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Effect of chlorphentermine on the lipids of rat lungs

JOSEPHINE GLOSTER, DONALD HEATH, PHILIP HASLETON, and PETER HARRIS

Cardiothoracic Institute, 2 Beaumont Street, London W1N 2DX and Department of Pathology, University of Liverpool, Liverpool

Gloster, Josephine, Heath, D., Hasleton, P., and Harris, P. (1976). *Thorax*, **31**, 558–564. **Effect of chlorphentermine on the lipids of rat lungs.** Chronic administration of chlorphentermine to rats resulted in a reduction of body weight compared to a normal control group. The weight of the heart, liver, kidney, and spleen was less in the treated group while the weight of the lungs was increased significantly. There was no change in the ratio of right ventricular to left ventricular weight in the rats treated with chlorphentermine, supporting the views that this drug does not cause pulmonary hypertension.

Biochemical analysis showed that the increase in the weight of the lungs was due to the accumulation of phospholipid. All classes of phospholipid were affected, but particularly phosphatidyl choline, the tissue concentration of which increased nine times. Chlorphentermine also increased the proportion of palmitate present in pulmonary phosphatidyl choline. Histological examination of the lung after treatment with chlorphentermine showed evidence of this drug-induced lipidosis.

No conclusion can as yet be reached as to the mechanism involved in the accumulation of phospholipid in the lung after chlorphentermine.

Chronic administration to rats of certain drugs including chlorphentermine (p-chloro-α, α-dimethyl phenethylamine hydrochloride) brings about a disorder of phospholipid metabolism (Schmien, Seiler, and Wassermann, 1974; Karabelnik and Zbinden, 1975; Seiler and Wassermann, 1975). This is characterized by the accumulation in several organs of lipids which appear ultrastructurally as intracellular myelin figures. Such inclusions are especially common in the lung and have been reported in association with the administration to rats of chlorphentermine (Smith, Heath, and Hasleton, 1973; Smith, Heath, and Hasleton, 1974a) and of the antidepressant iprindole which is an iminodibenzyl derivative (5- (3-dimethyl-aminopropyl) -6,7,8,9,10,11-hexahydrocyclo-oct(b) indole).

The reason for the predilection of phospholipidosis for the lung is the rapid turnover of phospholipid in that organ associated with the formation of pulmonary surfactant by granular (type II) pneumocytes and possibly by Clara cells (Smith, Heath, and Moosavi, 1974b). Hence the lung and especially these cells are particularly susceptible to the effects of chlorphentermine. A pulmonary phospholipidosis is rapidly induced with the appearance of myelin figures in not only the granular pneumocytes and Clara cells but also membranous pneumocytes and the endothelial cells of pulmonary capillaries (Smith *et al.*, 1973). Many of the lamellar inclusions are shed into alveolar spaces where they lie enmeshed in phospholipid lattices or become engulfed by large numbers of pulmonary histiocytes which pass into the alveolar spaces (Vijeyaratnam and Corrin, 1972; Smith *et al.*, 1973). The purpose of the investigation described in this paper was to determine the magnitude and nature of the pulmonary phospholipidosis induced by chlorphentermine.

METHODS

ANIMALS Seventeen young male Sprague Dawley rats weighing 175–200 g were used in these studies. The animals were allowed free access to both food and water. They were weighed at the outset

of the experiment and twice weekly thereafter.

Twelve of them were given intraperitoneal injections of chlorphentermine hydrochloride in a dosage of 50 mg per kg body weight daily for six days in each week until 50 injections had been given. The remaining five rats were kept as controls and received intraperitoneal injections of similar volumes of physiological saline.

REMOVAL OF ORGANS At the end of the experiment the animals were weighed and then killed by cervical dislocation. The heart, lungs, liver, spleen, and kidneys were quickly removed and rinsed in ice-cold saline to remove blood. All visible fat and connective tissue were removed, and the organs were blotted dry with fillter paper and weighed. The heart was weighed after the great vessels had been dissected off. The atria were removed, and the free wall of the right ventricle was dissected, blotted, and weighed separately. The left ventricle was weighed together with the interventricular septum.

