Brit. J. Pharmacol. (1966), **26**, 723–729.

HEART PHOSPHOLIPIDS IN THE ELECTROLYTE-STEROID CARDIOPATHY

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(*Received January* 10, 1966)

Of the various experimental cardiac necroses the electrolyte-steroid cardiopathy has perhaps been the most extensively studied. Although its mechanism is not completely understood, it is known that in the prenecrotic stage there is a decrease of myocardial potassium (Nickerson, Karr & Dresel, 1961 ; Prioreschi, 1962), an increase of sodium (Prioreschi, 1963a) and alterations of the cardiac mitochondria (D'Agostino, 1964). It is also known that the protective action of various agents is related to their capacity for increasing the heart potassium level (Prioreschi, 1963b ; Prioreschi, 1964). The present quantitative study of the heart phospholipids during the development of the electrolyte-steroid cardiopathy was suggested by the role played by the compounds on the ion transport across the membrane (Rossiter & Strickland, 1960 ; Wolfe, 1964) and by the observation that during the development of the cardiac necroses produced in the rat by ligature of the abdominal aorta there is an alteration of the fatty acid composition in the cardiolipin fraction of the heart phospholipids (Prioreschi, Kuksis & Rogers, 1965).

METHODS

Male Sprague-Dawley rats with a mean initial body weight of 207 g (197–215 g) were divided into groups of five animals each. Some groups were used as controls while others received a micro-crystal suspension of 3 mg 9α-fluorocortisol in 0.2 ml. water subcutaneously once daily, and 2 mmole of Na_2HPO_4 in 2 ml. water by stomach tube twice daily. Three groups were killed on the second day of the experiment, three on the third, four on the fourth, six on the fifth and four on the sixth. At autopsy each animal was anaesthetized with chloroform, the chest was opened, and the heart was inspected for cardiac necroses, excised and blotted.

The atria were immediately removed and the ventricles were pooled in groups of five and homogenized in a mixture of chloroform-methanol (2:1, v/v) with a Virtis 45 blender. The homogenate was filtered and the residue washed with a 0.05 N NaCl solution and the lipids were extracted as described by Folch, Lees & Sloane Stanley (1957). The solvents were removed in a nitrogen atmosphere at room temperature and the total lipids separated into neutral lipids and phospholipids by silicic acid chromatography. The material was dissolved in chloroform and placed on columns (1×15 cm) of silicic acid (Mallinckrodt, 100 mesh) previously equilibrated with chloroform. The neutral lipids were eluted with 200 ml. chloroform and the phospholipids with 100 ml. methanol.

The volume of the phospholipid solution was reduced under nitrogen and the compounds were separated into classes by thin-layer chromatography using the Desaga apparatus. The glass plates (20×20 cm) were coated with a mixture of 30 g silica gel G (Merck and Co.) and 65 ml. distilled water. The plates were activated by heating in an oven at 100° C for at least three hours. The phospholipids were applied to the plates as a series of spots, with a 10 μl. Hamilton syringe. The chromatoplates were developed in glass-covered jars with a mixture of chloroform-methanol-acetic

acid-water (65:25:8:4, v/v). Three samples (one from control rats, one from animals killed on the fourth day, and one from animals killed on the fifth day) were each applied on four plates, of which two were developed with the above-mentioned mixture and two with a mixture of chloroform-methanol (65:25, v/v). The plates were then sprayed with 0.2% dichlorofluorescein solution (Eastman Kodak) and examined under ultraviolet light and the various spots identified by comparison with samples of egg-yolk phospholipids and beef cardiolipin standard (Sylvana Chemical).

The silica gel layers containing the lipids were scraped off the plates and the fatty acids transmethylated with methanol-sulphuric acid (10%, v/v) by heating the samples at 80° C for three hours in flame-sealed glass tubes. The methyl esters were then extracted with petroleum ether and concentrated under nitrogen.

The fatty acids were analysed in a gas chromatograph (Model 700, F and M Co.), equipped with a Minneapolis-Honeywell 1 mv recorder and a Model 240 Temperature Programmer (F and M Co.). The separations were performed starting at 100° C and increasing the temperature by 3° C per minute. Nitrogen was used as carrier gas and the two stainless steel columns (0.3×183 cm) were packed with 6% diethylene glycol succinate (DEGS) on 80–100 mesh diataport S. The fatty acids were identified by co-chromatography with known standards, by retention time in isothermal conditions and after hydrogenation performed with platinum oxide (Amend Drug and Chemical Co.) as catalyst.

As internal standard we used $C_{17:0}$ methyl ester, which was added in known quantities to each sample before transmethylation.

