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The Action of Digitonin on Rat Liver Mitochondria

PHOSPHOLIPID CONTENT

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1. The amount and types of phospholipid and the fatty acid composition of the various phospholipids were examined in intact rat liver mitochondria, in mitochondria devoid of their outer membrane (preparation A) and in very small pieces derived from the disruption of the inner-membrane complexes (preparation B). The latter two preparations were obtained by digitonin treatment and carry out oxidative phosphorylation. 2. The ratio μg.atoms of phospholipid P/mg. of protein was 0·163 for intact mitochondria, decreased to 0·118 on removal of the outer membrane and increased markedly to 0·292 on disruption of the inner-membrane complex. 3. Examination of the various types of phospholipid present showed that the molar proportions cardiolipin:phosphatidylcholine:phosphatidylethanolamine were approx. 1:6:6 for intact mitochondria and 1:3:3 for preparations A and B. 4. There was a correlation between the recovery of cardiolipin and adenosine triphosphatase activity in the conversion of intact mitochondria into preparations A and B. 5. The fatty acid contents of the various types of phospholipid purified by thin-layer chromatography were identical in all three preparations. Our results show a considerably higher content of arachidonic acid and lower content of oleic acid for phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol than have previously been reported for mitochondrial phospholipids.

The preceding papers (Hoppel & Cooper, 1968; Morton, Hoppel & Cooper, 1968) described the effects of repetitive treatment of rat liver mitochondria with digitonin on the enzyme content and structure of the resulting preparations. In the present paper we describe the effect of this treatment on the phospholipid composition and the fatty acid content of each phospholipid class of the preparations examined.

EXPERIMENTAL

Chemicals. All solvents were of analytical grade and were redistilled before use. The chloroform was shown to contain no carbonyl chloride by measuring the pH of the aqueous phase after extraction with water. DL-α-Tocopherol was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.).

Phospholipid standards. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were obtained from Supelco (Bellefonte, Pa., U.S.A.). Cardiolipin was obtained from General Biochemical Co. (Cleveland, Ohio, U.S.A.).

Samples. The mitochondria and preparations A and B were prepared as described by Hoppel & Cooper (1968).

Extraction. Samples (1ml.) containing 50–130mg. of protein/ml. were extracted overnight with 20ml. of chloroform–methanol (2:1, v/v) containing 1% DL-α-tocopherol/ml. of sample. The following day the samples were washed twice by the method of Folch, Lees & Sloane-Stanley (1957) and the solution was then diluted to 25ml. with chloroform.

Thin-layer chromatography. A sample from the 25ml. of extracted lipids in chloroform was taken for determination of total phosphorus. Another sample containing about 2mg. of phospholipid was concentrated to 250μl. under N_2 and streaked on thin-layer plates (CAMAG Kieselgel, type D–O, 0·5mm. thick) as a short band. The plates, containing standards, were developed by a method similar to that of Skipski, Peterson & Barclay (1964), but chloroform–methanol–acetic acid–water (50:30:8:3, by vol.) was employed and DL-α-tocopherol was added to a final concentration of 0·01%. After development and evaporation of volatile solvents either the entire plate (phospholipid class determination) or the ends of the bands (fatty acid content determination) were sprayed for phosphorus-positive material by the method of Dittmer & Lester (1964). For the determination of phosphorus in the different phospholipid bands the bands were scraped into 125mm.×15mm. Pyrex screw-cap tubes. After the addition of 0·5ml. of conc. H_2SO_4 to each sample the tubes were heated for 3hr.

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at 190° in a heating block. The tubes were then cooled, 10 drops of 30% H_2O_2 were added and the tubes again heated for 1 hr. Phosphorus was then determined by the method of Parker & Peterson (1965). For the determination of the fatty acid composition of the various phospholipids the sample areas corresponding to phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine and a blank area were scraped from the plate and transmethylated (described below). The plate was then rotated through 90°, and the remaining cardiolipin band was rechromatographed to remove neutral lipid contaminants. The solvent system (Mangold & Malins, 1960) was Skellysolve B–diethyl ether–acetic acid (80:20:1, by vol.) containing 0·01% DL-α-tocopherol. After development, the cardiolipin and a corresponding blank area were scraped off and transmethylated.