Two portions of lung, weighing between 0·2 and 0·5 g, were taken and dried by heating in an oven at 50°C, and by desiccation until a constant weight had been obtained. The dried portions of lung were then reweighed to determine the amount of water during the drying procedure.

HISTOLOGICAL EXAMINATION Part of the right upper lobe was removed and fixed in 10% buffered formalin for histological examination, as described previously (Heath, Smith, and Hastleton, 1973). The remainder of the lungs was used for biochemical examination.

EXTRACTION OF LIPID The remaining lung tissue was weighed, minced finely with scissors, and homogenized in methanol using a Teflon in glass homogenizer. The lipids were extracted according to the procedure described by Folch, Lees, and Sloane-Stanley (1957). Sufficient chloroform was added to the methanol homogenate to give a 2 : 1 mixture by volume of chloroform and methanol. A total volume of 60 ml chloroform: methanol (2 : 1 v/v) was used for the extraction of the control lungs, and 100 ml of the same mixture was used for the extraction of lung tissue from the chlorphentermine treated rats. After standing overnight at room temperature the extract was filtered, and the residue was re-extracted for a second time. After filtration the extracts were combined and 20 vol % of 0·1 M NaCl was added to form a two-phase system. The lower lipid-containing chloroform phase was taken to dryness using a rotary evaporator. The total lipids from the lungs of the control rats were immediately redissolved in 3–4 ml chloroform, and the lipids from the lungs of the test rats were redissolved in 20 ml chloroform.

LIPID SEPARATION Preliminary separation of the total lipid extract from the lungs of both groups of rats was made on small columns (1·5 g) of 100–200 mesh silicic acid (Mallincrodt) activated at 110°C overnight before use. The neutral lipids were eluted from the column by the use of 30 ml chloroform and the phospholipids by the addition of 30 ml methanol. All the lipid extract from the control lungs and one-tenth of the total extract (2·0 ml) from the chlorphentermine lungs was separated in this way.

The eluates from the columns were taken to dryness with the use of a rotary evaporator and immediately made up to volume with chloroform. The neutral lipids were made up to a volume of 1·0 ml for the test lungs, or to a volume of 2·0 ml in the case of the control lungs. The phospholipids were dissolved in a total volume of 5·0 ml chloroform in both groups.

THIN-LAYER CHROMATOGRAPHY Separation of the lipid classes was carried out on 0·25 mm thick layers of silica gel H (Merck). The plates were prewashed in methanol : diethyl ether (1 : 1 v/v) for several hours, allowed to dry, and then activated for one hour at 110°C immediately before use.

The neutral lipid classes were separated using the solvent mixture petroleum ether (BP 40–60°C): diethyl ether : acetic acid (85 : 15 : 2 v/v). Duplicate 100 μl aliquots were applied to the plates as bands about 2·5 cm wide. A standard mixture was also run on each plate to aid identification of the separated lipid classes. The neutral lipids were located by spraying the plates with 10% phosphomolybdic acid in methanol. The lipid-containing zones and corresponding blank areas were scraped off the plates. Each lipid fraction was eluted by being shaken with two 5 ml portions of diethyl ether, and centrifuging between each extraction. The combined extracts were taken to dryness. Cholesterol and cholesterol esters were determined by the method of MacIntyre and Ralston (1954). Triglyceride was determined by the modification of Van Handel (1961) and free fatty acids by the colorimetric method of Duncombe (1964).

Duplicate 50 μl aliquots of the phospholipid containing extract were taken for the direct determination of total phospholipid phosphorus by the method of Bartlett (1959). Duplicate 100 μl aliquots were then separated on thin layers of silica gel H using a solvent system of chloroform : methanol : acetic acid : water (65 : 45 : 1 : 4 v/v) and solvent tanks lined with filter paper. The position of the lipids was visualized by iodine vapour. The lipid zones were scraped off the plates, and the phospholipids eluted with two successive 5 ml portions of a mixture of chloroform : methanol : water : acetic acid (50 : 50 : 15 : 1 v/v). The volume of the extract was reduced by evaporation using a current of air and the phosphorus content of each phospholipid class was determined by the method of Bartlett (1959). Appropriate blank areas from a plate were processed in the same way.