The areas of the peaks were estimated by triangulation. The quantity of each fatty acid was calculated from the ratio of the area of the corresponding peak and the area of the internal standard peak.

The quantity of each phospholipid was calculated from the following formula suggested by Kuksis, Petermann & Beveridge (1965).

$$X = \frac{\frac{WFAMe}{MWFAMe} MWX}{NFA}$$

Where X is the phospholipid, the quantity of which is to be determined, WFAMe is the total weight of the fatty acid methyl esters calculated as described before and MWFAMe is the molecular weight of the fatty acid methyl ester with the calculated average chain length characteristic of each sample. The average chain length was calculated from the percentage of each fatty acid in each gas-chromatogram. MWX is the molecular weight of the phospholipid. The value is calculated by attributing the average chain length to each fatty acid in the molecule. Finally, NFA is the number of fatty acids in the molecule.

The average chain length was used to facilitate the calculations. Obviously the molecular weight of the average fatty acid could also have been calculated from the molecular weight and the percentage of each fatty acid in the sample.

The quantities expressed in mg/100 g of fresh tissue (±S.E.) and the incidence of cardiac necroses are reported in the tables.

RESULTS

Sphingomyelin, phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin were identified as major components of heart phospholipids and analysed. Small quantities of lysophosphatidyl choline, phosphatidyl serine and phosphoinositides are known to be present in the rat myocardium. They account for a total of about 13% of the total phospholipid phosphorus (Marinetti, Erbland & Stotz, 1958). In the present work, no attempt was made to isolate these compounds as their quantities were thought to be too small for reliable comparative study under our experimental conditions.

