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THIN LAYER AND GAS-CHROMATOGRAPHIC ANALYSIS OF ALVEOLAR LIPIDS IN SILICOTIC RATS

by

R. BATTI, B. ARNOUX, G. LAMY and R. MASSE

1974

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ABSTRACT

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KEY WORDS

LIPIDS
GAS CHROMATOGRAPHY
THIN-LAYER CHROMATOGRAPHY
RATS LUNGS
CARBO 14 COMPOUNDS
ACETATES
PNEUMOCONIOSIS
UPTAKE
TRACER TECHNIQUES
BIOCHEMISTRY

CONTENTS

1.- Introduction

It has been shown by Baily (1963) that the administration of silica at toxic dose leads to an increase in alveolar lipids. This increase has also been observed in various toxic dustingsand especially after Plutonium oxide administration, Masse (1971).

This anomaly is observed in the rat during the blockage of the physiological alveolar clearance, Nenot (1971), Le Bouffant (1971). It might either express a decrease in the turnover of alveolar lipids, or it might be primitive and therefore condition the cellular motility which converges to the cellular mobilization.

Alveolar lipids are in great part produced by Pneumocyte II Buckingham et al (1966), but alveolar macrophages which are numerous in silicotic intoxication have also a synthetic activity, Pfleger et al (1969).

In the present work, we compare the lipid composition of the alveoli in normal and silicotic rats, with the tendency in alveolar synthesis from ^{14}C acetate introduced in the alveoli.

2.- Materials and Methods

2.1. Animals

Ten male OFE rats kept in an heteroxenic state and weighing 350 g before being sacrified were used. After anesthesia with Nembutal experimental silicosis was induced by an intratracheal injection of 40 mg of Ni silica in 0.4 ml of a sterile physiological saline solution. 95% of the injected particles

were less than 5 μ in size. The rats were kept in a conventional environment for two months and compared to ten controls.

2.2. ^{14}C Acetate Incorporation

150 μCi ^{14}C Acetate having 50 - 100 mCi/mM were deeply injected into the trachea. The reaction was blocked 15 minutes after the injection of the labelled acetate by an intratracheal injection of 5 ml of a solution 10^{-2}M of sodium acetate.

2.3. Preparation of Total Lipid Samples

Extraction of the alveolar content was performed by lung washing according to the method of Brain (1968). A solution of 50 ml was collected. This solution contained: intraalveolar cells, lipidic micellae showing the picture of malta cross when observed with crossed nicols and centrifuging with the cells, free lipids or combined with proteins Pasquier (1970). The lipids were extracted by the Folch (1957) procedure.

Total lipids were determined by weight. For the determination of phospholipids, the lipidic phosphorus was determined by the method of Fiske and Subbarow, with about 3.8% mean concentration of phosphorus in the phospholipids of the lung.

3.- Lipid Analysis

3.1. Thin-Layer Chromatography (T.L.C.)

Lipid analysis was carried out by thin-layer chromatography

without any prior separation of neutral lipids and phospholipids.

All thin-layer chromatographic procedures were authenticated with lipid standards (> 98% pure) of known composition. For the separation of the lipids, a two dimensional T.L.C. was performed with a 250 µ- thick layer of silica gel HR: (35 g Merck silica in 70 ml H_2O). Resolution of the lipids was attained in the following solvent systems:

I	1st	Development solvent 1	Chloroform: Methanol: water 75 : 25 : 4, v/v
	2nd	Development solvent 3	Pentane: Diethyloxide: Formic Acid 90 : 30 : 1, v/v
II	1st	Development solvent 2	Tetrahydrofuran: dimethoxy methane: methanol: ammonia
	2nd	Development solvent 3	10 : 6 : 4, 1 v/v

Lipid areas were identified by exposing standards and sample areas to iodine vapours. Individual components were visualized by exposure to the following reagents:

- 50% Sulfuric Acid for Cholesterol
- Anthrone for Glycolipids
- Schiff-periodate (Nicols 1964) for diglycerides, monoglycerides and for identification of phosphatidylglycerol (Pfleger 1969) in the phosphatidyl dimethanolamine spot.

- Draggendorf for phosphatidylcholine and lipophosphatidylcholine.

Figure 1 and 2 reproduce the thin-layer chromatograms of the lipid separation.

3.2. Gas-Liquid Chromatography

The lipids were refluxed in methanolic KOH for 2 hours to transmethylate the fatty-acyl groups.

The free fatty acids were methylated with diazomethane according to the method of Schlenk, Gellerman (1960). The methyl esters of fatty acids were analyzed on a Perkin Elmer (Model 900) gas chromatograph using stainless steel colums (6 ft x 1/8 in) packed with 10% ethylene glycol succinate methyl silicone polymer (EGSSX) coated on chromosorb W(H P) 80/100 Mesh. Columns were prepared and conditioned according to the procedure of Wood and Snyder (1966). The columns were maintained at 170 $\pm$ 0.1 °C, the dual hydrogen flame detectors at 290 °C and the flash heater at 270° C. The flow rates for the FID detectors were air 400 ml/min and hydrogen 42 ml/min. The flow rate of helium carrier gas was 20 ml/min.