GAS LIQUID CHROMATOGRAPHY The fatty acid composition of the lecithin fraction was determined by gas liquid chromatography. Two hundred microlitres of phospholipid extract was applied to a thin-layer plate and the phospholipids were separated as described above. The plates were sprayed with 10% phosphomolybdic acid to locate the phospholipids. The lecithin was scraped off the plate and methylated directly by refluxing with 5% H_2SO_4-methanol for 6 h at 72°C. The methylated fatty acids were extracted with petroleum ether (BP 40–60°C). A Perkin Elmer Model 881 gas chromatograph with a flame ionization detector was used for the analysis. The separations were carried out on dual stainless steel columns (2 m long and $\frac{1}{8}$-inch OD) packed with 10% polyethylene glycol adipate on 80–100 mesh Chromosorb W. The initial temperature of the column was 165°C programmed for a temperature rise of 1°C/min to a maximum of 200°C. The area under each peak was determined by means of an Infotronics electronic integrator (Model CRS-100).

RESULTS

HISTOPATHOLOGY OF THE LUNG AFTER CHLORPHENTERMINE In all the rats the alveolar spaces were packed with large ovoid cells, 30×15 μm, which had foamy cytoplasm. Many of the cells were single and had an eccentric nucleus. Some contained two or more nuclei, and it seems likely that in these the cytoplasm of two or more cells had fused to form a syncytium-like mass. In some instances, the cells were dumb-bell-shaped where there was a linking strand of cytoplasm extending through a Cohn's pore. The foamy appearance of the cells was due to numerous clear intracytoplasmic vesicles which had aggregated in some cells to produce larger clear areas. In many alveolar spaces the foam cells had fused together to produce a granular pale-staining mass. At higher magnification this apparently amorphous material still showed the vesicular pattern characteristic of the cytoplasm of the viable cells.

Some of the intra-alveolar cells in tissue fixed in formalin gave a vivid magenta colour on staining with the periodic acid-Schiff reaction. Other foam cells, usually smaller, in the same section gave a negative reaction with this stain. The granular debris within the alveoli which had resulted from breakdown of groups of the intra-alveolar cells gave a cherry-pink reaction with the PAS stain. None of the intra-alveolar cells gave a positive reaction for haemosiderin with the Prussian blue reaction. There was no evidence of fibrosing alveolitis. Around many of the bronchi was a cellular exudate of plasma cells, lymphocytes, and mononuclears.

EFFECT OF CHLORPHENTERMINE ON THE BODY AND ORGAN WEIGHTS OF THE RAT The body weights of the rats at the start of the experiment and at the end, together with the wet weights of the lung, heart, liver, kidneys and spleen, are given in Table I. The starting body weights of the two groups of rats were similar. After 50 days of chlorphentermine treatment the mean body weight of the test group was 83 g less than the mean weight of the control rats. This difference in body weight was reflected in the smaller size of the heart, liver, kidneys, and spleen in the chlorphentermine group. The notable exception to this was in the

TABLE I

BODY WEIGHTS AND ORGAN WEIGHTS IN THE RAT AFTER TREATMENT WITH CHLORPHENTERMINE

	Control n=5 (wt in g ± SD)	Test n=12 (wt in g ± SD)
Body weight—start	184±6	180±8
end	405±36	322±29*
Lung	2·366±0·4	3·701±0·5*
Whole heart	1·204±0·2	0·833±0·1*
Left ventricle	0·802±0·1	0·576±0·1*
Right ventricle	0·179±0·02	0·136±0·01†
Liver	14·539±2·7	11·792±0·9*
Kidneys	3·198±0·4	2·974±0·1
Spleen	0·720±0·2	0·512±0·1†

*P < 0·001
† P < 0·01

case of the lungs which weighed significantly more than in the control rats ($P<0.001$).

So that any possible changes in organ weight unrelated to the differing body weights could be determined, the ratios of organ weight to body weight were calculated and are shown in Table II. The ratio of lung to body weight in the chlorphentermine treated rats was double that occurring in the control group. The kidney/body weight ratio was also significantly higher after chlorphentermine ($P<0.01$) while the heart/body weight ratio was reduced ($P<0.01$). There was no difference, however, in the ratio of the weight of the right ventricle to that of the left ventricle.