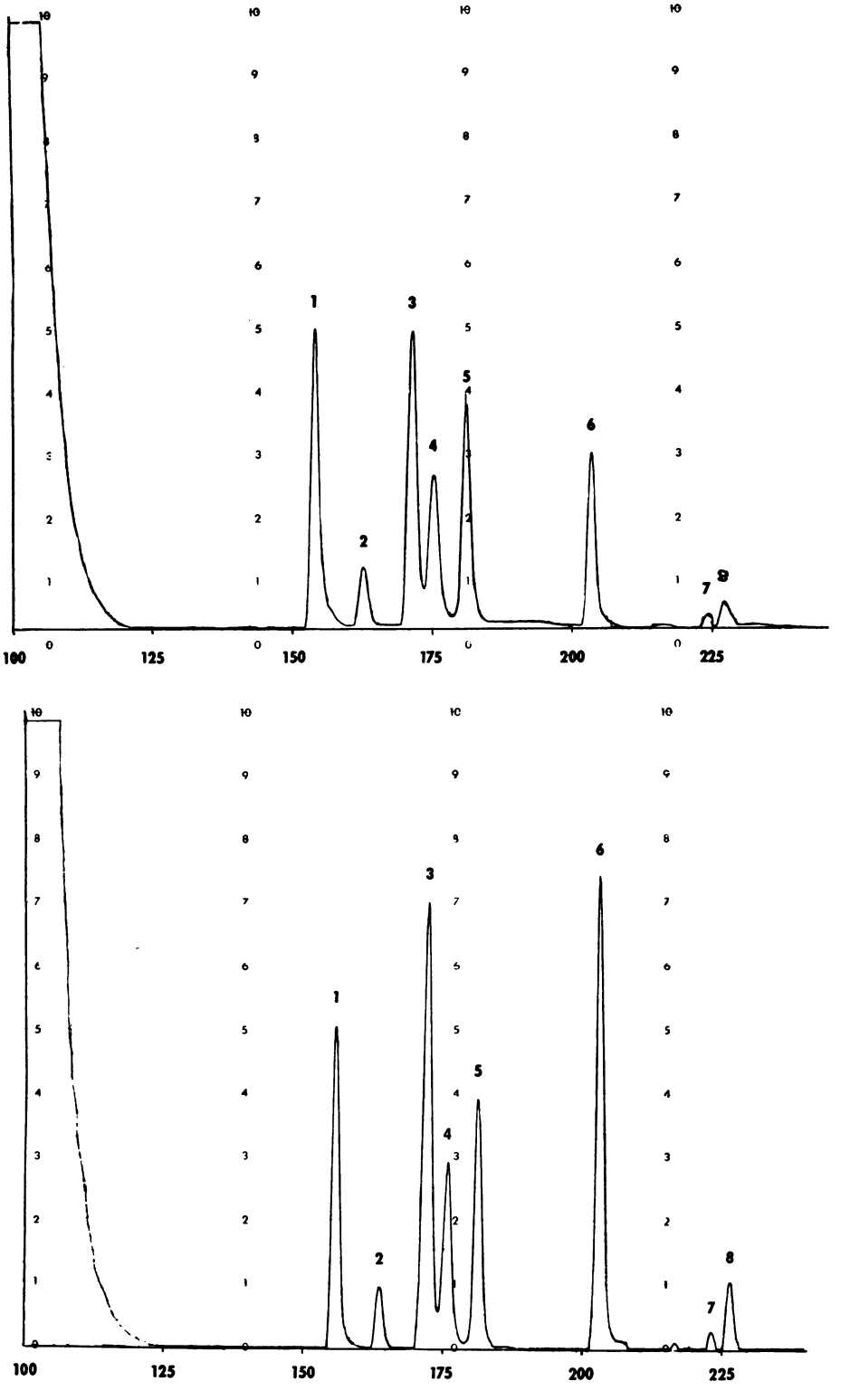

Fig. 1. Gas-chromatograms of heart phosphatidyl choline fatty acids from normal rats (top) and during the development of electrolyte-steroid cardiopathy (bottom). The numbers below the baseline indicate the temperature (C).

1=$C_{16:0}$; 2=$C_{17:0}$ (added to the sample); 3=$C_{18:0}$; 4=$C_{18:1}$; 5=$C_{18:2}$; 6=$C_{20:4}$; 7=$C_{22:5}$; 8=$C_{22:6}$.