Amino phosphatides were detected by the ninhydrin method of Skipski *et al.* (1964). A positive reaction from the mitochondrial fractions was only obtained for the band corresponding in R_F to phosphatidylethanolamine. Molybdate spray by the method of Dittmer & Lester (1964) showed bands corresponding in R_F to phosphatidylcholine, phosphatidylethanolamine and cardiolipin in all mitochondrial fractions and to phosphatidylinositol in intact mitochondria. The Dragendorf spray as prepared by Skidmore & Entenman (1962) showed orange bands corresponding in R_F to phosphatidylcholine. The Schiff test for plasmalogens showed no violet colour on the plates (Skidmore & Entenman, 1962).

Gas–liquid chromatography. The material scraped from the plates was transmethylated by placing it into 5 ml. ampoules and adding 1 ml. of a mixture of 2% (v/v) H_2SO_4 in methanol. The ampoules were flushed with N_2, sealed and refluxed in a 65° oven overnight. The ampoules were allowed to cool and then opened as used. Then 1 ml. of

Table 1. *Total phospholipid content*

Results are given as means ± S.D., with the numbers of experiments in parentheses. Each experiment was done with a preparation made from 18 livers; determinations were made in duplicate in each experiment and the average was used.

Preparation	Phospholipid (μg. atom of P/mg. of protein)
Mitochondria	0·163 ± 0·013 (5)
Prep. A	0·118 ± 0·009 (4)
Prep. B	0·292 ± 0·012 (5)

Table 2. *Phospholipid distribution*

Results are given as means ± S.D., with the numbers of experiments in parentheses. Each experiment was done with a preparation made from 18 livers; determinations were made in duplicate in each experiment and the average was used.

	Phospholipid distribution (% of phospholipid P)		
Quantity measured	Mitochondria (6)	Prep. A (5)	Prep. B (4)
Phosphatidylcholine	41·0 ± 0·5	36·7 ± 4·7	33·3 ± 2·8
Phosphatidylethanolamine	35·6 ± 5·6	32·7 ± 4·4	39·2 ± 0·7
Cardiolipin	12·7 ± 3·5	21·3 ± 5·5	22·7 ± 2·7
Phosphatidylinositol	4·6 ± 3·8	4·3 ± 2·2	0·8 ± 0·8
Lysolecithin + sphingomyelin	2·7 ± 2·4	1·7 ± 1·6	1·5 ± 1·0
Recovery (% of total phospholipid)	99·8 ± 1·6	95·3 ± 5·8	97·6 ± 4·1

Table 3. *Comparison of phospholipid distribution in liver mitochondria*

		Phospholipid distribution (% of phospholipid P)				
Reference	Species	Cardiolipin	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-inositol	Others
Parsons *et al.* (1967)	Guinea pig	22·5	40·0	28·4	7·0	2·3
Fleischer *et al.* (1967)*	Ox	17·2	43·4	34·5		4·9
Strickland & Benson (1960)	Rat	12	49	30	8	1
MacFarlane, Gray & Wheeldon (1960)	Rat	9	51	31	6	
Getz, Bartley, Stirpe, Notton & Renshaw (1962)	Rat	8·4	38·6	42	9·9	6·6
Schwarz, Dreisbach & Kleschick (1963)	Rat	7·7	49·6	26·4	4·4	6·1
This paper	Rat	12·7	41·0	35·6	4·6	2·7

* Measured by charring.

water and 1 ml. of Skellysolve F were added and the contents mixed to extract the fatty acids from H_2SO_4. The Skellysolve F extract was transferred to a 2 ml. vial and evaporated under N_2 to 1 μl. for injection on to the gas–liquid-chromatographic column.

An F and M model 402 dual-column gas chromatograph with flame ionization detectors standardized with National Institutes of Health standards A, B and C and a methyl arachidonate–methyl stearate mixture was used. The 6 ft. glass columns had external diam. $\frac{1}{4}$ in. and were packed with Chromosorb W (AW) coated with 12% ethylene glycol adipate (Applied Science, State College, Pa., U.S.A.). The temperature of the injection port was 220°, the oven 180° and the detector 210°. The carrier gas was N_2 at a flow rate of 65 ml./min. All samples were run at a minimal attenuation of 8×10 and all blanks were run at a maximal attenuation of 4×10. The area of each peak was calculated by measurement of the height times width at half height.

RESULTS AND DISCUSSION

Phospholipid content. The phospholipid contents of the preparations examined are shown in Table 1. Our value of 0·163 μg.atom of phospholipid P/mg. of protein for rat liver mitochondria is within the range of values found by others. Caplan & Greenawalt (1966) reported 0·178, Schnaitman, Erwin & Greenawalt (1967) 0·154 and DePury & Collins (1967*b*) 0·20 for rat liver mitochondria. Parsons, Williams, Thompson, Wilson & Chance (1967) found a value of 0·217 for guinea-pig mitochondria and 0·182 was reported by Fleischer, Rouser, Fleischer, Casu & Kritchevsky (1967) for ox liver mitochondria.