TABLE II

ORGAN WEIGHT/BODY WEIGHT RATIOS IN THE RAT AFTER TREATMENT WITH CHLORPHENTERMINE

	Control n=5	Test n=12
Lung/body weight	0·0058 ±0·0008	0·0116 ±0·0016*
Heart/body weight	0·0030 ±0·0003	0·0026 ±0·0002†
Liver/body weight	0·0357 ±0·0046	0·0368 ±0·0025
Kidney/body weight	0·0078 ±0·0006	0·0093 ±0·0010†
Spleen/body weight	0·0017 ±0·0003	0·0016 ±0·0003
RV/LV	0·2235 ±0·010	0·2382 ±0·030

*P < 0·001
†P < 0·01

The lungs of the control group contained a mean of 80·5±1·7% water, while the figure was reduced to 76·6±1·6% water in the test rats given chlorphentermine ($P<0.001$).

LIPID CONTENT OF THE LUNGS AFTER CHLORPHENTERMINE The results shown in Table III are expressed as the lipid contained in the total lung mass. The amount of cholesterol, both free and esterified, and of free fatty acid was increased, while there was a significant decrease in the amount of triglyceride present. The most striking finding, however, was the sevenfold increase of from 41 mg to 284 mg in the phospholipid content in the lungs from the chlorphentermine treated rats. The greatly increased total lipid content of the lungs in the test rats was, therefore, due in the most part to this increase in phospholipid. The proportion of phospholipid to total lipid was increased from 59% in the controls to 92% in the test group. When the amount of lipid was related to the dry weight of lung it was found that 15% of the dry weight of the control lung was lipid, while lipid made up as much as 35% of the dry weight after chlorphentermine.

TABLE III

LIPID CONTENT OF RAT LUNGS AFTER TREATMENT WITH CHLORPHENTERMINE

	Control n=5 (mg lipid ± SD)	Test n=12 (mg lipid ± SD)
Cholesterol ester	1·3 ±0·3	2·4 ±0·5*
Cholesterol	7·1 ±1·0	15·8 ±3·5*
Free fatty acid	0·4 ±0·1	1·1 ±0·3*
Triglyceride	19·7 ±9·3	6·3 ±3·2*
Phospholipid	41·1 ±8·4	283·7 ±62·5*
Total lipid present in lung	69·6 ±18·1	309·2 ±66·9*

*P < 0·001
The content has been expressed as mg lipid present in both lungs.

The individual phospholipid classes were separated by thin-layer chromatography. All the phospholipid classes in the lung were increased after chlorphentermine treatment ($P<0.001$). A ninefold increase in the lecithin content from 18·5 mg in the lungs of the controls to 171·9 mg in the test group was observed.

The percentage distribution of the phospholipid classes is illustrated in the Figure. Phosphatidyl choline is the major component forming 44% of the total phospholipids in the lungs in the control group. After treatment with chlorphentermine the

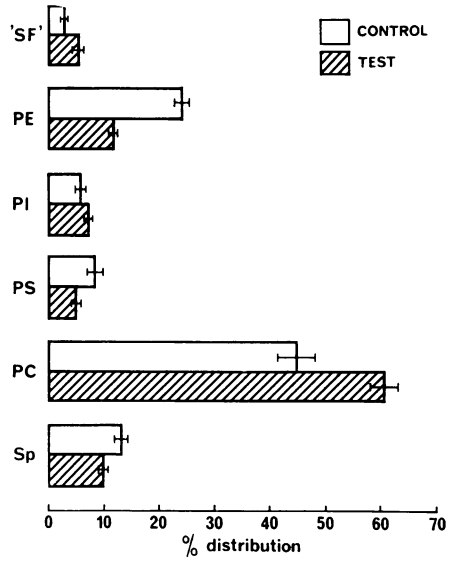

FIGURE *Percentage distribution of phospholipid classes in rat lung after treatment with chlorphentermine. SF=material migrating at the solvent front. Tentatively suggested to be a mixture of phosphatidic acid and phosphatidyl glycerol; PE=phosphatidyl ethanolamine; PI=phosphatidyl inositol; PS=phosphatidyl serine; PC=phosphatidyl choline; Sp=sphingomyelin.*

