyl ester/phosphorus molar ratio of 6. Thus the evidence is conflicting and does not exclude the possibility that both phosphatidic acids and bisphosphatidic acids may occur in animal tissues. Hence the material labelled 'phosphatidic acid' in Table 2 may be either of these materials or a mixture of both.

The fraction X described by Collins (1959) has been shown to be distributed in a similar manner to cardiolipin and this is strong evidence for the identity of this fraction. In microsomes the lipids contain a very small amount of cardiolipin-like material and of the 'phosphatidic acid', but in the mitochondria both fractions are well defined. The cardiolipin predominates and represents 5% of the total lipid phosphorus. The high content of phosphatidic acid-like material in mitochondria is in good agreement with the results of Marinetti *et al.* (1958) for pig-heart-cell fractions and of Macfarlane *et al.* (1960) for rat-liver-cell fractions.

Collins (1960) has presented evidence that the complex phospholipids are easily broken down by the analytical procedures in general use to produce diester phospholipids, which are identical with the classical phospholipids. It is also suggested that the finding of unusual compounds such as lysolecithin could be due to splitting of these complex phospho-

Fig. 3. Countercurrent distribution of the acetone-insoluble-phospholipid derivatives in carbon tetrachloride-chloroform-methylene chloride-methanol-water. Every fifth tube was analysed for phosphorus (—). (a) Mitochondrial lipids (92 $\mu\text{g. atoms}$ of phosphorus); (b) microsomal lipids (212 $\mu\text{g. atoms}$ of phosphorus) and the distribution of a sample of lysolecithin (---).

Table 2. *Phospholipids of mitochondria and microsomes*

(1) Present results; (2) Marinetti, Erbland & Stotz (1958) for pig-heart-cell fractions; (3) Macfarlane, Gray & Wheeldon (1960) for rat-liver-cell fractions. —, Signifies not detected.

	Phosphorus (molar % of total lipid phosphorus)					
	Mitochondria			Microsomes		
	(1)	(2)	(3)	(1)	(2)	(3)
'Phosphatidic acid'	2			1		
'Cardiolipin'	5	13*	9†	1	2*	1†
Phosphatidylethanolamine and phosphatidylserine	20	28	31	12	25	26
Complex phospholipids	37	—	—	39	—	—
Lecithin and sphingomyelin	31	42	51	39	58	62
Inositol and minor phospholipids	5	14	6	9	4	10
Lysolecithin	—	—	—	—	8	—

* Described as polyglycerolphosphatide.

† Described as cardiolipin.

lipids. Collins (1960) has proposed that the complex phospholipids may be phosphate triesters and that this structure is consistent with the above-mentioned facts.

In the present study of the phospholipids of rat-liver mitochondria and microsomes it is apparent that the difference in the complex phospholipids in these two fractions is due to the presence of a component in microsomes which is absent from mitochondria. The fact that one component of the complex phospholipids seems to be associated with the endoplasmic reticulum raises speculation about the role of triester phospholipids in the functioning of the cell.

SUMMARY

1. The phospholipids from rat-liver mitochondria and microsomes have been examined by the countercurrent distribution of the dinitrophenylated and methylated derivatives.

2. The mitochondrial lipids contain a higher proportion of cardiolipin than do the microsomes.

3. The mitochondria lipids lack a component of the complex amino-phospholipids which is present in microsomes. No lysolecithin could be detected.

We wish to thank Miss H. Doery of the Commonwealth Serum Laboratory, Australia, for a gift of lysolecithin,

Dr P. Plackett of the Animal Health Division, Commonwealth Scientific and Industrial Research Organization, for a gift of cardiolipin, the Standard Vacuum Refining Co. Aust. Pty. Ltd. for a gift of *n*-heptane and the National Health and Medical Research Council for financial support. One of us (V. L. S.) held a Commonwealth Post-graduate Award and a grant from the University of Melbourne. We wish to express our appreciation for the technical assistance of Miss T. B. G. Kaak.

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Mammalian Glycosidases

3. THE INTRACELLULAR LOCALIZATION OF β -GLUCURONIDASE IN DIFFERENT MAMMALIAN TISSUES*

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(Received 27 September 1960)

Kerr & Levy (1951) and Walker & Levy (1951) showed that a large part of the β -glucuronidase activity in water homogenates of mouse liver was associated with the cytoplasmic granules, and was apparently spread over particles of all sizes. The enzyme was fully active in water homogenates, and the fraction associated with the granules could be brought into a soluble state by incubation in acetate buffer, mechanical disintegration, and freezing and thawing, as well as by treatment with acetone or with the non-ionic, surface-active agent Triton X-100 (Rohm and Haas Co.). Walker (1952) extended this work to mouse liver homo-

genized in sucrose solution (0.25M), and found that nearly all the enzyme was then associated with the cytoplasmic granules. The enzyme within the granules now displayed only a fraction of its total activity, owing apparently to slow diffusion of the substrate into the particles, since the activity rose when the substrate concentration was increased. The enzyme could be released by the measures already described, and, in addition, by dilution of the medium with water. These methods made the enzyme fully active, the most convenient being the use of Triton X-100. It was concluded that semi-permeable membranes must play a considerable part in regulating the activity of the enzyme *in vivo*.

* Part 2: Conchie & Hay (1959).