The removal of outer membrane from rat liver mitochondria by the use of digitonin as described by Hoppel & Cooper (1968) gives a relatively pure inner-membrane complex still containing approx. 80% of the 'soluble' mitochondrial enzymes. The preparation had a phospholipid P/protein ratio that was 73% of that of intact mitochondria. This decline would be expected because Parsons *et al.* (1967) found that the outer membrane of guinea-pig liver mitochondria is rich in phospholipid and Schnaitman *et al.* (1967) have made the same observation with rat liver mitochondria.

The particulate fraction B, consisting of relatively small pieces obtained from preparation A by retreating it with digitonin, contains very little of the 'soluble' enzymes but still retains a considerable amount of the cytochromes and bound enzymes of preparation A (Hoppel & Cooper, 1968). Table 1 shows that it was enriched in phospholipid relative to both intact mitochondria (179%) and preparation A (247%).

Phospholipid distribution. Table 2 shows the relative amounts of the various types of phospholipids in each of the preparations. The most notable change observed was the marked increase in the fraction of the total phospholipid that is cardiolipin

Table 4. *Fatty acid content of phospholipids*

1 and 2 refer to two different experiments, each with preparations made from 18 livers, and were single estimations. All area percentages below 1% were deleted and the remaining fatty acid methyl ester areas normalized to 100%.

		Fatty acids (area percentages)													
		$C_{16:0}$ acid		$C_{16:1}$ acid		$C_{18:0}$ acid		$C_{18:1}$ acid		$C_{18:2}$ acid		$C_{20:4}$ acid		$C_{22:6}$ acid	
Phospholipid	Preparation	Expt.... 1	2	1	2	1	2	1	2	1	2	1	2	1	1
Cardiolipin	Mitochondria	4·1	4·2	3·4	2·8			22·1	14·6	70·2	74·9				
	Prep. A	2·3	3·9	3·4	2·0			14·4	12·6	79·4	70·5				
	Prep. B	2·0	2·7	3·1	2·1			16·4	16·1	76·6	75·6				
Phosphatidyl-choline	Mitochondria	23·4	21·2			18·1	23·6	5·4	5·3	19·3	15·8	28·4	29·4	5·1	4·3
	Prep. A	23·4	23·0			19·0	19·9	5·3	4·9	17·1	14·7	30·9	31·6	4·1	5·6
	Prep. B	23·0	22·8			20·5	14·4	5·7	6·0	16·8	16·9	29·5	34·6	4·3	4·9
Phosphatidyl-ethanolamine	Mitochondria	21·1	20·4			22·9	24·7	3·6	3·1	7·2	7·6	34·9	35·9	10·0	7·9
	Prep. A	22·6	23·3			17·7	25·4	4·7	2·8	6·8	7·1	38·1	35·8	9·8	5·2
	Prep. B	20·4	18·7			26·7	26·4	2·9	3·1	5·2	6·7	35·0	35·3	9·6	9·5
Phosphatidyl-inositol	Mitochondria	8·1				36·4				4·0		51·3			

for both preparations A and B. This is in accord with the findings of Parsons *et al.* (1967) that the outer mitochondrial membrane is low in cardiolipin. However, Parsons *et al.* (1967) found that the molar proportions cardiolipin P:phosphatidylcholine P:phosphatidylethanolamine P were 1:3·5:2·5 for intact guinea-pig liver mitochondria and remained relatively unchanged (1:4·1:2·6) after removal of the outer membrane by swelling in phosphate buffer, whereas our values were 1:6·4:5·6 for the rat liver mitochondria and these changed to 1:3·4:3·1 after removal of the outer membrane by digitonin treatment (preparation A) and remained essentially unchanged on further treatment, i.e. 1:2·9:3·4 for preparation B. Our findings are very similar to those of Bartley, Getz, Notton & Renshaw (1962), who reported proportions 1:4·6:5·0 for rat liver mitochondria and found that they changed to 1·2:3:2·3 on treatment with digitonin. However, Bartley *et al.* (1962) also found that all of the phospholipids of their digitonin preparations were 'lysophosphatides', whereas we have found very little, if any, lysolecithin in any of our preparations.