percentage of phosphatidyl choline was increased to 61% of the total. Proportionally this increase occurred at the expense of phosphatidyl ethanolamine, phosphatidyl serine, and sphingomyelin whose percentage distribution was, therefore, lower. The percentage of phosphatidyl inositol present in the lungs after chlorphentermine treatment was increased slightly. In all the thin-layer separations there was material migrating above phosphatidyl ethanolamine at the solvent front. This material was believed to be a mixture of phosphatidic acid and phosphatidyl glycerol although no definite identification could be made. After chlorphentermine treatment the percentage of this material was also increased. The above figures refer to the percentage composition; the absolute quantity of each class of phospholipid per gram dry weight of lung was increased after treatment with chlorphentermine.

Gas liquid chromatography was used to examine the fatty acid composition of the phosphatidyl choline fraction of the lung. The percentage fatty acid composition determined in this way is given in Table IV. A characteristic feature of lung tissue is the high proportion of saturated fatty acids occurring in the phospholipids. Thus in the lungs from the control rats palmitic acid formed 68% of the total fatty acids in phosphatidyl choline. After chlorphentermine treatment the amount of palmitic acid present in phosphatidyl choline was increased to 80·0% of the total ($P<0.001$). The total percentage of saturated acids was increased from a figure of 74·8% in the lungs of the control rats to a figure of 85·1% in the lungs of the test rats after chlorphentermine treatment.

TABLE IV

PERCENTAGE FATTY ACID COMPOSITION OF LECITHIN FROM RAT LUNG AFTER TREATMENT WITH CHLORPHENTERMINE

		Control n=5 (%±SD)	Test n=12 (%±SD)
Myristic acid	[14:0]	1·5±0·2	3·3±0·5
Palmitic acid	[16:0]	68·0±3·3	80·0±3·0
Palmitoleic acid	[16:1]	6·4±0·9	5·9±0·9
Stearic acid	[18:0]	5·3±0·9	1·8±0·3
Oleic acid	[18:1]	10·1±1·0	3·9±1·0
Linoleic acid	[18:2]	5·4±1·6	3·4±1·0
Arachidonic acid	[20:4]	3·1±0·5	1·8±0·7
Total saturated acids		74·8	85·1

DISCUSSION

As would be expected, chronic administration of the anorectic drug chlorphentermine by intraperitoneal injection in rats caused a reduction in body weight compared to a normal control group. Lüllmann *et al.* (1972) found a similar reduction whether the chlorphentermine was given by mouth or intraperitoneally. Young adult male rats were chosen for these studies so it would be expected that they would be in a phase of active growth at this period. Anorectic drugs lead to a suppression of appetite, so it is important to distinguish those changes in organ weights due to the action of the drug from those occurring due to the effect of the reduced food intake. A previous study on the effect of restricted food intake on the rat (Gloster, Heath, and Harris, 1972a) showed a decrease in weight of all organs studied compared with free-fed animals. After chlorphentermine, however, the weight of the rat lung was found to be significantly higher than in the controls. This increase in weight could not be accounted for by the presence of oedema as the percentage of water present in the lungs from the test animals was in fact decreased. The lung/body weight ratio found in the previous study (Gloster *et al.*, 1972a) was the same in both free-fed and food-restricted rats. The doubling of the lung/body weight ratio observed after chlorphentermine can thus be accounted for by the action of the drug and not simply by diet.

Similarly, a restricted diet had no effect on the lipid content of the lungs of the rat (Gloster, Heath, and Harris, 1972b). After chlorphentermine, however, the total lipid content was increased, the biggest rise occurring in the phospholipids. The histological changes occurring after treatment with chlorphentermine show evidence of this drug-induced lipidosis. The histological appearances of the lung were identical with those seen in a previous study in which chlorphentermine was administered to rats (Heath *et al.*, 1973).