A 2.5 mV, 250 mm strip-chart recorder was used at a chart speed of 10 mm/min. The fatty acid methyl esters from the biological samples were identified by comparing their retention time with those of known standards. Percentage composition was determined by half-height analysis of the chroma-

tographic peaks.

3.3. Radioactive Measurements

^{14}C radioactivity was measured in the following scintillator: POPOP : 0.3 g - PRO : 5 g - Toluene: 11 with a liquid scintillation counter model SL 40 Intertechnique. The areas of the identified spots were scrapped and analyzed directly without any prior extraction. The radioactivity was measured by desintegration per minutes, and the results were compared afterwards.

4.- Results and Discussion

The results reproduce the mean values of the pool of ten animals in each batch.

Table 1 - Composition of Lipids

	Control	Silicotic
Total lipids	5.7 mg	76.6 mg
Phospholipids	4.6 mg	17.4 mg
% $\frac{\text{Phospholipids}}{\text{Total lipids}}$	80.7%	22.5%

Table 2 - Rate of ^{14}C Acetate Incorporation into Lipids

Control	Silicotic
2%	0.1%

In the solvent system 3-1 phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl inositol are partially resolved and the scraping of the sample areas is therefore difficult. The whole represent 24.4% of the incorporation of ^{14}C Acetate into the phospholipids. In the solvent system 3-2, these three components are completely resolved. The radioactivity of the scraped areas correspond to 28.4% of that of phospholipids. On the other hand, lysophosphatidyl cholines, sphingolipids and phosphatidyl choline are not resolved in this solvent system.

The solvent system 3-1 gives on the contrary a good resolution. The rate of incorporation of the ^{14}C Acetate into Lipids is shown in table 3 which represent the values obtained in the two chromatograms for the highly resolved spots.

Table 3 - Rate of incorporation of Radioactive Percursor into Lipids

Lipids	Control	Silicotic
Cholesterol Ester	5.1	3.6
Triglycerides	6.6	13.2
Fatty Acids	3.7	33.6
Rf 0.62 unidentified	11.7	2.2
Cholesterol	8.3	2.5
Monoglycerides	7.2	3.2
Dyglycerides	7.1	14.2
Glycolipids	6.6	7.1
Phosphatidyl ethanolamine	5.2	5.4
Phosphatidyl glycerol + Phosphatidyl dimethanolamine	4.8	5.3
Phosphatidyl choline	19.2	5.7
Phosphatidyl inositol	5.6	1.4
Sphingolipids	1.8	2.9
Lysophosphatidyl cholines	3.4	1.0

The fatty acid composition of the lipid from pulmonary surfactant is shown in table 4.

Table 4 - Fatty Acid Composition of Pulmonary Surfactant Percentage of Total Fatty Acids(x)

Fatty Acids	Control	Silicotic
14:0	7.22	15.90
16:0	64.51	56.03
16:1	8.33	10.13
18:0	9.68	5.09
18:1	6.62	8.35
18:2	3.62	4.47
20:4	+	+
% Saturated in Surfactant	81.41	77.02
% Unsaturated in Surfactant	18.57	22.95

The number before the colon represents the number of carbon atoms.

The number after the colon represents the number of double bonds.

+ Indicates the presence of a component in amounts less than 1%.

(x) Values are mean percentage from four samples

The analysis of the alveolar lipid in the silicotic and in the control rat shows a marked difference in composition though a slight variation is observed in the fatty acid composition. Nevertheless a high increase of the short chain fatty acid is observed in the silicotic rat.

We observe in the system used to evidence the synthesis of alveolar lipids:

1) a low incorporation of ^{14}C acetate into lipids;

2) the importance of the pool of labelled free fatty acids;

3) the evident decrease of the incorporation of these fatty acids in the phosphatidyl cholines.

Although the preceeding does not represent the physiological pathway through which the alveolar lipids are renewed since the precursors are introduced via the blood stream, we observe that the incorporation of ^{14}C acetate is as follows:

	Control	Silicotic
Neutral Lipids	45%	71%

of the activity found in the chromatogram.

If this incorporation does not reflect the composition of the alveolar lipids for the control rat

Control	Neutral Lipids
Composition	19.3%
Incorporation	45%

for the Silicotic rat on the contrary the values are much closer.

Silicotic	Neutral Lipids
Composition	77.5%
Incorporation	71%

this would mean that the lipids secreted by Pneumocytes II undergo an alveolar turn over which modifies the initial composition of the surfactant.