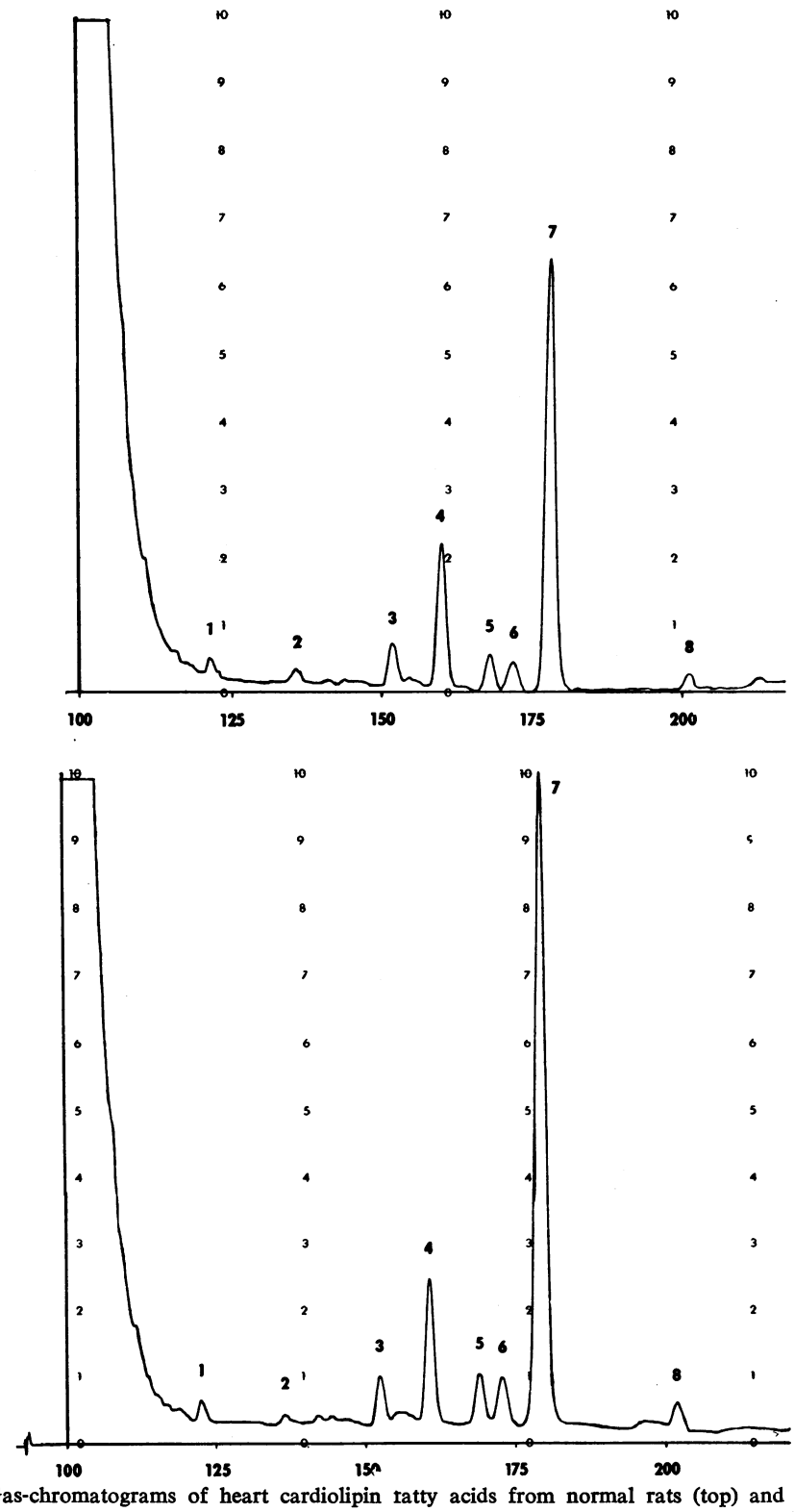

Fig. 2. Gas-chromatograms of heart cardiolipin fatty acids from normal rats (top) and during the development of the electrolyte-steroid cardiopathy (bottom). The numbers below the baseline indicate the temperature (C).

1=$C_{12:0}$; 2=$C_{14:0}$; 3=$C_{16:0}$; 4=$C_{17:0}$ (added to the sample); 5=$_{18:0}$; 6=$C_{18:1}$; 7=$C_{18:2}$; 8=$_{20:4}$.

Table 1 shows the average intensity of cardiac necroses and the quantities of phosphatidyl choline with its fatty acid composition at various intervals during the experiment. The cardiac necroses started to be visible on the fifth day and about half of the animals were affected on the sixth day. The quantity of phosphatidyl choline increases from the beginning of the experiment and the difference becomes significant

TABLE 1

HEART PHOSPHATIDYL CHOLINE CONCENTRATION AND ITS FATTY ACID COMPOSITION DURING DEVELOPMENT OF ELECTROLYTE-STEROID CARDIOPATHY

Concentrations are in mg/100 g fresh tissue (± S.E.). The values in bold figures are significantly ($P < 0.05$) different from the control values. Parentheses enclose the number of animals used for each determination

	Controls (50)	9α-Fluorocortisol + Na_2HPO_4				
		2nd day (15)	3rd day (15)	4th day (20)	5th day (30)	6th day (15)
$C_{16:0}$	121·42± 9·44	106·92± 2·60	97·90±18·98	142·08±32·42	130·56± 9·16	116·20±10·90
$C_{18:0}$	147·00±10·44	137·66± 6·48	144·98±24·00	**208·96±19·26**	**221·48± 6·22**	**230·30± 7·22**
$C_{18:1}$	71·72± 6·52	65·52± 6·68	87·34±22·42	95·06±16·22	90·42± 9·56	84·12± 1·84
$C_{18:2}$	85·04± 9·48	99·08±18·32	110·42±14·14	**132·72±17·64**	**121·16±11·06**	**126·72± 2·66**
$C_{20:4}$	131·26±14·60	122·04±31·46	128·78±12·66	169·06±38·58	**178·76±19·68**	**184·18± 8·02**
$C_{22:5}$	7·12± 1·34	9·12± 1·16	8·12± 0·70	10·80± 1·52	**15·22± 1·46**	**15·38± 3·80**
$C_{22:6}$	25·42± 3·58	19·44± 6·22	19·04± 1·56	34·14± 4·52	18·88± 3·86	32·76± 9·94
Phosphatidyl choline	776·40± 56·74	734·44± 94·78	781·80± 105·74	**1,029·76± 109·22**	**988·60± 76·40**	**1,019·60± 95·44**
Cardiac necroses (%)	0	0	0	0	20·00	53·33

on the fourth day of treatment before the cardiac necroses become visible. There is also a distortion of the fatty acid pattern (Fig. 1) with increased quantities of $C_{18:0}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:5}$. No changes were detected for $C_{16:0}$, $C_{18:1}$ and $C_{22:6}$.

The results of the analysis of the cardiolipin fraction are reported in Table 2. The amount of cardiolipin is significantly increased starting on the fourth day of treatment. The fatty acid composition shows an increase of $C_{18:1}$, $C_{18:2}$ and $C_{20:4}$ in the last days of the experiment (Fig. 2). No significant changes were found in the quantities of sphingomyelin and phosphatidyl ethanolamine nor in their fatty acid patterns.

DISCUSSION

The results of the present experiment indicate that before the necroses become visible there is an increase in the cardiac concentration of phosphatidyl choline and cardiolipin. In both phospholipids the concentration of $C_{18:2}$ and $C_{20:4}$ are increased in addition to the increase of $C_{18:0}$ and $C_{22:5}$ in the phosphatidyl choline and of $C_{18:1}$ in the cardiolipin fraction. There seems to be no relation between the incidence of the necroses and the changes in heart phospholipid level.