There was a striking parallel between the effect of digitonin treatment on the content of cardiolipin and adenosine triphosphatase. In the conversion of mitochondria into preparation A there was 121% enrichment in the μg.atoms of cardiolipin P/mg. of protein and 144% enrichment in the specific activity of adenosine triphosphatase (Hoppel & Cooper, 1968). The recovery was 75% for cardiolipin and 73% for adenosine triphosphatase. Preparation B was enriched 310% in concentration of cardiolipin and 303% in the specific activity of adenosine triphosphatase relative to mitochondria. The recovery was 42% for cardiolipin and 39% for adenosine triphosphatase.

The values for intact mitochondria are compared in Table 3 with those reported by others for liver mitochondria. There is reasonably good agreement between the values reported for rat liver mitochondria, but those of bovine and guinea-pig liver have a relatively higher content of cardiolipin.

Fatty acid content of phospholipid fractions. Table 4 shows the distribution of fatty acids in the different phospholipid classes. There were no significant changes in the distribution of fatty acids on repetitive treatment of mitochondria with digitonin. This indicates that the fatty acid compositions of the various phospholipids in both the inner and outer mitochondrial membranes were identical even though the relative amounts of the types of phospholipids in the two membranes were different. These findings are in contrast with those of Bartley *et al.* (1962), who reported that digitonin treatment produced changes in the fatty acid composition of some phospholipids. Table 4 also shows that each phospholipid class had a unique

Table 5. *Comparison of fatty acid contents of purified rat liver mitochondrial phospholipids*

Reference	Phospholipid	Fatty acids (area percentages) $C_{16:0}$ acid	$C_{16:1}$ acid	$C_{18:0}$ acid	$C_{18:1}$ acid	$C_{18:2}$ acid	$C_{20:4}$ acid	$C_{22:6}$ acid
Getz & Bartley (1961)	Cardiolipin	2·0	1·9	0·4	11·9	79·5		
Gray (1964)		0·9	3·3	Trace	10·0	83·6	Trace	
Getz *et al.* (1962)		3·9	2·5	1·4	12·8	74·0	1·6	0·4
This paper		4·2	3·1		18·9	72·6		
Getz *et al.* (1962)	Phosphatidylcholine	19·7	1·5	18·7	12·2	20·0	19·5	3·4
MacFarlane *et al.* (1960)		13·2	3·5	21·9	14·1	19·6	13·8	7·9
DePury & Collins (1967*a*)		21·8	1·1	29·4	9·0	17·7	18·6	
Glende & Cornatzer (1966)*		34		19	10	15·5	19	
This paper		22·3		20·9	5·4	18·1	28·9	4·7
Getz *et al.* (1962)	Phosphatidylethanolamine	20·2	0·8	19·5	10·4	16·3	21·0	8·7
MacFarlane *et al.* (1960)		17·5	1·0	30·4	4·8	4·3	21·0	14·0
Bartley *et al.* (1962)		18·7	0·8	21·4	9·7	16·1	20·9	7·7
Glende & Cornatzer (1966)*		32		28	9	9	22	
This paper		20·8		23·8	3·4	7·4	35·4	9·0
Getz *et al.* (1962)	Phosphatidylinositol	12·1	0·8	32·0	6·8	16·2	21·5	3·4
Glende & Cornatzer (1966)*		13		45	5	4·5	23	
This paper		8·1		36·4		4·0	51·3	

* Data for 252-day-old rats.

distribution of fatty acids. Phosphatidylethanolamine had a lower percentage of $C_{18:2}$ acid and slightly higher percentage of $C_{20:4}$ and $C_{22:6}$ acids than did phosphatidylcholine. Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol had a ratio of unsaturated to saturated fatty acids almost 1:1, whereas cardiolipin contained unsaturated fatty acids almost exclusively with the major fatty acid being $C_{18:2}$ acid. The only saturated fatty acid in cardiolipin was palmitic ($C_{16:0}$) acid.

The fatty acid composition in each purified phospholipid fraction agreed reasonably well with similar reports in the literature. The one clear difference however between our findings and those of workers in other Laboratories was that we found a much higher content of arachidonic ($C_{20:4}$) acid and a much lower content of oleic ($C_{18:1}$) in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. The other literature values are shown in Table 5. The reason for these discrepancies is not clear, but may result from the fact that we used DL-α-tocopherol to prevent lipid peroxidation during handling (Witting, Harvey, Century & Horwitt, 1961). Another possible explanation is variations in the degree of microsomal contamination. Both Getz *et al.* (1962) and MacFarlane *et al.* (1960) have shown that microsomal phospholipids have high oleic acid and low arachidonic acid content. Getz *et al.* (1962) have also shown that microsomes contain approx. 24% of lysophosphatides, but our preparations contained very little lysolecithin.

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