In this medium of low metabolic activity but with prolonged effect due to alveolar stasis we can presume that the enzymatic equipment of lengthening or condensation of the fatty acids as well as the stages of transfer Diglycerides CDP choline are in part deficient and consequently would explain the decrease of lecithin and the low increase of glycerides and fatty acids. The pathway of synthesis by methylation of the lysolecithin does not appear to be a major factor, as the relation $\frac{\text{Lecithins}}{\text{Lysolecithins}}$ remains almost constant in both control and silicotic rats.

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lipid constituents of normal and quartz-dusted guinea pig lungs. Nature 198, 856-857.

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LIST OF FIGURES

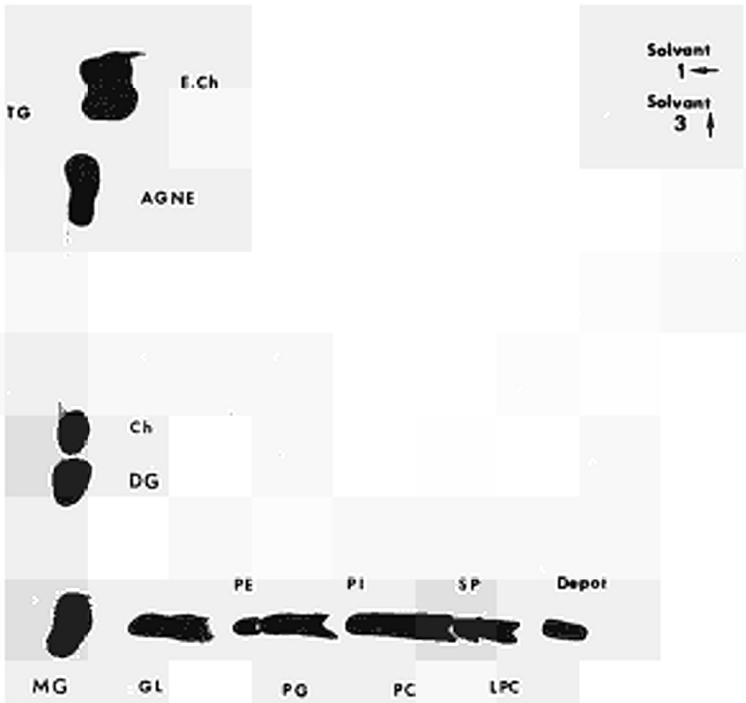

Figure 1 Two-dimensional thin-layer chromatogram on silica gel HR (250 µ thick) of pulmonary surfactant lipids from silicotic rat in the solvent system 3-1.

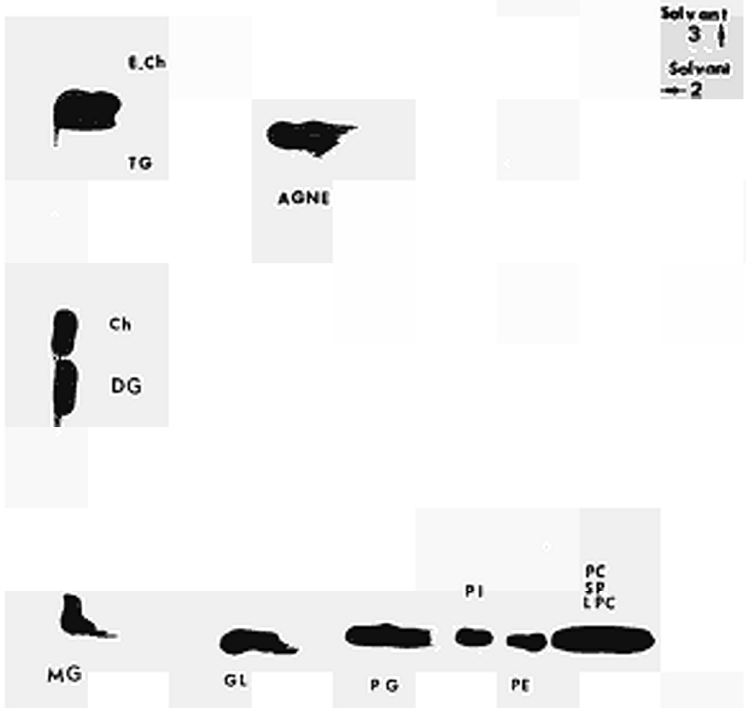

Figure 2 Two-dimensional thin-layer chromatogram on silica gel HR (250 µ thick) of pulmonary surfactant lipids from silicotic rat in the solvent system 3-2.

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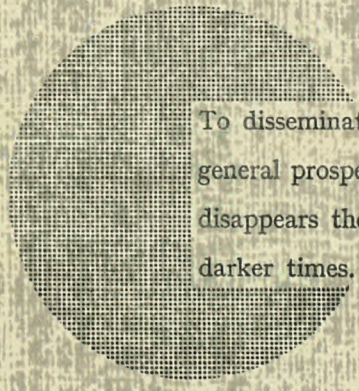

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Alfred Nobel

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