With the solvent system chloroform-methanol-acetic acid-water (65:25:8:4, v/v), the various phospholipid fractions were well separated and cardiolipin was carried near the solvent front in the chromatoplate (Prioreschi *et al.*, 1965). This could conceivably have resulted in contamination of the compound with traces of neutral lipids and other impurities. To check on this possibility, in three instances we used the solvent mixture chloroform-methanol (65:25, v/v), as described under Methods. With this solvent the more polar phospholipids are poorly separated and remain near the starting point but

TABLE 2

HEART CARDIOLIPIN CONCENTRATION AND ITS FATTY ACID COMPOSITION DURING DEVELOPMENT OF ELECTROLYTE-STEROID CARDIOPATHY

Concentrations are in mg/100 g fresh tissue (± S.E.). The values in bold figures are significantly ($P < 0.05$) different from the control values. Parentheses enclose the number of animals used for each determination

	Controls (45)	9α-Fluorocortisol + Na_2HPO_4				
		2nd day (15)	3rd day (15)	4th day (20)	5th day (30)	6th day (20)
$C_{12:0}$	0·77±0·19	1·39±0·73	1·91±0·56	0·85±0·50	0·70±0·34	1·32±0·18
$C_{14:0}$	0·48±0·16	0·96±0·49	0·96±0·48	0·72±0·42	0·64±0·32	0·49±0·31
$C_{16:0}$	3·42±0·88	3·50±0·74	3·68±0·66	3·82±0·70	3·14±0·49	3·16±0·39
$C_{18:0}$	2·88±0·60	2·83±0·89	3·43±2·00	4·24±1·60	3·50±0·91	2·92±0·39
$C_{18:1}$	1·99±0·24	2·48±0·68	2·63±0·52	**3·26±0·68**	**3·22±0·42**	**3·07±0·43**
$C_{18:2}$	22·97±2·72	25·01±3·38	34·34±6·54	**35·22±2·72**	**41·31±7·43**	**35·54±3·29**
$C_{20:4}$	Traces	Traces	**1·48±0·93**	**2·65±1·54**	**1·30±0·78**	**0·72±0·46**
Cardiolipin	39·95±4·32	44·87±5·04	55·32±2·33	**61·94±6·18**	**64·80±9·22**	**60·71±5·71**
Cardiac necroses%	0	0	0	0	20·00	53·33

cardiolipin has an R_F of 0.93. In each case the fatty acid patterns of the same cardiolipin isolated with the two different solvent systems were identical.

The significance of the alterations observed in the fatty acid pattern and in the concentration of cardiolipin and phosphatidyl choline cannot be immediately assessed. It is known that phospholipids play a role in various transport and permeability phenomena (Rossiter *et al.*, 1960; Wolfe, 1964) but no detailed correlation appears to have been made between changes in function and alteration in composition.

The changes in the cardiolipin fraction would suggest some metabolic alterations at the mitochondrial level in view of the fact that cardiolipin seems to be exclusively associated with the mitochondria (Ansell & Hawthorne, 1964) and that mitochondrial alterations have been shown to be associated with electrolyte-steroid cardiopathy (D'Agostino, 1964).

It is of interest that changes in the fatty acid pattern of cardiolipin were found in the cardiopathy produced by ligature of the abdominal aorta in the rat (Prioreschi *et al.*, 1965). In that study, however, we did not measure the quantities of the various phospholipids or fatty acids, but only the fatty acid pattern of the various phospholipids.

SUMMARY

1. The concentration of various heart phospholipids separated by thin-layer chromatography and the gas-chromatographic pattern of their fatty acids were studied at various intervals during the development of electrolyte-steroid cardiopathy in the rat.

2. Before the cardiac necroses become visible there is an increase in the concentrations of phosphatidyl choline and cardiolipin.

3. The fatty acid pattern of phosphatidyl choline and cardiolipin were distorted because of increased quantities of $C_{18:0}$, $C_{18:2}$, $C_{20:4}$ and $C_{22:5}$ in the phosphatidyl choline and increased quantities of $C_{18:1}$, $C_{18:2}$ and $C_{20:4}$ in the cardiolipin fraction.

4. No changes were observed in the sphingomyelin and phosphatidyl ethanolamine fractions.

5. The role of phospholipids in the development of cardiac necroses is discussed.

This work was supported by research grants from the Ontario Heart Foundation and the Medical Research Council of Canada. The author wishes to thank the Upjohn Company for the generous supply of 9α-fluorocortisol.

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