The occurrence of the large foam cells with lamellated inclusions in the alveoli of chronically treated rats was first reported by Franken, Lüllmann, and Siegfriedt (1970) and has since been confirmed by other workers (Lüllmann-Rauch *et al.*, 1972; Smith *et al.*, 1973). Electron microscopy was not carried out on lung tissue from the present animals but a previous study (Smith *et al.*, 1973) demonstrated that the alveolar 'foam cells' contained lamellar inclusions. They proved to be pulmonary histiocytes which had ingested lamellar material extruded from granular pneumocytes and bronchiolar Clara cells. The intra-alveolar debris was found to contain fragments of

lamellar material and phospholipid lattices. Similar laminated inclusions are found in granular pneumocytes, membranous pneumocytes, bronchiolar Clara cells, pulmonary macrophages, and endothelial cells of pulmonary capillaries. Lamellar inclusions of similar appearance have been found in other organs of rats and guinea-pigs (Lüllmann, Lüllmann-Rauch, and Reil, 1973a). These lamellations have a periodicity of between 4 and 5 nm, which is the same as described by Stoeckenius (1962) for phospholipid micelles in an aqueous phase.

Biochemically, chlorphentermine treatment was found to result in a large increase in the phospholipids of rat lungs while the total neutral lipid content was almost unchanged. The neutral and phospholipid contents in the control rats were similar to the values reported by Rooney, Canavan, and Motoyama (1974). Neutral lipids made up 41% of the total lipids, while phosphatidyl choline was the major component of the phospholipids. After chlorphentermine the proportion of neutral lipids was reduced to only 8% of the total lipids. The percentage changes in the distribution of phospholipid classes observed in this study after giving chlorphentermine were similar to those reported by Karabelnik and Zbinden (1975). The major increase was in the amount of phosphatidyl choline present.

Whole lung was used in the present study. No differentiation can thus be made between lung tissue and alveolar fluid. It has been well documented that the pulmonary surfactant making up the active lining layer of the alveolar walls is rich in dipalmitoyl phosphatidyl choline. Chevalier and Collett (1972) suggested that phosphatidyl choline synthesized in the endoplasmic reticulum of the type II alveolar cell was transferred through the Golgi complex, incorporated into the lamellar bodies, and eventually released to the surface. The lamellar body is thus probably composed largely of surfactant. Hallman and Gluck (1975) have provided evidence that the lamellar inclusion bodies have the same phospholipid content as surfactant. Another possible site of pulmonary surfactant production could be the bronchiolar Clara cells which show a marked hyperplasia with accumulation of what appears to be phospholipid lattices after treatment with chlorphentermine (Smith *et al.*, 1974b).

The mode of action of chlorphentermine in causing an accumulation of phospholipid in the lung is still unknown. It could be due to an increase in synthesis or to a decrease in catabolism. The fact that triglyceride is reduced after chlorphentermine could be an indication that the synthetic pathway involving 1,2 diglycerides is directed towards phospholipid synthesis rather than for neutral glyceride formation. The particular effect of chlorphentermine on pulmonary phospholipid may be due not only to the high rate of phospholipid turnover in the lung but also to the high concentration of the drug in that organ. Lüllmann, Rossen, and Seiler (1973b) found that the lung displays a very marked binding affinity for chlorphentermine with a low rate of exchange of the bound material. Nuclear magnetic resonance studies by Seydel and Wassermann (1973) showed that chlorphentermine was bound specifically to lecithin.

There appear to be considerable species differences regarding the histological and biochemical changes occurring after treatment with chlorphentermine. The lungs of rats are greatly affected, those of guinea-pigs less so, while the lungs of mice showed no significant increase in lipid (Seiler and Wassermann, 1975). It is of interest that the closely related drug, phentermine, is not accumulated by the lung and does not cause the same histological changes as are shown by chlorphentermine (Lüllmann *et al.*, 1973b).

The lack of change in the ratio of right ventricular to left ventricular weight in the rats treated with chlorphentermine speaks against the occurrence of pulmonary hypertension. The muscular pulmonary arteries of the test rats had appearances identical with those described by Smith *et al.* (1974a) in normal rats. Lüllmann *et al.* (1972) also reported that the pulmonary vasculature was unaffected although they observed an increased pulmonary arterial pressure in rats treated with chlorphentermine.

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Requests for reprints to: Professor P. Harris, Cardiothoracic Institute, 2 Beaumont Street, London W1N 2DX.